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Stefan Offermanns
Walter Rosenthal
Editors

Encyclopedia of Molecular Pharmacology

Third Edition

Springer

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Stefan Offermanns • Walter Rosenthal
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Encyclopedia of Molecular Pharmacology

Third Edition

With 440 Figures and 175 Tables

Springer

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Preface to the Third Edition

More than a decade has passed since the second edition of the *Encyclopedia of Molecular Pharmacology* was published. During this time, our knowledge in molecular pharmacology has increased and the way scientific information is presented and made accessible has changed. The third edition therefore puts an emphasis on essays, covering important aspects of molecular pharmacology and written by leading experts. We have removed short definitions from the encyclopedia, as they are now available from numerous internet resources. Similarly, the appendix, which listed drugs and drug targets, has been omitted. For systematic up-to-date information on established or potential drug targets such as receptors, channels, transporters, and enzymes, we now refer to the well-curated web-based databases provided by national and international pharmacological societies such as the Guide to Pharmacology (www.guidetopharmacology.org) or the biannually published *Concise Guide to Pharmacology* of the *British Journal of Pharmacology.*

It has again been a pleasure and a great learning experience to interact with the 400 authors of the encyclopedia, who have done a great job to briefly describe important topics of molecular pharmacology for a broad readership. We thank the new and old authors for their excellent work and many helpful and constructive suggestions, which went into the third edition. On the side of Springer Nature, we would like to thank Susanne Dathe and Andrew Spencer, for initiating and guiding the project, as well as Kate Emery, who tirelessly managed the project for the last two years. Finally, our special thanks go to Svea Hümmer, who provided excellent and invaluable assistance during all stages of this project.

Bad Nauheim/Frankfurt am Main/Jena
May 2021

Stefan Offermanns
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Preface to the Second Edition

The first edition of the *Encyclopedic Reference of Molecular Pharmacology* was well received by its readers, thanks to the excellent work done by the authors, of whom most have contributed to the second edition as well. The basic structure of the Encyclopedia has remained unchanged. It is primarily based on essays, which have been updated, and their number has been increased to 225 to include many new exciting areas. These essays cover important drugs and drug targets, but also general principles of pharmacology as well as cellular processes and pathological situations which are relevant for drug action. In addition, there are about xy key words linked to the essays. The Encyclopedia is complemented by an Appendix, which has been greatly enlarged, listing more than 700 drugs and more than 4,000 proteins that act as receptors, membrane transport proteins, transcription factors, enzymes or adhesion molecules.

During the preparation, we greatly enjoyed the interaction with all our colleagues who contributed to this reference work. It has been a pleasure and an enriching experience to deal with so many facets of pharmacology. We are very thankful to the contributing authors for the careful updating of their essays, and, in particular, we would like to express our gratitude to the more than xy new authors who have written excellent essays on novel topics. Finally, we would like to thank Dr. Michaela Bilic and Simone Giesler from Springer for their enthusiasm throughout the project and their constant support.

Heidelberg/Berlin
November 2007

Stefan Offermanns
Walter Rosenthal

Preface to the First Edition

The era of pharmacology, the science concerned with the understanding of drug action, began only about 150 years ago when Rudolf Buchheim established the first pharmacological laboratory in Dorpat (now, Tartu, Estonia). Since then, pharmacology has always been a lively discipline with "open borders", reaching out not only to other life sciences such as physiology, biochemistry, cell biology and clinical medicine, but also to chemistry and physics. In a rather successful initial phase, pharmacologists devoted their time to describing drug actions either at the single organ level or on an entire organism. Over the last few decades, however, research has focused on the molecular mechanisms by which drugs exert their effects. Here, cultured cells or even cell-free systems have served as models. As a consequence, our knowledge of the molecular basis of drug actions has increased enormously. The aim of *Encyclopedic Reference of Molecular Pharmacology* is to cover this rapidly developing field.

The reductionist approach described above has made it increasingly important to relate the molecular processes underlying drug actions to the drug effect on the level of an organ or whole organism. Only this integrated view will allow the full understanding and prediction of drug actions, and enable a rational approach to drug development. On the molecular or even atomic level, new disciplines such as bioinformatics and structural biology have evolved. They have gained major importance within the field but are particularly relevant for the rational development and design of new drugs. Finally, the availability of the complete genome sequence of an increasing number of species provides a basis for systematic, genome-wide pharmacological research aimed at the identification of new drug targets and individualised drug treatment (pharmacogenomics and pharmacogenetics). All these aspects are considered in this encyclopedia.

The main goal of the *Encyclopedia* is to provide up-to-date information on the molecular mechanisms of drug action. Leading experts in the field have provided 159 essays, which form the core structure of this publication.

Most of the essays describe groups of drugs and drug targets, with the emphasis not only on already exploited drug targets, but also on potential drug targets as well. Several essays deal with the more general principles of pharmacology, such as drug tolerance, drug addiction or drug metabolism. Others portray important cellular processes or pathological situations and describe how they can be influenced by drugs. The essays are complemented by more than 1600 keywords, for which links are provided. By looking up the

keywords or titles of essays highlighted in each essay, the reader can obtain further information on the subject. The alphabetical order of entries makes the Encyclopedia very easy to use and helps the reader to search successfully. In addition, the names of authors are listed alphabetically, together with the title of their essay, to allow a search by author name.

Apart from very few exceptions, the entries in the main text do not contain drug names in their titles. Instead, drugs that are commonly used all over the world are listed in the Appendix. Also included in the Appendix are four extensive sections that contain tables listing proteins such as receptors, transporters or ion channels, which are of particular interest as drug targets or modulators of drug action.

The *Encyclopedia* provides valuable information for readers with different expectations and backgrounds (from scientists, students and lecturers to informed lay-people) and fills the gap between pharmacology textbooks and specialized reviews.

All the contributing authors as well as the editors have taken great care to provide up-to-date information. However, inconsistencies or errors may remain, for which we assume full responsibility. We welcome comments, suggestions or corrections and look forward to a stimulating dialog with the readers of the *Encyclopedic Reference of Molecular Pharmacology* whether their comments concern the content of an individual entry or the entire concept.

We are indebted to our colleagues for their excellent contributions. It has been a great experience, both personally and scientifically, to interact with and learn from the 200 plus contributing authors. We would also like to thank Ms. Hana Deuchert and Ms. Katharina Schmalfeld for their excellent and invaluable secretarial assistance during all the stages of this project. Within Springer-Verlag, we are grateful to Dr. Thomas Mager for suggesting the project and to Frank Krabbes for his technical expertise. Finally, we would like to express our gratitude to Dr. Claudia Lange for successfully managing the project and for her encouraging support. It has been a pleasure to work with her.

Heidelberg/Berlin
June 2003

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About the Editors

Stefan Offermanns After finishing medical studies, Stefan Offermanns did postdoctoral work at the Free University Berlin and the California Institute of Technology. From 2000 to 2009, he held the Chair of Pharmacology at the University of Heidelberg. Since 2008 he has been Director at the Max Planck Institute for Heart and Lung Research and Professor at the Goethe University, Frankfurt. He has combined basic science with clinically oriented research to study the role of various cellular signaling mechanisms in the cardiovascular and metabolic systems as well as in cancer.

Professor Walter Rosenthal is an MD and molecular pharmacologist. He is recognized as a specialist in signal transduction (G-proteins, G-protein-coupled receptors, and anchoring proteins). In 1996, he became founding Director of the Leibniz Institute of Molecular Pharmacology (FMP) in Berlin, and in 1998, he was appointed Full Professor at the Charité-Universitätsmedizin Berlin. He headed the FMP for 12 years before serving as Chair of the Board and Scientific Director of the Max Delbrück Center for Molecular Medicine in Berlin-Buch (2009–2015). Since 2014 he has been President of Friedrich Schiller University Jena.

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A

A Receptors

► Adenosine Receptors

AADs, Antiarrhythmic Agents

► Antiarrhythmic Drugs

ABC Proteins

► ABC Transporters

ABC Transporters

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Synonyms

ABC proteins; ATP binding cassette proteins

Definition

The ABC transporters represent one of the largest families of transmembrane proteins in most organisms including plants, bacteria, and eukaryotic cells. They perform numerous key physiological processes directly coupled with the hydrolysis of ATP. From the pharmacological point of view, ABC transporters are of special interest because of their relevance in transmembrane transport of various drugs, thereby modifying intracellular concentrations and hence effects of these compounds. Furthermore, they represent potential target structures to interfere with (patho-)physiological processes in which they are involved.

Basic Characteristics

ATP-binding cassette (ABC) proteins are present in plants, bacteria, and eukaryotic organisms including mammalians. In humans the ABC gene superfamily comprises about 50 members, which are organized in 7 subfamilies called ABCA to ABCG on the basis of their domain organization and phylogenetic similarities. Besides the systematic gene nomenclature alternative names for the proteins are widely used, which are based on the first functional description such as P-glycoprotein for ABCB1 (which are also used for the proteins in this article). ABC proteins are involved in various physiological processes including the transport of lipids such as cholesterol (e.g., ABCA1, ABCG1/5/8) or fatty acids (ABCD1/2), presentation of peptides by MHC class I molecules (ABCB2/ABCB3, which are better known as transporter associated

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with antigen processing (TAP)), or regulation of ion channels. For example, the regulatory subunit of the K_{ATP} channel of the pancreatic beta-cells and target structure of the antidiabetic sulfonylureas SUR1 is encoded by the ABCC8 gene, and ABCC7 encodes an important chloride channel (cystic fibrosis transmembrane conductance regulator, CFTR) in epithelial cells of the lung and pancreas. In addition, some ABC transporters are important components of physiological barriers like the intestinal epithelium or the blood-brain barrier and in excreting organs as the liver and kidney. Here, they are acting as efflux pumps protecting the organism against endogenous metabolites as well as environmental toxins including several pharmacological important drugs. Some of these transporters, namely, ABCB1 (P-glycoprotein, P-gp, MDR1), ABCG2 (breast cancer resistance protein, BCRP), and several members of the multidrug resistance protein (MRP) family (ABCC1-C6; ABCC10-ABCC12), are of high pharmacological relevance, because they have been identified as one underlying mechanism of a cancer-related phenotype called multidrug resistance (MDR) (Chen et al. 2016; Gottesman and Ling 2006; Slot et al. 2011). This phenomenon is characterized by the resistance of cancer cells against anticancer drugs caused by reduced intracellular drug concentrations. Furthermore, these transporters can affect pharmacokinetic parameters of various drugs in general (Chan et al. 2004).

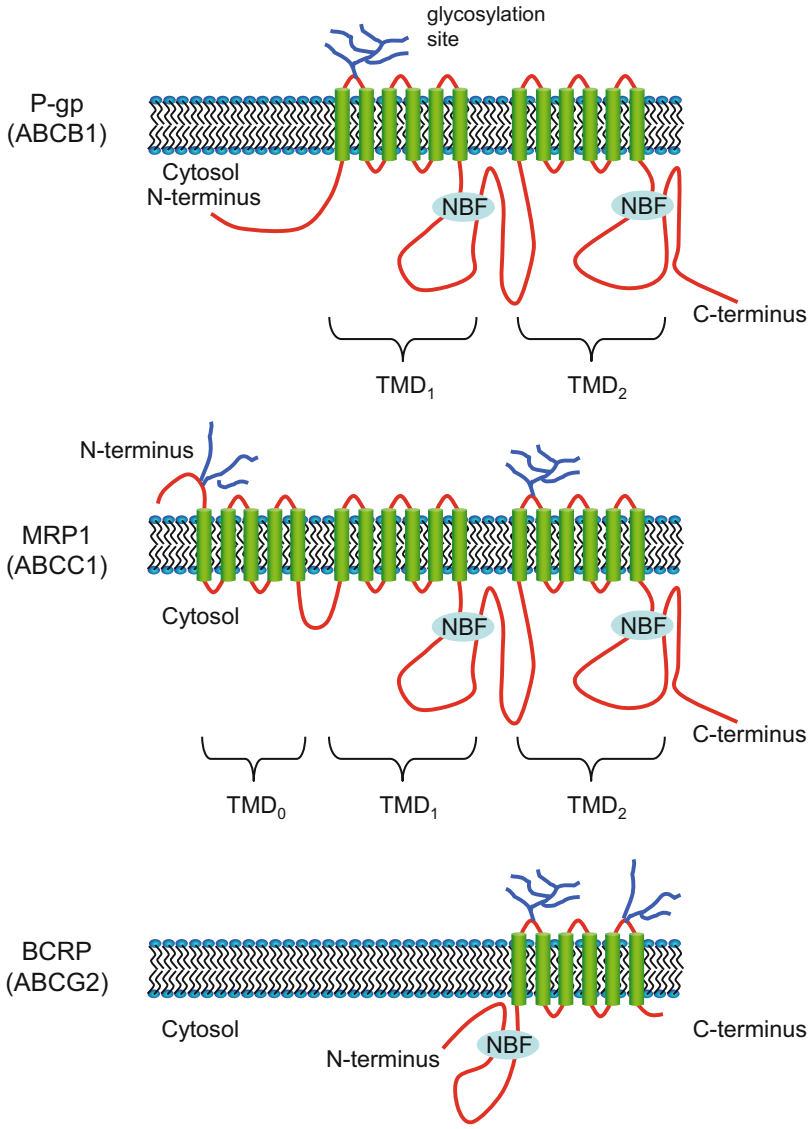
Topology and Structure

Most ABC transporters, especially those located in the plasma membrane, are phosphorylated and glycosylated transmembrane proteins of different molecular weights (e.g., P-gp/ABCB1, 170 kDa; BCRP/ABCG2, 72 kDa). In general, active ABC transporters consist of two nucleotide-binding folds (NBF) and two transmembrane domains (TMD, each consisting of six α-helices), which can be localized on a single polypeptide (full transporter, e.g., P-gp) or formed by dimerization of a so-called half transporter (e.g., BCRP). This core structure can be modified by additional TMDs (e.g., MRP1/ABCC1) (Fig. 1). The NBFs contain the highly conserved Walker A and B consensus motifs as well as the LSGGQ signature motif, which is unique for ABC transporters and catalyzes the ATP hydrolysis – a prerequisite for substrate binding and transport against a substrate gradient. For further details on structure and mechanism of ABC transporters, see Beis (2015).

Tissue Distribution and Localization

Although initially detected in cancer cell lines, ABC transporters show a wide tissue distribution. Several drug transporting ABC proteins are highly expressed in physiological barriers such as the luminal membrane of enterocytes, the endothelial cells of the blood-brain barrier, or the maternal facing (apical) membrane of the placental syncytiotrophoblast. In all of these organs, they protect sensible tissues like the brain or the growing fetus against potentially toxic compounds. In addition, ABC transporters are highly abundant in the kidney and liver (Fig. 2). In hepatocytes they are involved in the elimination of many endogenous and exogenous compounds and therefore expressed in both the canalicular and sinusoidal membranes. The canalicular expression is a prerequisite for biliary elimination. For example, the bile salt export pump (BSEP/ABCB11) transports bile salts, MRP2 (ABCC2) is involved in the elimination of organic anions like bilirubin glucuronides or glutathione conjugates, MDR3 (ABCB4) is essential for secretion of phospholipids, and P-gp (ABCB1) eliminates a number of drugs into bile. Other ABC transporters like MRP3 (ABCC3) and MRP4 (ABCC4) are located at the sinusoidal membrane of hepatocytes. They transport xenobiotics and several conjugates back into the blood and are of special relevance under pathophysiological conditions. For example, hepatic expression of both transporters is induced during cholestasis, thereby protecting the hepatocytes against toxic bile acid concentrations by transport into the blood followed by increased renal elimination (Keppler 2011; Slot et al. 2011).

ABC transporters are also expressed in other organs like the heart, lung, pancreas, or blood cells. Here, they may be important for both physiological processes and local drug concentrations. In this context it is noteworthy that many of these transporters not only eliminate xenobiotic and

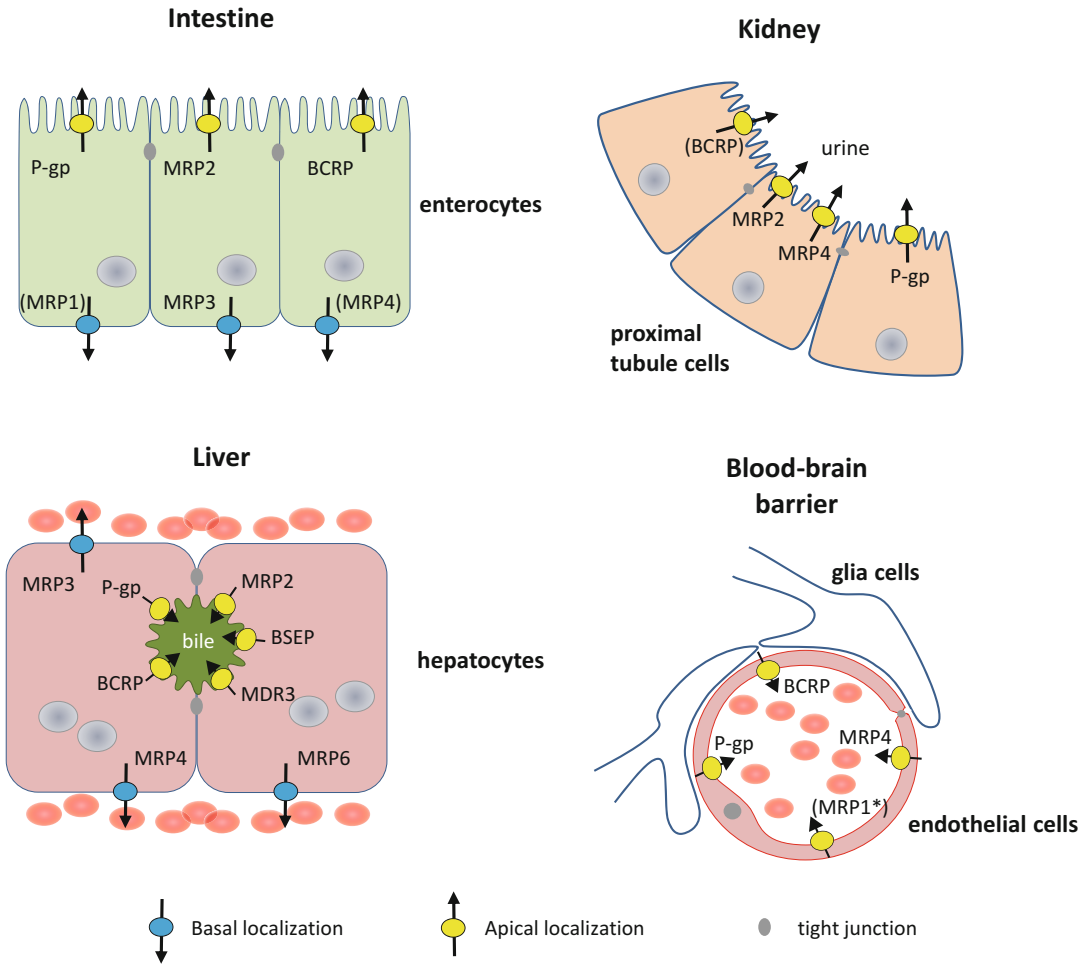


ABC Transporters, Fig. 1 Predicted membrane topologies of P-gp (ABCB1), MRP1 (ABCC1), and BCRP (ABCG2), exemplifying full (P-gp and MRP1) and half (BCRP) transporters, as described in the main text (NBF, nucleotide-binding fold; TMD, transmembrane domain; scheme modified according to Borst and Elferink 2002)

toxic compounds from the cell but export also physiological important metabolites. Finally, the intracellular localization of ABC transporters is not restricted to the plasma membrane, but they have been also detected in intracellular structures. For example, ABCD1/2 is localized in peroxisomes and ABCB2/3 in the endoplasmic reticulum. For further reading, see Borst and Elferink (2002).

Physiological and Pathophysiological Aspects

Based on their physiological function, it is not surprising that genetic variants affecting expression and function of ABC proteins have been identified as the underlying mechanisms for several diseases. For example, loss-of-function mutations in the gene encoding the cholesterol transporter ABCA1 have been associated with the Tangier disease (patients show a severe reduction of high-density lipoprotein, HDL), and genetic variants of ABCA7 have been identified as risk factors for Alzheimer's disease (Aikawa et al. 2018). Cystic fibrosis is caused by genetic defects in the CFTR (ABCC7) gene, and certain polymorphisms in the SUR1 (ABCC8) gene are risk factors for the development of diabetes. In



ABC Transporters, Fig. 2 ABC transporter expression and localization in pharmacokinetically important tissues, such as the intestine, liver, kidney, and brain microvascular endothelial cells forming the blood-brain barrier. Details on localization and function of the depicted transporters are given in the main text (∗the localization of MPR1 at the blood-brain barrier is still unclear)

view of physiological processes, also several MRPs are of interest. The block of biliary elimination of bilirubin glucuronides caused by the absence of MRP2 (ABCC2) from the canalicular membrane leads to the Dubin-Johnson syndrome. Genetic defects of MRP6 (ABCC6) cause the ectopic mineralization disorder pseudoxanthoma elasticum (characterized by fragmentation and mineralization of elastic fibers) probably through a diminished hepatocellular export of ATP, which is extracellularly rapidly converted into AMP and the mineralization inhibitor pyrophosphate (Jansen et al. 2013).

MRP4 (ABCC4), which is expressed in many tissues and cancer cells, transports not only xenobiotics like nucleotide-based anticancer drugs but also endogenous signaling molecules such as cyclic nucleotides and has been established as an independent regulator of especially cAMP levels in several cell types including vascular smooth muscle cells and platelets (Belleville-Rolland et al. 2016). Genetic variations of MRP8 (ABCC11) determine the human cerumen type (wet or dry ear wax) and presence of underarm osmidrosis but are also involved in therapy resistance in breast cancer (Ishikawa et al. 2013).

Based on their pathophysiological roles, ABC proteins represent drug targets of established and potential novel drugs. For example, the widely used antidiabetic sulfonylureas and glinides inhibit the regulatory subunit SUR1 (ABCC8) of the ATP-sensitive potassium channels (K_{ATP}), thereby inducing the insulin secretion. Drugs such as ivacaftor have been developed, which can at least partially restore the function of CFTR (ABCC7) in certain genetic defects and thus treat cystic fibrosis (Berical et al. 2019). Moreover, in order to treat this disease, several approaches have already been evaluated to replace the defective ABCC7 by gene therapy. Other physiological relevant transporters like MRP4 may also represent novel targets, e.g., to regulate platelet function (Belleville-Rolland et al. 2016).

Drugs

From the pharmacological point of view, ABC transporters represent drug targets (pharmacodynamics relevance, see above) and – even more important – affect the bioavailability of other compounds including various drugs (pharmacokinetic relevance).

Drug Transport

The pharmacokinetic relevance of ABC transporters becomes evident because of their high expression at physiological barriers and excretion organs like the intestine, blood-brain barrier, liver, and kidney. Substrates of these transporters, especially for P-gp, BCRP, and several MRPs, are intensively eliminated into the bile and the urine or transported back to the intestine, thereby limiting oral bioavailability. In this context, it is not surprising that ABC transporters are involved in drug-drug interactions in analogy to metabolizing enzymes. Besides inhibition and in turn enhanced bioavailability after coadministration of transporter substrates or inhibitors, ABC transporters can also be induced by certain mechanisms leading to a reduced bioavailability of their substrates. For example, similar to the P450 enzyme CYP3A4, the promoter of ABCB1 (P-gp) contains a transcription factor binding site for the pregnane X receptor (PXR). PXR, which is expressed in high abundance in the liver and intestine, functions as a xenosensor detecting potential toxic compounds. Its activation leads to an enhanced expression of metabolizing enzyme (e.g., CYP3A4) and ABC transporters (mainly P-gp), thereby protecting the organisms. Of note, not only drugs like rifampicin, barbiturates, or carbamazepine but also apparently harmless compounds like St. John's wort (active compound hyperforin) can activate PXR and in turn upregulate transporter expression. Besides PXR, binding sites for other nuclear receptors like the constitutive androstane receptor (CAR), the farnesoid X receptor (FXR), the steroid and xenobiotic receptor (SXR), or the peroxisome proliferator-activated receptor (PPAR) have also been described for ABC transporters.

Besides pharmacokinetic important organs, ABC transporters are expressed in drug target structures, thereby affecting local drug concentrations. Many anticancer drugs are ABC transporter substrates, and tumor cells often show an enhanced transporter expression resulting in a multidrug resistance phenotype, an unsolved problem in chemotherapy. Inhibitors of ABC transporters, especially P-gp, have been developed as chemosensitizers to overcome resistance, but so far clinical trials revealed only limited or no benefits. The problem of limited drug availability at its target site is not restricted to cancer therapy. As already mentioned, drug transporting ABC transporters like P-gp and BCRP are also expressed at the blood-brain barrier, thereby limiting the access of drugs to the brain. While this is useful for drugs like loperamide, a morphine-based drug against diarrhea, it is a problem for central acting drugs like antipsychotics. A list of pharmacological relevant ABC transporters and their substrates is given in Table 1.

In pharmacotherapy, a different response of patients to a certain drug is often observed. A part of this interindividual variability can be attributed to pharmacokinetic differences of the substances in the different patients. Besides the abovementioned drug-drug interaction on transporter level, genetic variability of ABC

ABC Transporters, Table 1 Substrates of ABC transporters involved in multidrug resistance and drug transport according to (Keppler 2011; Ishikawa et al. 2013; Lund et al. 2017; Mao and Unadkat 2015; Slot et al. 2011; Zhou et al. 2008)

ABC transporter	Transporter substrates
P-gp (ABCB1)	**Endogenous substrates:** steroids, lipids, bilirubin, bile acids **Drugs:** anticancer drugs (e.g., docetaxel, doxorubicin, etoposide, erlotinib, imatinib, irinotecan, paclitaxel, topotecan, vinblastine); antibiotic and virostatic drugs (e.g., abacavir, clarithromycin, elvitegravir, erythromycin, indinavir, levofloxacin, maraviroc, nelfinavir, ritonavir, sofosbuvir); immunomodulatory drugs (e.g., cyclosporin A, everolimus, methylprednisolone, sirolimus, tacrolimus); cardiovascular drugs (e.g., apixaban, atorvastatin, dabigatran etexilate, digoxin, simvastatin, talinolol, ticagrelor); and others (e.g., canagliflozin, cimetidine, domperidone, fexofenadine, glibenclamide, ivermectin, lansoprazole, loperamide, morphine, oxcarbamazepine, ondansetron, saxagliptin)
MRP1 (ABCC1)	**Endogenous substrates:** cysteinyl leukotrienes (LTC4, LTD4); reduced (in co-transport) and oxidized glutathione (GSH, GSSG); glutathione, glucuronate, and some sulfate conjugates of endogenous lipophilic compounds such as steroids (e.g., estradiol 17β-glucuronide); folic acid; sphingosine 1-phosphate; and others **Drugs (resistance profile):** many cytostatic drugs (e.g., methotrexate, anthracyclines, vinca alkaloids, etoposide, cyclophosphamide, paclitaxel; transport often GSH-dependent), colchicine; HIV protease inhibitors (e.g., indinavir ritonavir); some antibiotics (e.g., ciprofloxacin); and others
MRP2 (ABCC2)	**Endogenous substrates:** bilirubin glucuronides, cysteinyl leukotrienes, estradiol 17β-glucuronide, estrone-3-sulfate, dianionic conjugated bile salts, and others **Drugs:** glutathione, glucuronate, and sulfate conjugates of drugs; statins (e.g., pravastatin); HIV protease inhibitors (e.g., indinavir, ritonavir); antibiotics (ampicillin, azithromycin, ceftriaxone); resistance to cytostatic drugs (e.g., irinotecan, cisplatin, anthracyclines, vinca alkaloids, methotrexate, mitoxantrone); valsartan; and others
MRP3 (ABCC3)	**Endogenous substrates:** estradiol-17β-glucuronide, LTC4, bile salts (e.g., cholylglycine), bilirubin glucuronides, dehydroepiandrosterone-3-sulfate (DHEAS), and others **Drugs:** methotrexate, clopidogrel metabolites, acetaminophen glucuronide, phytoestrogen conjugates, and others
MRP4 (ABCC4)	**Endogenous substrates:** cyclic nucleotides (cAMP, cGMP), urate, estradiol-17β-glucuronide, DHEAS, bile acids (+GSH), folate, LTC4, LTB4 (+GSH), prostanoids (PGE1/2, TXB2), sphingosine 1-phosphate, and others **Drugs:** nucleoside-based antiviral drugs (e.g., adefovir, zidovudine, acyclovir, ganciclovir); some HIV protease inhibitors (e.g., nelfinavir, tenofovir); resistance to purine-based anticancer drugs (e.g., 6-mercaptopurine, 6-thioguanine), methotrexate; statins, furosemide, hydrochlorothiazide, olmesartan, and others
MRP5 (ABCC5)	**Endogenous substrates:** cyclic nucleotides (cGMP, cAMP, cCMP), folate, glutamate conjugates, and others **Drugs:** nucleoside-based antiviral drugs (e.g., adefovir), methotrexate and several other anticancer drugs (resistance to cladribine, cladarabin, gemcitabine, cytarabine, 5-FU, 6-mercaptopurine, thioguanine), and others
MRP8 (ABCC11)	**Endogenous substrates:** cyclic nucleotides (cAMP, cGMP), estradiol-17β-glucuronide, DHEAS, LTC4, folate, GSH-conjugated sulfanylalkanols (odorant precursors), and others **Drugs:** fluoropyrimidine-based anticancer drugs, eribulin, tenofovir, and others
BCRP (ABCG2)	**Endogenous substrates:** flavonoids, porphyrins, folate, sulfate conjugates (e.g., estrone-3-sulfate) **Drugs:** anticancer drugs (e.g., topotecan, irinotecan, SN-38, methotrexate, imatinib, mitoxantrone); antibiotics/anti-retrovirals (e.g., zidovudine, lamivudine, sulfasalazine, nitrofurantoin); prazosin, pantoprazole, rosuvastatin, and others

transporter is also involved. Besides genetic loss-of-function variants leading to the abovementioned inherited diseases, several more common polymorphisms have been identified in ABC transporter genes. Some of these variants have been associated with a reduced transporter

expression or function (Bruhn and Cascorbi 2014; Wolking et al. 2015). For example, an amino acid exchange in the BCRP transporter (rs2231142, 421C>A, Q141K) leads to reduced transporter function and in turn causes enhanced plasma levels of certain drugs (e.g., statins). In the case of MRP2, a polymorphism (rs717620, -24C>T) in the promoter region of the ABCC2 gene has been identified. This variant is associated with a reduced transporter expression and in turn pharmacokinetic differences of some MRP2 substrates in the respective patients. Interestingly, the relevance of genetic variation within the ABCB1 (P-gp) gene is still less clear. Even if ABCB1/P-gp is probably the best characterized transporter in this field and various synonymous and nonsynonymous polymorphisms as well as deletions and insertions have been identified, the clinical relevance of these polymorphisms is still unclear.

Taken together, ABC transporters represent a large family of proteins affecting the pharmacokinetic parameters of various drugs. Here, P-gp is currently the best characterized member, and it may also be one of the most important ABC transporters with regard to drug transport. However, ABC transporters act in a coordinated fashion with other detoxification systems like P450 enzymes and uptake transporters. In particular, P-gp and CYP3A4 are closely intertwined in terms of regulation and function. Thus, further reviews have to address the combined action of various systems.

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7-ACA Derivatives

► Cephalosporins

ACE Inhibitors

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Synonyms

Angiotensin-1 converting enzyme inhibitors

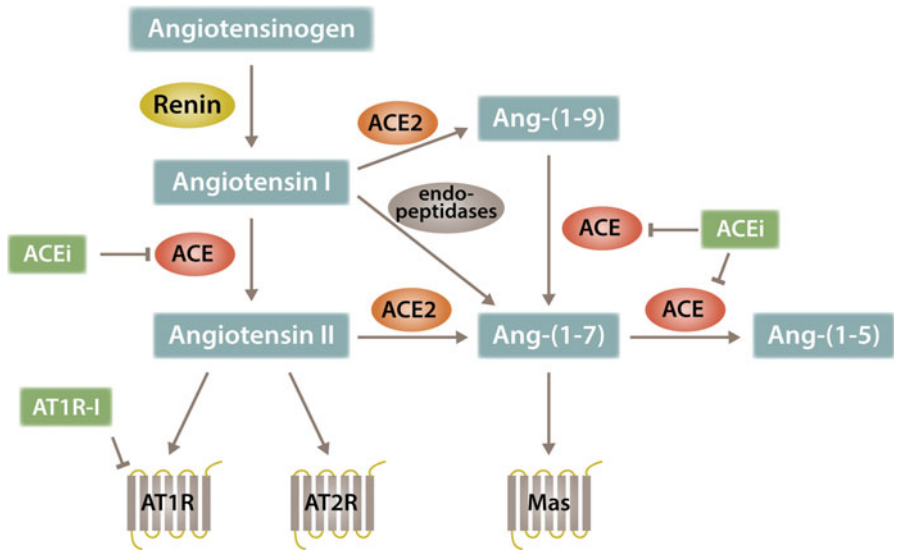
Angiotensin-1 Converting Enzyme (ACE)

Angiotensin-1 converting enzyme (ACE, EC 3.4.15.1, also known as peptidyl dipeptidase) is a key zinc metallopeptidase in cardiovascular regulation that acts against angiotensin peptides (Ang I, Ang II), as well as other vasoactive peptides, including kinins (e.g., bradykinin), substance P, and the acetylated tetrapeptide Ac-SDKP. Becasue of its promiscuity as an enzyme, ACE and its peptide substrates and products affect many physiologic processes, including blood pressure control, hematopoiesis, reproduction, renal development, renal function, and the immune response (for reviews see Acharya et al. 2003; Bernstein et al. 2012; Arendse et al. 2019).

ACE is a critical component of the renin-angiotensin aldosterone system (RAAS) (Fig. 1), which controls blood pressure and strongly influences the function of the heart and the kidneys, as well as the walls of blood vessels. For these reasons, drugs that target the RAAS, such as ACE inhibitors, are among the most important therapeutic agents available today for the treatment of hypertension, heart failure, coronary artery disease, renal insufficiency, and general atherosclerosis.

Mechanism of Action of ACE Inhibitors

ACE inhibitors competitively inhibit the enzymatic activity of ACE with K_i values between



ACE Inhibitors, Fig. 1 An overview of the renin-angiotensin-aldosterone system (RAAS). A complex pathway of peptides converted to active hormones by peptidases and key receptors provide a number of targets for therapeutic intervention. AT2R, angiotensin type 2 receptor; AT1R, angiotensin type 1 receptor; Mas, Mas receptor for Ang 1–7; ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; ACEi, ACE inhibitor; AT1R-I, angiotensin type 1 receptor blocker

10^{-10} and 10^{-11}. ACE cleaves a wide range of pairs of amino acids from the carboxy-terminal end of a plethora of peptide substrates. The conversion of Ang I to Ang II and the degradation of bradykinin to inactive fragments are considered the most important functions of ACE. Ang II (a powerful vasoconstrictor) primarily circulates in the blood and causes the muscles surrounding the blood vessels to contract, thereby narrowing the vessels. This increases the pressure within the vessels, causing an increase in blood pressure (known as hypertension). Ang II also stimulates the release of the hormone aldosterone from the adrenal glands, which in turn signals the kidneys to retain salt and water, which further raises blood pressure. Furthermore, Ang II acts as a growth factor that stimulates thickening of the blood vessel walls, aggravating the process of atherosclerosis, or hardening of the arteries. Drugs acting on ACE and the RAAS reduce blood pressure, improve the function of the heart, and slow down the progression of atherosclerosis and kidney disease. However, Ang II is not the only peptide metabolized by ACE.

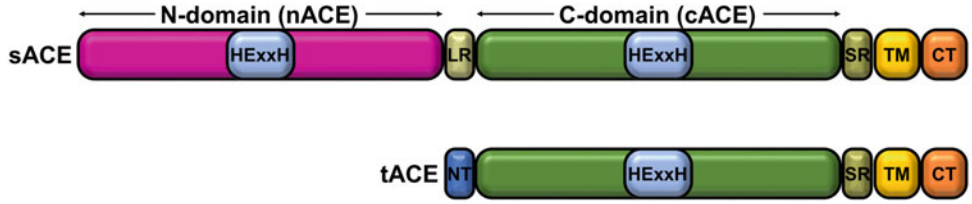
ACE also acts on other peptide hormones, notably bradykinin, which has the opposite effect of Ang II and acts as a vasodilator. ACE inhibition results in reduced levels of Ang II (therefore, less vasoconstriction) but increased levels of bradykinin (therefore, more vasodilatation). These effects together cause a greater reduction in blood pressure than Ang II alone. Bradykinin also has anti-inflammatory effects, all of which explains why ACE inhibitors have a different therapeutic profile than drugs that only block the Ang II receptor. Ang I in the blood is formed from angiotensinogen, a protein produced by the liver and released into the blood. Ang II is formed from Ang I in the blood by ACE. Current clinically available ACE inhibitors are nonpeptide analogues of Ang I that reduce (inhibit) the enzymatic activity ACE by decreasing the production of Ang II. As a result, blood vessels enlarge or dilate, and blood pressure is reduced. This lower blood pressure makes it easier for the heart to pump blood and can improve the function of a failing heart. In addition, the progression of kidney disease due to high blood pressure or diabetes is slowed.

Molecular Properties of ACE

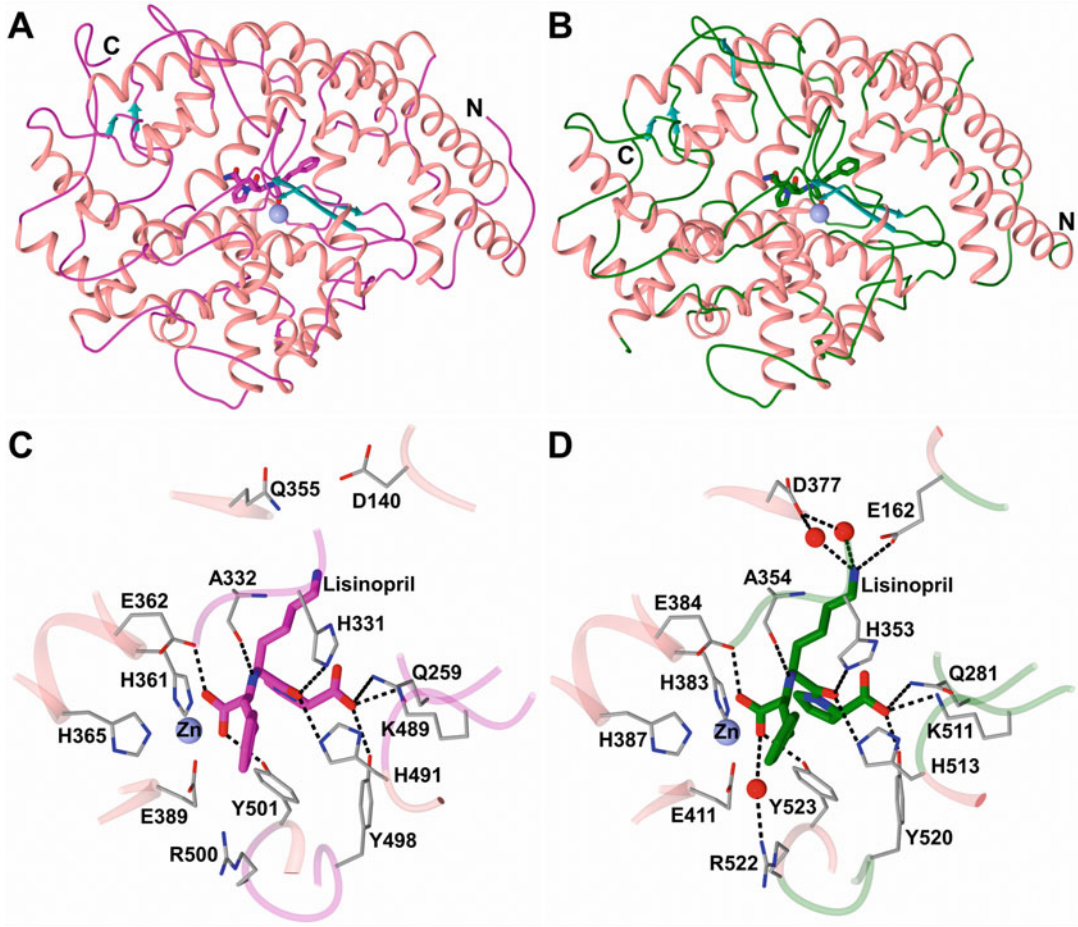
Human ACE is a type I transmembrane protein comprised of 1306 amino acids when processed to its mature form. A sequence of 22 hydrophobic amino acids located near the carboxy terminus of the protein serves as the transmembrane domain that anchors ACE to the cell surface. This creates a 28-residue cytosolic domain and a 1277-residue glycosylated extracellular domain. ACE is member of a large family of proteins that undergo cleavage with release of their ectodomain as a soluble form of the protein. This solubilization or shedding is a controlled process that can be upregulated by protein kinase C activation and other mechanisms, resulting in the release of ACE into the systemic circulation.

There are two isoforms of human ACE: in somatic tissues, it exists as a glycoprotein composed of a mature single large polypeptide chain of 1277 amino acids (sACE), corresponding to the extracellular domain of the full-length membrane protein. sACE is localized on the plasma membrane of endothelial and absorptive epithelial and neuroepithelial cells (Soubrier et al. 1988). The approximate concentration of sACE in human plasma is between 36 and 288 ng/ml (260–2076 pM), which is ~200-fold molar excess compared with Ang I. However, sACE has limited impact on tissue Ang-II levels, which might be more dependent on the local conversion of Ang-I to Ang-II by endothelial ACE in direct proximity to type I angiotensin II receptor (AT1R). sACE consists of two homologous catalytically active centers on each N and C domain, known as nACE and cACE (Soubrier et al. 1988) (Fig. 2). In germinal cells, ACE is synthesized as a lower molecular mass form and is thought to play a role in sperm maturation and the binding of sperm to the oviduct epithelium. Testis ACE (tACE, 701 amino acids long) is identical to cACE, except for a unique 36-residue sequence constituting its amino terminus.

Our understanding of the function of ACE has been aided by detailed molecular structures, based on high-resolution crystal structures of cACE and nACE with a clinical ACE inhibitor such as lisinopril (Fig. 3, Natesh et al. 2003; Corradi



ACE Inhibitors, Fig. 2 Schematic representation of the domain structure of sACE and tACE. *LR* linker region, *NT* N-terminus, *SR* stalk region, *TM* transmembrane region, *CT* C-terminus, *HExxH* Zinc binding histidine and catalytic glutamate conserved motif



ACE Inhibitors, Fig. 3 Structures of nACE (PDB2C6N) and cACE (PDB1O86) in complex with lisinopril. Schematic representation of the overall structures of (**a**) nACE and (**b**) cACE inhibitor complexes (loop regions are shortened for clarity), with close-up view of bound lisinopril in the active site of (**c**) nACE and (**d**) cACE. Zinc ions and water molecules are depicted as lilac and red spheres, respectively, with helices and β-strands colored in rose and dark cyan, respectively. Lisinopril and loop regions are colored magenta for nACE and dark green for cACE

et al. 2006). The catalytic site with the zinc ion is buried deep inside the groove of the molecule. It contains the conserved HEXXH zinc binding motif, formed by two histidine residues that coordinate the zinc ion together with a conserved glutamate residue. Two functionally important chloride ion binding sites, two with cACE and one with nACE, have also been identified.

Both nACE and cACE possess distinct but overlapping substrate specificity and physiologic functions, differences in chloride dependence, and distinct glycosylation patterns. Both domains catalyze the degradation of bradykinin with similar efficiencies. Knockout mouse models for these domains show similar bradykinin plasma levels as wild-type mice (Bernstein et al. 2011), suggesting that bradykinin cleavage by one domain can effectively compensate for the absence of the other domain. In contrast, cACE is the primary site for Ang-II formation and is essential and sufficient for controlling blood pressure in vivo (Fuchs et al., 2008; Bernstein et al. 2011). On the other hand, nACE is the primary site for the clearance of the tetrapeptide Ac-SDKP, a potent anti-inflammatory and antifibrotic peptide. nACE is thermally more stable than cACE, more resistant to proteolysis under denaturing conditions, and is less dependent on chloride activation as compared to cACE.

Design of First-Generation ACE Inhibitors

In 1977 Cushman et al. first described the synthesis of a series of potent inhibitors of ACE designed on the basis of an assumed mechanistic homology with carboxypeptidase A (Cushman et al. 1977). This ushered in a generation of drugs including captopril, lisinopril and enalapril, many of which are still used today in the clinic. Remarkably, these inhibitors were developed without the benefit of any detailed chemical, kinetic, or structural information on human ACE.

There are currently over 20 ACE inhibitors available which belong to three different chemical classes: thiolate compounds such as captopril; carboxylate compounds, such as enalapril; and phosphinate zinc binding group compounds, such as fosinopril (Fig. 4). The thiolate compounds exert more undesired, but also desired effects, since they additionally interact with endogenous sulfhydryl groups. Carboxylate group compounds are in general more potent than captopril. The phosphinate compounds are usually characterized by the longest duration of action.

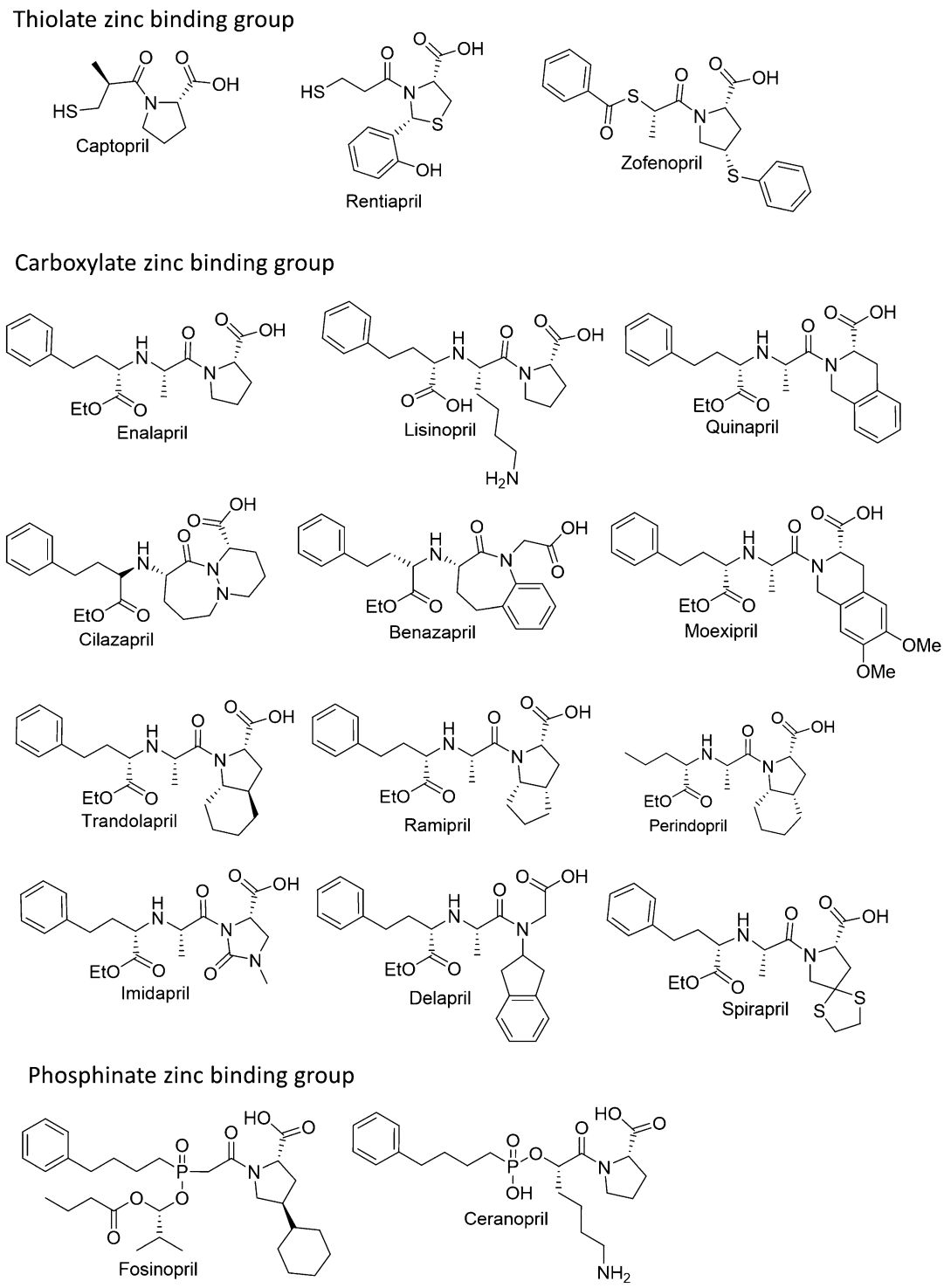
The majority of these ACE inhibitors are prodrugs which improve their oral bioavailability, but need to be converted to active compounds in the liver, kidneys, and/or intestinal tract. In effect, converting enzyme inhibitors have quite different kinetic profiles with regard to half-life, onset and duration of action, or tissue penetration. However, in general, ACE inhibitors at the doses currently used in the clinic are safe drugs with good glucose tolerance.

Adverse Effects of Present ACE Inhibitors

ACE inhibitors are commonly prescribed because they seldom cause adverse effects. Despite the success of ACE inhibitors, many patients (~20–25%) are unable to tolerate long-term treatment with current-generation ACE inhibitors because of undesired side effects. These side effects may include:

- Dry cough
- Skin rash
- Increased blood potassium levels (hyperkalemia)
- Dizziness from blood pressure decreasing too much
- Headaches
- Loss of taste

In rare cases, particularly in certain populations, ACE inhibitors can cause issues in the throat and tongue to swell (this is called angioedema) which is potentially life threatening. It is now widely accepted that these effects are caused by increased bradykinin or substance P and stimulation of the vagal fibres. Additionally, ACE inhibitors can result in lower aldosterone



ACE Inhibitors, Fig. 4 ACE inhibitors. The structures of 17 approved ACE inhibitors categorized according to their respective zinc binding groups

levels which lead to hyperkalemia in patients with poor kidney function. ACE inhibitors containing sulfhydryl groups, such as captopril, have been linked to rash, neutropenia, and nephrotic syndrome and these effects have been associated with renal insufficiency.

Chronic use of ACE inhibitors can lead to ACE inhibitor escape, where Ang-II is not reduced to normal levels. The hydrolysis of Ang-I by chymase is a possible mechanism for ACE inhibitor escape. Studies in rodents have shown that chronic ACE inhibitor treatment caused a marked increase in chymase activity in the left ventricle mediated by the bradykinin receptor (Wei et al. 2010).

Thus, some of these limitations of ACE inhibitors involving the wide substrate specificity of ACE and the ability of other enzymes to metabolize Ang II have necessitated the design of a next-generation ACE inhibitor that is specific for the cACE catalytic site of the enzyme.

ACE Inhibitors: Clinical Implications

Essential Hypertension

Essential or primary hypertension is a type of elevated blood pressure that does not have an identifiable cause, but is usually associated with poor diet, obesity, genetic factors, smoking, and lack of exercise. The first-choice drugs for the treatment of hypertension are ACE inhibitors or Ang II receptor blockers (ARBs), thiazide or thiazide-like diuretics, and calcium channel blockers. ACE inhibitors as initial therapy alone, or in combination with thiazide diuretics, were recommended in the landmark ALLHAT study (ALLHAT Officers and Coordinators for the ALLHAT Collaborative Research Group. The Antihypertensive and Lipid Lowering Therapy in Heart Attack Trial 2002). However, there were no differences between ACE inhibitor (lisinopril), diuretics (chlorthalidone), and calcium channel blockers (amlodipine) in the primary endpoint of combined fatal coronary heart disease or nonfatal or fatal myocardial infarction. In other trials (HOPE and EUROPA), ACE inhibitors ramipril and perindopril produced better outcomes than placebo in patients at increased cardiovascular risk. ACE inhibitor and ARBs have a cardioprotective effect independent of blood pressure lowering in patients at high risk for a cardiovascular event, however the attained blood pressure, not the drug used, is of primary importance in such patients.

Congestive Heart Failure and Myocardial Infarction

Several large randomized trials have shown that ACE inhibitors reduce mortality in patients with congestive heart failure (CHF). The reduction in mortality has been seen in patients with asymptomatic left ventricular dysfunction, and mainly results from a reduction in the progression of CHF, although the incidence of myocardial infarction (MI) and sudden death may also decrease. While ACE inhibitors improve the outcome in patients with systolic dysfunction, a lot of patients with hypertension develop CHF because of diastolic dysfunction related to left ventricular hypertrophy. ACE inhibitors reverse left ventricular hypertrophy in hypertensive patients, but have not been shown to reduce adverse outcomes in patients with heart failure and preserved ejection fraction.

Changes in the shape, size, and function of the heart due to cardiac injury (cardiac or ventricular remodeling) following MI is attenuated with ACE inhibitor treatment. A number of large clinical trials have demonstrated that ACE inhibitors reduce short- and long-term mortality, prevent onset of heart failure, and reduce the risk of stroke following MI. Trials such as SAVE, AIRE, and TRACE showed a 20% reduction in mortality in high-risk patients with left ventricular dysfunction or heart failure after MI. Moreover, ACE inhibitor or ARB treatment after acute MI is linked to long-term survival, regardless of underlying renal function (Evans et al. 2016).

Diabetic Nephropathy

The burden of diabetes has increased dramatically over the last three decades and the number of adults living with diabetes is projected to rise from 463 million (2019) to 700 million (2045) (https://diabetesatlas.org/en/). Over time,

diabetes can damage the blood vessels, heart, eyes, kidneys, and nerves and it is one of the leading causes of kidney disease. One of the reasons ACE inhibitors are so effective in the treatment of diabetes is their inhibition of bradykinin metabolism. Bradykinin acts as a vasodilator generating signals that lead to increased production of nitric oxide. Additionally, bradykinin increases insulin sensitivity, most notably during insulin resistance (Henrikson and Jacob 2003). ACE inhibitors also regulate the production of the vasoconstrictor Ang II and high blood pressure (more specifically, increased glomerular capillary pressure) that contribute to the acceleration of the complications associated with diabetic nephropathy. Finally, there is evidence for a local RAAS that directly affects the pancreas and can lead to decreased insulin secretion by regulating islet perfusion (Stump et al. 2006).

ACE Inhibitors and Cancer

The RAAS also plays a critical role in cancer biology and affects tumor growth and metastasis. Angiotensin II has an antiapoptotic role that involves AT1R/phosphatidylinositol 3-kinase/Akt activation and the suppression of caspase-9 and -3 activation. Ang II also reduces cell adhesion and invasion via Ang II interaction with the AT1 receptor, and this effect may be due to reduced expression of integrins. However, there have been conflicting results from epidemiological studies assessing whether ACE inhibitors and ARBs have protective effects against cancer risk. The response to ACE inhibitor and ARB therapy might depend on tumor type or stage, as studies have shown that specific cancer types, such as renal cell carcinoma, hepatocellular carcinoma, certain lung cancers, and pancreatic cancer, are more responsive to inhibition of the RAAS than others (Rosenthal and Gavras 2019). Furthermore, the high ACE activity DD genotype is associated with increased susceptibility to develop certain cancers. ACE inhibitors and ARBs have also been suggested to prevent cardiotoxicity and improve patient outcomes in different cancers when administered as coadjuvants with chemotherapy.

ACE Inhibitors as Modulators of Atherosclerosis

ACE inhibitors play an important role in the regulation of oxidative stress. Ang II stimulates NADPH oxidase in the vascular wall resulting in the production of superoxide anion leading to smooth muscle cell hypertrophy. ACE inhibitors enhance nitric oxide activity and limit the formation of hydrogen peroxide which is involved in smooth muscle cell proliferation and this retards the progression of carotid artery thickening. The benefits of the ACE inhibitor ramipril on the thickness of the carotid artery, administered with or without antioxidant vitamin E, were investigated in the SECURE clinical trial (Lonn et al. 2001). The progression of atherosclerosis was significantly less in the ramipril treated patients, but there was no significant decrease in progression rates in the vitamin E group. In the randomized Quinapril Ischemic Event Trial (QUIET, Cashin-Hemphill et al. 1999), there was no overall benefit of quinapril on coronary angiography-measured progression of atherosclerosis. This outcome could be due to the dosage or that almost 20% of the patients were simultaneously taking lipid-lowering drugs. ACE inhibitors should be considered in appropriate patients to slow atherosclerosis or prevent its development.

New Generation of Domain-Specific ACE Inhibitors

Until the molecular cloning of the ACE gene, expression of the full-length protein and nACE and cACE in isolation, ACE was assumed to comprise a polypeptide chain with a single active site and this was the basis for the design of all currently used ACE inhibitors. Animal studies using transgenic mice that express ACE with inactivated nACE or cACE have provided valuable insights regarding the in vivo functions and synergy of these structurally similar but nonidentical substrate binding sites (Fuchs et al. 2008). We now know that nACE and cACE play distinct physiological roles (with minor differences in potency and pharmacokinetic properties). Concomitantly, domain-selective ACE inhibitors as a single drug, or in combination with other inhibitors or receptor blockers, offer the promise of safer

and more effective treatment for hypertension and cardiovascular disease.

Based on what we now know, inhibitors of cACE will affect cardiovascular function similar to those of current-generation ACE inhibitors, but with improved side effect profiles largely due to decreased bradykinin levels. Moreover, we cannot rule out the possibility that cACE-selective inhibitors have a different therapeutic spectrum than current-generation inhibitors, all of which are essentially mixed nACE/cACE inhibitors. This, together with reduced side effects, will enable clear marked differentiation from current strategies.

RAAS inhibitors, for example ACE inhibitors, are among the few treatment options for slowing cardiovascular disease (CVD) end-organ damage, but the benefits seen with normal clinical doses of these drugs are modest. This has been ascribed, in part, to incomplete blockade of the RAAS. A novel approach to limiting CVD end-organ damage is increasing plasma levels of the acetylated tetrapeptide Ac-SDKP, a thymosin β4-derived tetrapeptide that reduces target-organ inflammation and fibrosis in relevant animal models. Ac-SDKP degradation is almost entirely dependent on hydrolysis by nACE, and current ACE inhibitors lead to a fivefold increase in plasma Ac-SDKP levels. It has been shown that inactivation of nACE significantly reduces bleomycin-induced lung fibrosis, implicating Ac-SDKP in the mechanism of protection. This represents a strong case for the use of nACE-selective ACE inhibitors for increasing tolerance to bleomycin in cancer therapy and treatment of fibrosing lung diseases (Li et al. 2010). Highly selective nACE inhibitors will substantially increase Ac-SDKP levels, providing cardio- and reno-protective effects, without leading to excessive RAAS inhibition. AnnACE-selective inhibitor will leave the cACE active site free to metabolize angiotensin and bradykinin peptides and will therefore not induce hypotension, hyperkalemia, and renal impairment that are observed with excessive RAAS inhibition.

There are currently no domain-selective ACE inhibitors commercially available. However, several domain-specific residues within the active site of each domain have been identified that are important for conferring domain selectivity. A number of experimental compounds with up to 3–4 orders of magnitude selectivity for one domain over the other have been identified (Dive et al. 1999; Watermeyer et al. 2008, 2010; Kröger et al. 2009; Douglas et al. 2014). In particular, a cACE-selective ACE inhibitor, lisinopril-tryptophan (Lis-W) reduces blood pressure and Ang II levels similarly to a conventional ACE inhibitor without increasing bradykinin levels in a hypertensive mouse model (Burger et al. 2014). Moreover, nACE-selective phosphinic-based inhibitors RXP407 (Dive et al. 1999) and 33RE (Douglas et al. 2014), and cACE inhibitors RXPA380 (Georgiadis et al. 2004) and carboxylate and ketone derivatives have proved to be invaluable in elucidating the molecular basis of the selectivity of nACE and cACE. The phosphinic peptides RXP407 and RXPA380 are good starting points for further development, but are not suitable for use as pharmacological lead compounds due to their poor pharmacokinetic profiles. These compounds (delivered as a single IV dose in rats) are rapidly cleared, unchanged, via renal excretion, probably because of their highly polar nature. Compounds with similar pharmacological profiles, but substantially improved PK properties, are required to deliver the benefits of a domain-selective ACE inhibitor.

Dual ACE/NEP or Vasopeptidase Inhibitors

Maximal suppression of the RAAS by ACE inhibitors and ARBs does not always lead to adequate reduction in blood pressure, and thus extensive efforts have gone into developing vasopeptidase inhibitors that target multiple structurally related peptidases within the different vasoactive systems controlling blood pressure and cardiovascular function. The natriuretic peptide system is an important peptide hormone system that influences blood pressure, fluid and electrolyte homeostasis, renal function, and cardiovascular function. Natriuretic peptides (NPs) comprise atrial, brain, and C-type natriuretic peptides (ANP, BNP, and CNP, respectively), which principally mediate natriuretic, diuretic, vasorelaxant, and antimitogenic responses largely directed to reduce blood

pressure and maintain fluid volume homeostasis (reviewed by Pandey (2005)). With the discovery and elucidation of the actions of NEP and its inhibitors, both the similarities and differences between the RAAS and the NP system have become clearer. The greater efficacy of dual ACE and NEP inhibitor therapy was initially confirmed in animal models of heart failure and cardiomyopathy, followed by the development of orally active molecules that inhibited both ACE and NEP, i.e., dual inhibitors (Fournie-Zaluski et al. 1994). The structural similarity between ACE, NEP, and endothelin converting enzyme (ECE-1) and their overlapping substrate specificity enabled the development of single molecules that target two or even three of these enzymes. Remarkably, the design of current-generation ACE inhibitors as well as vasopeptidase inhibitors that have entered clinical trials to date has been achieved with limited knowledge of the sequences and three-dimensional structures of the enzymes. Early dual inhibitors were designed rationally based on specific ACE and NEP inhibitors. Combining a P1′ benzyl group, known to be important for NEP inhibition, with a P2′ proline group, as seen in the first ACE inhibitors such as captopril, led to a series of potent mercaptoacyl dipeptides with dual inhibitory activity. Further structure–activity relationships (SAR) to optimize for in vivo activity led to conformationally restricted dipeptide mimetics and, eventually, to the development of omapatrilat. The dual ACE/NEP inhibitors were the first vasopeptidase inhibitors to enter clinical trials. They were developed to simultaneously block the ACE-mediated formation of the vasoconstrictor Ang II and the NEP-mediated degradation of vasodilator natriuretic peptides. Of the dual natriuretic peptides tested in the clinic, omapatrilat progressed the furthest but eventually failed to obtain approval after large stage III clinical trials due to an increased risk of vasodilator-mediated adverse effects. In the OVERTURE (Omapatrilat Versus Enalapril Randomized Trial of Utility in Reducing Events) trial, comparison of omapatrilat with enalapril in 5,770 patients with heart failure failed to show any superiority of omapatrilat in the primary endpoint (all cause mortality or hospitalization for heart failure) (Packer et al. 2002). Moreover, angioedema, which can obstruct the upper airways, occurred more frequently with omapatrilat (0.8%) than with enalapril (0.5%). In order to obtain a clearer understanding of the frequency of this complication, and, in particular, to compare omapatrilat with an ACE inhibitor, enalapril, the OCTAVE (Omapatrilat Cardiovascular Treatment vs. Enalapril) trial was conducted in 25,302 hypertensive subjects. As expected, omapatrilat was superior to enalapril in reducing blood pressure. However, the incidence of angioedema was again significantly higher and more severe in the subjects treated with omapatrilat (2.17%) than in those receiving lisinopril (0.68%). Based on observations of increased angioedema in the OCTAVE trial, efforts to gain approval for omapatrilat, and further clinical research on the entire class of vasopeptidase inhibitors were halted.

Despite decades of research on structure–function relationships on peptidases that metabolize vasoactive peptides, in preclinical models of cardiovascular diseases, and in clinical research, significant uncertainties remain regarding the physiology and pathophysiology of vasoactive peptide systems and their effect on cardiovascular function and diseases. It may be unnecessary to leave both nACE and NEP free to degrade bradykinin and other vasodilatory peptides. Since ACE is the primary bradykinin-metabolizing enzyme, nACE may compensate sufficiently for cACE in preventing the buildup of dangerous levels of bradykinin. Consequently, dual cACE–selective/NEP inhibitors are likely to offer a promising alternative for the treatment of hypertension and cardiovascular disease by potentiating natriuretic peptide levels in addition to blocking Ang II formation. Important insights from the crystal structures of cACE and NEP in complex with different inhibitors (Sharma et al. 2020) pave the way for the development of new leads with similar efficacy to omapatrilat, but with improved side effect profiles.

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Acetylcholine Acetylhydrolase

► Cholinesterases

Acetylcholine Hydrolase

► Cholinesterases

Acetylcholinesterase (EC 3.1.1.7) (AChE)

► Cholinesterases

Acetylthiocholinesterase

► Cholinesterases

Acetyl-β-Methylcholinesterase

► Cholinesterases

Acid-Sensing Ion Channels

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Definition

Acid-sensing ion channels (ASICs) are non-voltage-gated sodium channels transiently activated by extracellular protons, selective for sodium, and belong to the epithelial sodium channel (ENaC)/degenerin (DEG) family of ion channels. The members of this ion channel superfamily share a similar topology with short intracellular amino and carboxy termini and two membrane-spanning domains connected by a large extracellular domain. The ASIC ion channel group consists of four genes encoding at least six ASIC subunits including 1a, 1b, 2a, 2b, 3, and 4.

Basic Characteristics

Channel family, structural organization and function

Channel Family

The ENaC/DEG superfamily of cation channels encompasses more than 60 members including the ASICs (Wemmie et al. 2006, 2013; Kellenberger and Schild 2015, 2002; Stockand et al. 2008). ASICs have been cloned in the mid-1990s based on sequence homology to ENaC and DEGs. Extracellular acidification opens ASICs only transiently, because of rapid desensitization, indicating that ASICs can exist in the three functional states closed, open, and desensitized (Waldmann et al. 1997; Carattino 2011; Kellenberger and Schild 2015) (Fig. 1c). ASICs are expressed in all vertebrates and responsible for acid-evoked currents in many neurons of the nervous system.

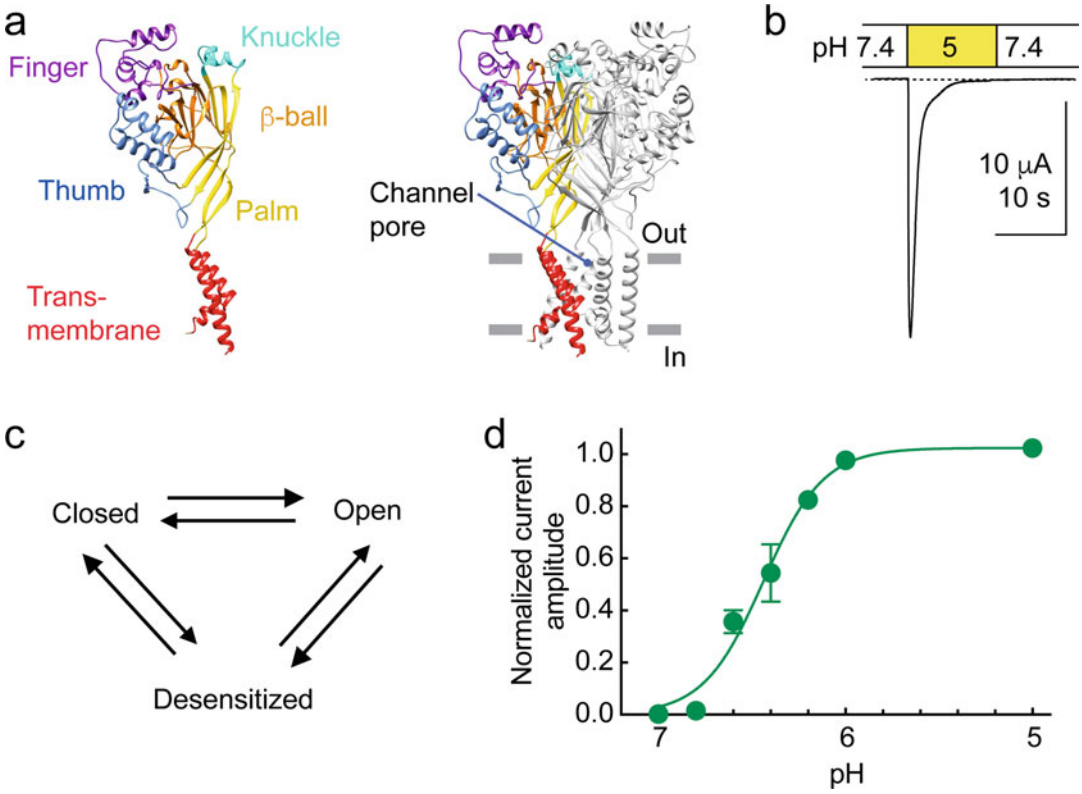
Structural Organization

The ASIC group consists of four ASIC genes resulting in six isoforms in rodents, termed ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3 and ASIC4 with a length of 500 to 560 amino acids (Kellenberger and Schild 2015). The members of ENaC/DEG superfamily share the same topology characterized by a large extracellular domain (~370 amino acids), short intracellular amino and carboxy termini (~35 to 90 amino acids), and two transmembrane regions (~20 amino acids). Individual ASIC subunits assemble to form homo- or heterotrimeric channels, except for ASIC2b and ASIC4. ASIC2b is not functional as homomeric channel, but forms functional

heteromeric channels together with other ASIC subunits. So far, no role for ASIC4 in homo- or heteromeric channels has been observed. In 2007, Jasti et al. described the first crystal structure of one of the ASIC proteins, the chicken ASIC1a channel (Jasti et al. 2007) which shares 90% sequence identity with human ASIC1a. This structure showed that the channel is formed by three subunits. The shape of the single subunit was compared to that of a hand, and sub-domains were named accordingly (Fig. 1a). Subsequently, structures obtained from chicken ASIC1a representing likely the closed and the toxin-opened conformation were published (Baconguis et al. 2014; Yoder et al. 2018).

Basic Functional Properties

ASICs are transiently activated by a rapid drop in extracellular pH (Fig. 1b). Protons are the main physiological activators of ASICs. As mentioned above, ASICs exist in three different functional states termed closed, open, and desensitized (Fig. 1c). The transient peak current lasts hundreds of milliseconds and is terminated by desensitization. In ASIC3 and some heteromeric ASICs, the desensitization is not complete and allows a small sustained current. Return to physiological pH 7.4 brings the channel into the closed state, allowing subsequent activation by protons. The molecular details of the activation of ASICs are currently not completely understood. Several studies indicate that protonation events close to



Acid-Sensing Ion Channels, Fig. 1 ASIC structure and function. (**a**) Structural model of a single ASIC subunit (left) and of a channel trimer (right), based on the crystal structure of chicken ASIC1a opened by mit-toxin (Baconguis et al. 2014). The subdomains are color-coded in one subunit and labeled. (**b**) Typical ASIC1a current trace, obtained with two-electrode voltage-clamp to −60 mV of a *Xenopus* oocytes expressing ASIC1a. The extracellular pH was changed as indicated on the top of the Fig. (**c**) Kinetic scheme of ASIC function. Upon a pH change from 7.4 to 5.0, the channels pass from the closed to the open and then into the desensitized state. (**d**) pH – current response curve of ASIC1a. The normalized current amplitude is plotted as a function of the pH

the thumb (the "acidic pocket"), in the palm, and in the extracellular pore entry contribute to pore opening (see Kellenberger and Schild 2015).

ASIC1a opens at pH 7.0 and reaches its half-maximal activation (pH_{50}) at pH 6.2–6.6 (Fig. 1d). The pH_{50} values of other ASICs are 5.9–6.3 (ASIC1b), 4.0–4.9 (ASIC2a), and 6.4–6.7 (ASIC3) (Waldmann et al. 1999; Wemmie et al. 2013; Kellenberger and Schild 2015). Many endogenous and exogenous compounds were shown to modulate ASIC function (reviewed in Wemmie et al. 2013; Kellenberger and Schild 2015), as discussed below.

Physiological Roles

ASICs are expressed in all vertebrates; even organisms with rudimentary nervous system have been shown to express at least one of the ASICs. ASICs contribute to acid-evoked currents in many neurons of both the central and peripheral nervous systems (CNS and PNS). ASIC1a, ASIC2a, and ASIC2b are expressed throughout the CNS and PNS (Wemmie et al. 2013; Kellenberger and Schild 2015). In the CNS, ASIC1a, ASIC2a, and ASIC2b show their highest expression in the hippocampus, the cortex, the cerebellum, the olfactory bulb, and the amygdala. ASIC1b and ASIC3 expression is restricted to the PNS (Wemmie et al. 2013). ASIC3 is most abundant in dorsal root ganglia. So far, ASIC4 has not been found in the PNS and shows a more dispersed expression compared to other ASICs in the CNS (rev. in Kellenberger and Schild 2015; Wemmie et al. 2013).

Local pH changes affect the function of almost any protein, including ion channels, and influence therefore many cellular processes. The release of the content of the synaptic vesicles that are acidic leads to a rapid lowering of the pH in the synaptic cleft. Tissue acidification occurs in situations such as inflammation or ischemia (rev. in Boscardin et al. 2016).

The activation of ASICs allows Na^+ entry into neurons, inducing depolarization of the neuronal membrane, generating action potentials, and, thus, exciting the neurons. The physiological and pathological roles of ASICs, demonstrated with mouse models deficient of specific ASIC isoforms or with pharmacological approaches using small molecule inhibitors or ASIC-specific toxins, include fear conditioning and anxiety, retinal function, neurodegeneration after ischemic stroke, synaptic plasticity, learning, and memory (Wemmie et al. 2013; Kellenberger and Schild 2015). In addition, studies with mice and rats showed evidence for a role of ASICs in the PNS (mostly ASIC3) and of ASICs of the CNS (mostly ASIC1a) in the sensation of different forms of pain. ASICs also have been proposed to play a role in mechanosensation. While such a role has been demonstrated on the level of the animal, it has so far not been possible to directly show activation of ASICs by mechanical stimuli in cell systems, suggesting that ASICs are part of a mechanotransduction complex that may not be intact in dissociated cells (rev. in Wemmie et al. 2013 and Kellenberger and Schild 2015).

ASIC Regulators

Ions and Polyamines

The activity of ASICs can be modulated by both divalent and trivalent metal ions, including Ni^{2+}, Cu^{2+}, Ca^{2+}, and Zn^{2+} ions (rev. in Wemmie et al. 2013; Kellenberger and Schild 2015). Ca^{2+} functions as allosteric modulator of ASIC pH dependence, likely by competing with protons for binding sites, and as a channel blocker (Paukert et al. 2004; Babini et al. 2002).

Depending on the ASIC type of channel, Zn^{2+} can potentiate or inhibit the channel. Zn^{2+} has been reported to potentiate proton-induced currents mediated by homomeric ASIC2a and heteromeric ASIC2a-containing channels at micromolar concentrations. In contrast, ASIC1a, ASIC1b, and ASIC3 are inhibited by Zn^{2+}; nanomolar concentrations inhibit homomeric ASIC1a channels, whereas ASIC1b and ASIC3 are only inhibited by micromolar concentrations of Zn^{2+} (rev. in Wemmie et al. 2013; Kellenberger and Schild 2015; Baron and Lingueglia 2015).

The endogenous polyamine spermine modulates proton-evoked ASIC1- and ASIC1a/ASIC2a-mediated currents (Babini et al. 2002; Baron and Lingueglia 2015).

Neuropeptides

FANaC, a member of the ENaC/DEG ion channel family, is activated by the neuropeptide FMRFamide (Phe-Met-Arg-Phe amide). FMRFamide has also been shown to modulate the function of ASIC1 and ASIC3 by potentiating their responses to acidification. The action on ASICs of FMRFamide and related neuropeptides involves amino acid residues in the palm and a linker between the palm and the thumb (Vick and Askwith 2015; Bargeton et al. 2019). FMRFamide and related neuropeptides increase ASIC activity in several ways, including an acidic shift of the pH dependence of desensitization, a slowing of the current decay, and the generation of a sustained current (Fig. 2).

Proteases

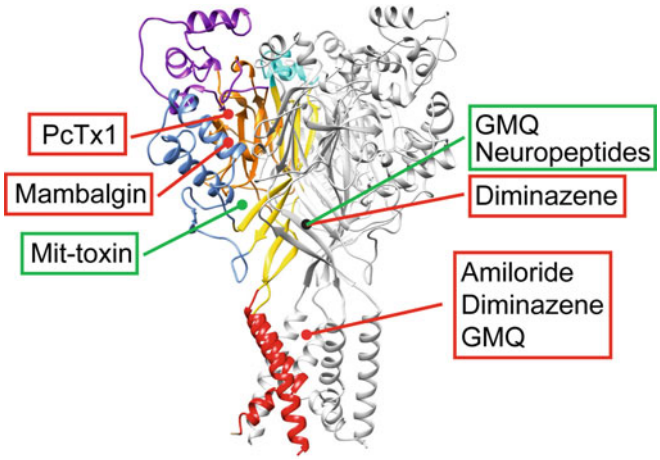
Channel regulation by different types of proteases (mainly serine proteases) is a topic that has been intensively described for the ENaC. There is also evidence for proteolytic regulation of ASICs. The serine proteases trypsin and matriptase led to an acidic shift of the pH dependence of the peak current of ASIC1a, but not ASIC1b. Furthermore, proteolytic cleavage of ASIC1a, but not ASIC2a, by tissue kallikrein was reported (Su et al. 2011). The relevant protease cleavage sites involved in this process are located in the finger domain of ASIC1a for trypsin and matriptase (Vukicevic et al. 2006; Clark et al. 2010), similar to the cleavage sites in α and γENaC. This points to a regulatory role of the finger domain for ASIC activity.

Protein Kinases

The activation of the tropomyosin-related kinase B (TrkB) has been shown to increase ASIC1a activity and its surface expression via the phosphatidylinositol 3-kinase (PI3-K)/protein kinase B (PKB or Akt) pathway. Moreover, the regulation of ASIC function by other protein kinases has been described in many studies (discussed in Wemmie et al. 2013; Kellenberger and Schild 2015).

Amphiphilic Substances

Bile acids have been shown to activate two members of the ENaC/DEG family of ion channels, the bile acid-sensitive ion channel (BASIC) and ENaC. It is conceivable that bile acids may play a role in ASIC regulation under physiological and pathophysiological conditions as increased ASIC activity in sensory neurons of the gastrointestinal tract may contribute to hyperalgesia and colonic hypersensitivity observed in patients with irritable bowel syndrome with increased bile acid concentrations in their gut. Indeed, it was reported that the function of ASIC1a heterologously expressed in *Xenopus laevis* oocytes is modulated by bile acids and also other amphiphilic substances like the detergent maltoside (Ilyaskin et al. 2017). In the presence of these bile acids, the whole-cell currents elicited by acidic pH were significantly



Acid-Sensing Ion Channels, Fig. 2 Sites of drug action. The figure shows the sites of action of toxins and small molecules that inhibit or modulate ASICs. Green lines mark positive modulation, while red lines indicate inhibition. Note that for mit-toxin, the interaction region with ASIC is larger than indicated and stretches over the whole thumb and up to the finger

increased. Molecular docking predicted binding of bile acids to the pore region near the DEG site (G433) in the open conformation of the channel.

Lipids

ASIC function can also be regulated by lipids, e.g., arachidonic acid (AA) (Kellenberger and Schild 2015). It has been proposed that AA acts directly on ASICs to increase channel activity. Recently it has been shown that AA together with the endogenous lipid lysophosphatidylcholine can activate ASIC3 in the absence of any extracellular acidification (Marra et al. 2016).

GMQ and Related Compounds

The synthetic compound 2-guanidine-4-methylquinazoline (GMQ) is an exogenous modulator of ASICs. GMQ has been described as first non-proton activator of ASICs that depends on extracellular Ca^{2+} and exclusively activates ASIC3 (Yu et al. 2010). Subsequent studies showed that GMQ affects the pH dependence of not only ASIC3 but also ASIC1a, ASIC1b, and ASIC2a, leading to activation of ASIC3, and a decrease of the H^+-activated current amplitude of other ASICs (Alijevic and Kellenberger 2012). Based on their structural similarity with GMQ, two natural polyamines, the arginine metabolite agmatine and its analog arcaine, have been proposed to be endogenous non-proton ligands for ASIC3. These two compounds lead to ASIC3 activation similar to GMQ however to a smaller extent. Agmatine also activated heteromeric ASIC3/ASIC1b channels, extending its potential physiological relevance (rev. in Kellenberger and Schild 2015; Baron and Lingueglia 2015).

Drugs

Toxins

Several peptide toxins have been identified that bind to ASICs with nano- or micromolar affinity and act as gating modifiers, inhibitors, or activators. The most important known ASIC toxins comprise psalmotoxin1 (PcTx1) from the venom of the spider *Psalmopoeus cambridgei*, Hi1a of the Australian funnel-web spider *Hadronyche infensa*, APETx2 of the sea anemone *Anthopleura elegantissima*, mambalgin of the black mamba (*Dendroaspis polylepis polylepis*), and mit-toxin of the Texas coral snake (*Micrurus tener tener*) (Baron and Lingueglia 2015; Chassagnon et al. 2017). These are peptide toxins composed of ≥40 amino acid residues. PcTx1 and Hi1a are structurally related, with Hi1a containing two tandem PcTx1-like sequences. From co-crystallization and mutagenesis studies, it is known that mit-toxin, PcTx1, and mambalgin bind to the thumb – acidic pocket region of ASIC1a (rev. in Kellenberger and Schild 2015; Baron and Lingueglia 2015). Mit-toxin activates ASIC1a, ASIC1b, and ASIC3 and potentiates ASIC2a opening by protons. PcTx1 inhibits mostly ASIC1a homomers, and mambalgin inhibits ASIC1a and ASIC1b homomers and ASIC1a-containing heteromers, while APETx2 inhibits ASIC3 homomers and ASIC3-containing heteromers. Except for APETx2, which inhibits besides ASICs also voltage-gated Na^+ channels (Peigneur et al. 2012; Blanchard et al. 2012), these toxins appear to be specific for ASICs. PcTx1 and mambalgin exert their inhibition by changing the pH dependence of ASICs. PcTx1 shifts the pH dependence of desensitization to more alkaline values, leading to desensitization of ASIC1a at physiological pH and preventing channel opening by subsequent acidification. Mambalgin shifts the pH dependence of activation to more acidic values, thereby leading to a smaller response to acidification. In contrast, the effects of Hi1a and APETx2 appear to be pH-independent. The ASIC toxins have been instrumental in defining some functional roles of ASICs in animal studies (Wemmie et al. 2013).

Small Molecule Inhibitors

Currently there are no ASIC-selective, high affinity small molecule inhibitors available. Amiloride and its derivative benzamil are pore blockers of ENaC/DEG channels. While amiloride inhibits ENaC with an IC_{50} of ~100 nM, its IC_{50} of ASIC peak currents is ≥10 μM, and it does not inhibit sustained ASIC currents. At concentrations ≥10 μM, amiloride also inhibits several transporters (Wemmie et al. 2013; Kellenberger

and Schild 2015). In spite of these limitations, amiloride has been used in experimental studies in humans. A-317567 inhibits the transient and sustained ASIC current fractions in dorsal root ganglion neurons with an IC_{50} of 2–10 μM and was shown to be more potent in treating pain than amiloride in animal models (rev. in Kellenberger and Schild 2015; Baron and Lingueglia 2015). Nafamostat mesylate is a synthetic protease inhibitor with potential use as anticoagulant or antitumor agent. It has been shown to inhibit ASIC currents, including the sustained current component of ASIC3, with IC_{50} values of 2–70 μM (rev. in Kellenberger and Schild 2015; Baron and Lingueglia 2015). The analysis of a number of anti-protozoal diarylamidines identified several compounds that inhibit ASICs with IC_{50} values of 0.3–40 μM. Among these, diminazene was further characterized in several studies and shown to inhibit ASICs in part by a pore block (Schmidt et al. 2017). Diminazene inhibits the related BASIC channel with similar affinity. Several nonsteroidal anti-inflammatory drugs (NSAIDs) were shown to inhibit ASICs, with IC_{50} values in the micromolar range, requiring thus much higher concentrations than clinically used. ASIC mRNA levels in sensory neurons are upregulated in inflammation. Interestingly, several NSAIDs, including aspirin, diclofenac, and ibuprofen, prevented this upregulation at therapeutic doses (Voilley et al. 2001). There have been several attempts in developing more specific and potent ASIC inhibitors, resulting in the identification of inhibitors with sub-micromolar affinities (reviewed in Kellenberger and Schild 2015; Baron and Lingueglia 2015; Rash 2017). However, so far, these compounds have not been further developed.

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Acylcholine Acylhydrolase

▶ Cholinesterases

Adaptor Proteins

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Synonyms

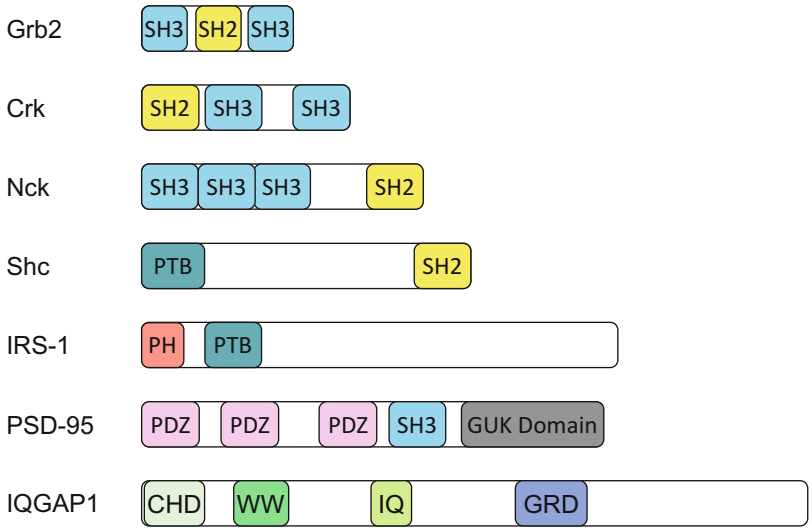
Anchoring protein; Docking protein; Scaffold

Definition

Adaptor proteins are multi-domain proteins (Fig. 1) that interact with components of signaling pathways. As a consequence of these interactions, adaptor proteins are able to regulate signaling events within the cell, providing spatiotemporal control and specificity and influencing how a cell responds to a particular stimulus.

Basic Mechanisms

Adaptor proteins function by simultaneously interacting with multiple components of a signaling pathway (Fig. 2). In order to be able to bind to more than one target protein at the same time, adaptor proteins contain at least two specific protein-protein interaction domains. These domains recognize specific motifs in the target proteins and can act



Adaptor Proteins, Fig. 1 Adaptor protein domains. A scheme of the domain structures of some well-characterized adaptor proteins is shown. Descriptions of domain characteristics are in main text except GUK domain, guanylate **kinase** domain; CHD, calponin homology domain; IQ, isoleucine/glutamine (IQ) domain; GRD, GAP-related domain. For clarity, not all domains contained within these proteins are shown

completely independently, like beads on a string, or interact with another domain within the same molecule. Such intramolecular interactions can regulate the ability of each domain to bind to its target (Pawson 2007).

Adaptor Protein Function

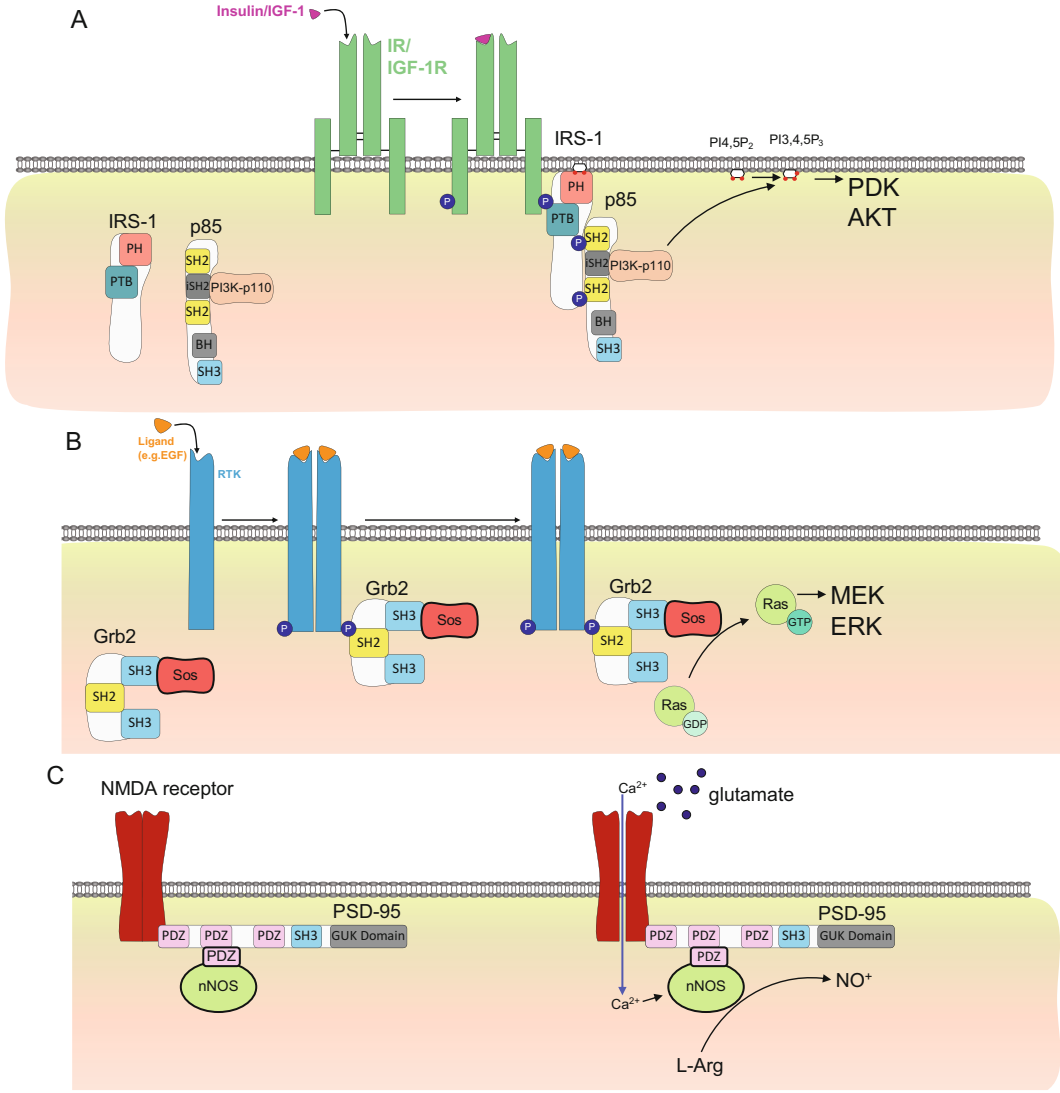
In their simplest form, adaptor proteins perform a straightforward function: the formation of multiprotein complexes. However, they often provide more than a static scaffold support for signaling components, instead enabling dynamic regulation to control propagation of pathways and networks. Consequently, adaptor proteins can act as signaling modules, directing propagation of the pathway, influencing downstream events, and even modifying the cellular response to a specific stimulus. Some of the different roles of adaptor proteins are described below. These functions are not mutually exclusive, and more than one of these roles can be performed by a particular adaptor protein at one time.

Assembly of Signaling Complexes

This is perhaps the simplest function provided by adaptor proteins and involves bringing together individual components of a pathway. These complexes promote propagation, and often amplification, of the signal. Examples of this are found in the **MAP kinase** pathway, where the adaptor proteins IQGAP1 and kinase suppressor of Ras-1 (KSR-1) bind to multiple **kinases**. Consequently, they enable efficient signaling from one **kinase** to the next (Brown and Sacks 2009; Langeberg and Scott 2015).

Spatial Regulation

Adaptor proteins can assemble the complexes in particular subcellular compartments. For example, following activation of a ligand-bound receptor, adaptor proteins can localize downstream signaling targets to the intracellular domains of the receptor (Fig. 2a, b), thereby facilitating propagation of the signal through the cell. Note that signaling events occur in all cell organelles and subcellular compartments and adaptor proteins appear to function throughout the cell. Through their ability to localize signaling targets to specific subcellular compartments, adaptor proteins not only facilitate signaling events but also influence how these signals are interpreted by the cell and consequently the cellular response. For example, in the **MAP kinase** pathway, signaling from the plasma membrane activates different signaling components to those activated when the signal originates from the Golgi (Brown and Sacks 2009).



Adaptor Proteins, Fig. 2 Examples of adaptor protein function. Selected examples of how adaptor proteins facilitate signaling are shown. (**a**) The insulin receptor contains an intrinsic tyrosine **kinase**. Following stimulation by insulin binding, the intracellular domains of the transmembrane receptor autophosphorylate (P) on key tyrosine residues. The adaptor protein IRS-1 contains a PH domain and PTB domain that recruit IRS-1 to the activated receptors. The receptor then catalyzes tyrosine phosphorylation of IRS-1 on its C-tail. These phosphorylated tyrosines are recognized by proteins containing **SH2** domains, such as the p85 regulatory subunit of phosphatidylinositol 3-**kinase** (PI3K). PI3K signaling can then generate lipid signals through the PDK-1 and AKT kinases to promote cell survival. (**b**) Analogous to the insulin receptor, many growth factor receptors contain an intrinsic tyrosine kinase that catalyzes receptor autophosphorylation. The **SH2** domain of the adaptor protein **Grb-2** associates with the phosphorylated tyrosine residues on the receptor, while the SH3 domain binds to the **Ras** activator **Sos**. Consequently, **Sos** is localized to the receptor at the plasma membrane where it activates the small GTPase **Ras**, which results in activation of the **mitogen-activated protein (MAP) kinase** cascade. By this mechanism, **Grb-2** facilitates the transduction of an extracellular stimulus to an intracellular signaling pathway. (**c**) The adaptor protein **PSD-95** associates through one of its three **PDZ** domains with the N-methyl-D-aspartic acid (NMDA) receptor. Another **PDZ** domain associates with a **PDZ** domain from neuronal nitric oxide synthase (nNOS). Through its interaction with **PSD-95**, nNOS is localized to the NMDA receptor. Stimulation of the NMDA receptor by glutamate induces an influx of calcium, which activates nNOS, resulting in the production of nitric oxide

Temporal Regulation

The duration of signaling influences how a cell responds to a particular stimulus. For example, brief activation of the **MAP kinase** cascade in the neuronal cell line, PC12, results in proliferation, while sustained **MAP kinase** signaling promotes differentiation of PC12 cells. Adaptor proteins are able to regulate the time course of signaling events and therefore the cellular outcome (Brown and Sacks 2009).

Activation of Signaling Components

The binding of an adaptor protein may activate the target molecule. An example of this is the adaptor protein insulin receptor substrate-1 (IRS-1), which is activated during insulin signaling. Following binding by insulin, the insulin receptor (which contains an intrinsic tyrosine kinase) catalyzes receptor autophosphorylation. The phosphotyrosine residues on the receptor are recognized by the PTB domain of IRS-1, and the activated receptor catalyzes phosphorylation of selected tyrosine residues on IRS-1 (Fig. 2a). A subset of these phosphorylated tyrosines act as docking sites for SH2 (see below) domains on p85, the regulatory subunit of phosphoinositide 3-**kinase (PI3K)**. As a consequence of the interaction between IRS-1 and p85, phosphoinositide 3-**kinase** is activated.

Inactivation of Kinases

A-kinase anchoring proteins (AKAP) are a well-studied class of adaptor proteins that regulate protein kinase A (PKA) signaling. PKA is activated in response to cyclic-adenosine monophosphate (cAMP). **AKAP**s provide spatiotemporal specificity for PKA activity by forming multi-protein complexes and localizing them to the appropriate subcellular compartment. These complexes contain kinases and phosphatases, as well as phosphodiesterases, which catalyze the hydrolysis of cAMP to AMP. Therefore **AKAPs** anchor both positive and negative regulators of PKA signaling (Langeberg and Scott 2015).

Sequestering Signaling Components

Specificity in signal transduction is also achieved by selective separation of signaling components. By associating with specific proteins and bringing them together, adaptor proteins can determine how the pathway propagates through the cell. This is important for **Ras** signaling, for example. **Ras**, which can be activated in response to numerous growth factors and signaling cues, regulates multiple pathways. Therefore, by sequestering specific proteins, adaptor proteins ensure that a particular stimulus activates only the appropriate pathway(s). Sequestration of proteins also enables adaptor proteins to negatively regulate signaling. An example of this can be found in T-cell activation. The adaptor protein, c-Cbl, sequesters the tyrosine **kinase**, Syk, preventing recruitment to IgE receptors. Consequently, T-cell activation is attenuated.

Domains

In order to bind to target proteins, adaptor proteins contain protein-protein interaction domains which recognize specific target motifs (Fig. 1). Through combinations of these domains, adaptor protein can interact with multiple target proteins, potentially forming large signaling complexes. While many different protein binding domains have been identified, adaptor proteins often utilize the well-characterized domains described below.

Src Homology 2 (SH2) and Src Homology 3 (SH3)

SH2 domains are common protein modules that recognize short motifs containing a tyrosine residue that has been phosphorylated by a tyrosine **kinase**. Other residues outside of this motif provide specificity to determine which **SH2** domain-containing protein associates with that particular site. **SH3** domains bind to polyproline motifs with the sequence PXXP. These two domains are often found independently in adaptor proteins such as **Src** homologous and collagen (Shc) and **PSD-95** (Fig. 1). However, the SH2/SH3 adaptors **Crk**, **Nck**, and **Grb-2** contain both **SH2** and **SH3** domains (Fig. 1). In these proteins, the **SH2** domain recognizes a binding motif in activated transmembrane receptors, while the **SH3** recognizes other signaling proteins, such as the **Ras** activator **Sos** (Fig. 2b). Consequently, these

proteins couple an activated receptor to signaling components downstream, thereby facilitating signal propagation.

Postsynaptic Density Protein/Drosophila Disk Large Tumor Suppressor/Zonula Occludens-1 Protein (PDZ)

PDZ domains bind to short peptide motifs at the C-terminal end of target proteins and are particularly important in spatial organization of receptors and ion channels. Many adaptor proteins contain multiple **PDZ** domains, which have important implications for their functions. By interacting with subunits from different receptors, adaptor proteins containing multiple **PDZ** domains can promote formation of homogenous receptor complexes. This clustering can be further enhanced through the ability of **PDZ** domains to self-associate, enabling oligomerization of the adaptor proteins. Conversely, individual **PDZ** domains can associate with different target proteins, enabling the formation of large heterogeneous complexes of proteins. Examples of **PDZ**-containing adaptor proteins include **PSD-95** (Fig. 1), **LIM kinase**, and membrane-associated guanylate kinases (MAGUKs) (Sugiura et al. 2011).

Phosphotyrosine Binding (PTB)

PTB domains recognize small peptides containing a phosphotyrosine, usually with the consensus sequence, NPXpY. Some **PTB**-containing proteins, such as Numb, are able to bind to the consensus peptide in the absence of phosphorylated tyrosine, suggesting phosphotyrosine is dispensable for the function of certain **PTB** domains. Hydrophobic residues N-terminal to the phosphotyrosine provide some specificity of target and distinction from **SH2** domains. **PTB** domains appear to be particularly important in docking proteins to activated receptors. Examples of **PTB**-containing proteins include Numb, IRS-1, and Shc (Fig. 1) (Wagner et al. 2013).

WW

WW domains (named after the one-letter abbreviation for the amino acid tryptophan) are small regions of around 30 residues, which, like **SH3** domains, bind to polyproline sequences. These sequences often contain the consensus sequence PPXY or PPLP. Examples of proteins that contain **WW** domains include the transcription coactivator YAP, Nedd4 E3 ubiquitin ligase, and IQGAP1 (Fig. 1).

Pleckstrin Homology (PH)

PH domains consist of about 120 amino acid residues. They do not interact with other proteins, but associate with specific phosphoinositides. Consequently, **PH** domains appear to be important for localizing target proteins to cellular membranes. Examples of **PH** domain-containing proteins include phospholipase C, IRS-1 (Fig. 1), and p120/RasGAP.

Regulation of Adaptor Proteins

The interactions between adaptor proteins and their targets are often regulated (see below). By these mechanisms, specific signals are able to control which adaptor proteins, and consequently which target proteins, are recruited to a particular signaling complex. Common methods of adaptor protein regulation are described here.

Phosphorylation

Phosphorylation is a common method of regulation. As described above, **SH2** domains bind to phosphorylated tyrosine residues. Conversely, phosphorylation of serines and threonines proximal to **SH3** and **PDZ** domains uncouples them from their target motifs. Therefore, modulation of protein **kinase** activity in cells regulates interactions between adaptor proteins and their target proteins.

Chemical Regulation

Cellular messengers, such as calcium, also regulate adaptor protein function. The adaptor protein IQGAP1 binds multiple members of the **MAP kinase** cascade, including B-Raf, MAPK/extracellular-regulated **kinase kinase** (MEK), and extracellular-regulated **kinase** (ERK). Binding of calcium to its effector, calmodulin, increases the association of calmodulin

with IQGAP1 and inhibits the interaction of IQGAP1 with B-Raf.

Intramolecular Interactions

Another way in which the function of adaptor proteins is regulated is through modulation of intramolecular interactions. Often one domain will bind to another domain in the same adaptor molecule, preventing further interactions with other proteins. An example of this is found in the adaptor protein, **Crk**. **Crk** contains an N-terminal **SH2** domain, followed by two **SH3** domains (Fig. 1). The **SH3** domains are separated by a linker region containing a tyrosine residue. When Crk is phosphorylated by a tyrosine **kinase**, such as Abl, the intrinsic **SH2** domain binds to the phosphotyrosine, attenuating **Crk** signaling activity (Kumar et al. 2014).

Conformational Changes

Changing the conformation of adaptor proteins can also alter their function. p130Cas, which is a target for **Src kinase**, serves as an example. The central region of p130Cas contains multiple **Src** phosphorylation sites that, when phosphorylated, promote the binding and recruitment of other targets proteins. Under resting conditions, these phosphorylation sites are hidden due to the folded conformation of p130Cas. When mechanical stress is applied to the cell, this central region is stretched exposing the **Src** phosphorylation sites. This alteration in conformation results in increased phosphorylation of p130Cas and recruitment of the adaptor protein **Crk**II, leading to activation of the Ras family GTPase, Rap1.

Pharmacological Intervention

Adaptor proteins are attractive targets for the design of new therapies for diseases in which signaling pathways are deregulated. For example, many cancers and inflammatory disorders display hyperactive **MAP kinase** signaling. This may be due to increased growth factor/cytokine stimulation or increased intracellular **kinase** activity. Adaptor proteins play well-established roles in controlling **MAP kinase** activation and so provide a potential target for novel therapies. For example, **Grb-2** has been the focus of research to identify compounds which target either its **SH2** or **SH3** domains. The importance of **Grb-2** in the activation of **Ras**, and therefore stimulation of the **MAP kinase** pathway, suggests that compounds that inhibit **Grb-2** function could potentially be useful in the treatment of many cancers.

At present no compounds targeting adaptor proteins have been approved for clinical use.

Therapies for treatment of diseases caused by hyperactive intracellular signaling may utilize inhibitors targeted against a specific **kinase**. However, this approach has several problems, such as inhibition of other nontarget **kinases** and inhibition of signaling events not related to the disease. Consequently, the use of **kinase** inhibitors is not always effective and often produces side effects. However, as described above, adaptor proteins provide specificity to signaling pathways. Therefore, disrupting adaptor protein function may allow more specific targeting of the aberrant cellular response which contributes to the disease, such as cell proliferation or cytokine production. By selectively interfering with adaptor interactions, these agents are likely to have fewer side effects and increased efficacy.

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ADC

► Antibody Drug Conjugates

ADCs

► Antibody Drug Conjugates

Adenosine Receptors

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Synonyms

A receptors; Ado receptors; P1 receptors

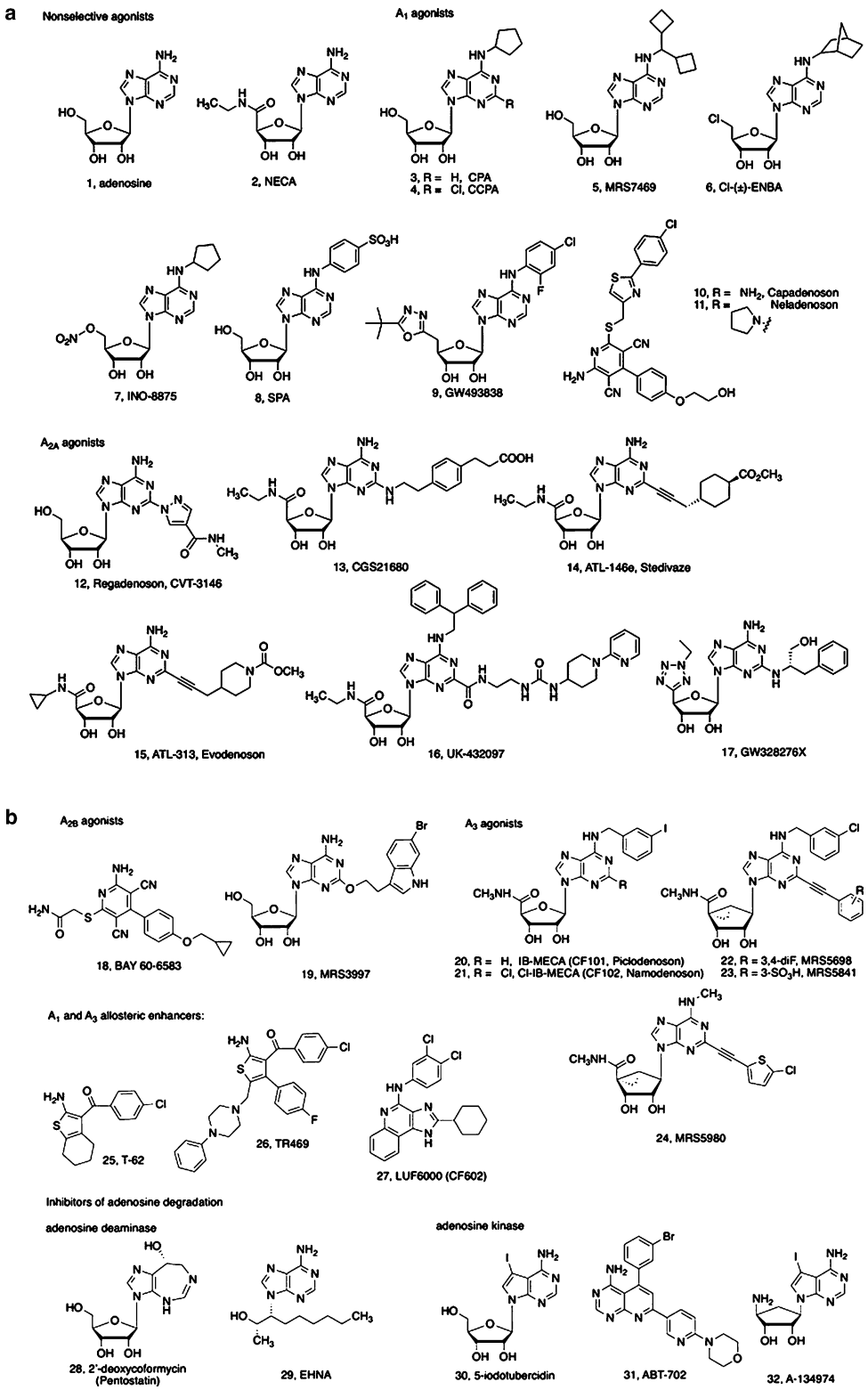
Definition

Extracellular adenosine acts through a class of G protein-coupled receptors (GPCRs), defined across mammalian species as A_1, A_{2A}, A_{2B}, and A_3ARs (adenosine receptors). Adenosine has a cytoprotective and allostatic role in the body, both in the periphery and in the central nervous system. Following binding of adenosine, or another naturally occurring agonist, the receptor interacts with heterotrimeric G proteins and β-arrestin to stimulate or inhibit downstream signaling cascades, or to induce receptor desensitization.

Basic Characteristics

The purine nucleoside adenosine, as a natural local modulator of cell action, increases the ratio of oxygen supply to demand, suppresses excessive inflammation, and promotes tissue protection against apoptosis or ischemic damage. ARs also have effects on proliferation and differentiation. Nearly every cell type in the body expresses one or more of these receptors, indicating the fundamental nature of adenosine as a cytoprotective mediator. A study of a mouse line in which all four ARs were knocked out indicated that ARs are not generally required for homeostasis of an animal, rather for allostasis (Xiao et al. 2019). In the cardiovascular system, ARs present on myocardial, vascular, and inflammatory cells respond to the stress of ischemia and other damaging conditions. Adenosine has been termed a "retaliatory" metabolite of ATP, in that its extracellular level rises in response to excessive energy demand in relation to the available energy supply, i.e., utilization of intracellular ATP. Adenosine aids in correcting this imbalance between energy supply and demand by causing vasodilation, increasing vascular integrity and angiogenesis, and counteracting the lethal effects of prolonged ischemia on cardiac myocytes. In the brain, activation of the presynaptic A_1AR suppresses the acute release of excitatory neurotransmitters and counteracts excitotoxicity by both presynaptic and postsynaptic mechanisms. However, in the aging brain, A_{2A}AR antagonists have cerebroprotective properties (Canas et al. 2018).

The affinity and selectivity of nucleosides as adenosine agonists has been extensively explored, resulting in thousands of selective agonists, of which many are useful in pharmacological studies (Müller and Jacobson 2011). Selected agonists of A_1, A_{2A}, and A_3 ARs are shown in Fig. 1. Highly selective antagonists of A_1, A_{2A}, A_{2B}, and A_3 ARs have been reported both as research tools and as experimental therapeutic agents (Fig. 2) (Chen et al. 2013; Jacobson et al. 2019). Caffeine and other naturally occurring alkylxanthines act as nonselective competitive antagonists of adenosine with respect to at least three of the four subtypes of the ARs. In fact, antagonism of ARs is the most likely mechanism of action of ingested caffeine. These simple xanthine antagonists are of micromolar affinity, but these lead molecules have been optimized for affinity and selectivity. Library screening has pointed the way for the identification of a variety of chemically novel lead structures that have been optimized by medicinal chemists for affinity and selectivity as adenosine antagonists.



Adenosine Receptors, Fig. 1 Structures of widely used AR agonists, both nonselective and selective. Affinities/potencies at the ARs are found in Table 2. (**a**) Nucleoside derivatives that are either nonselective (1, 2) or selective

Sources of Adenosine, Its Transport Mechanisms, and Metabolism

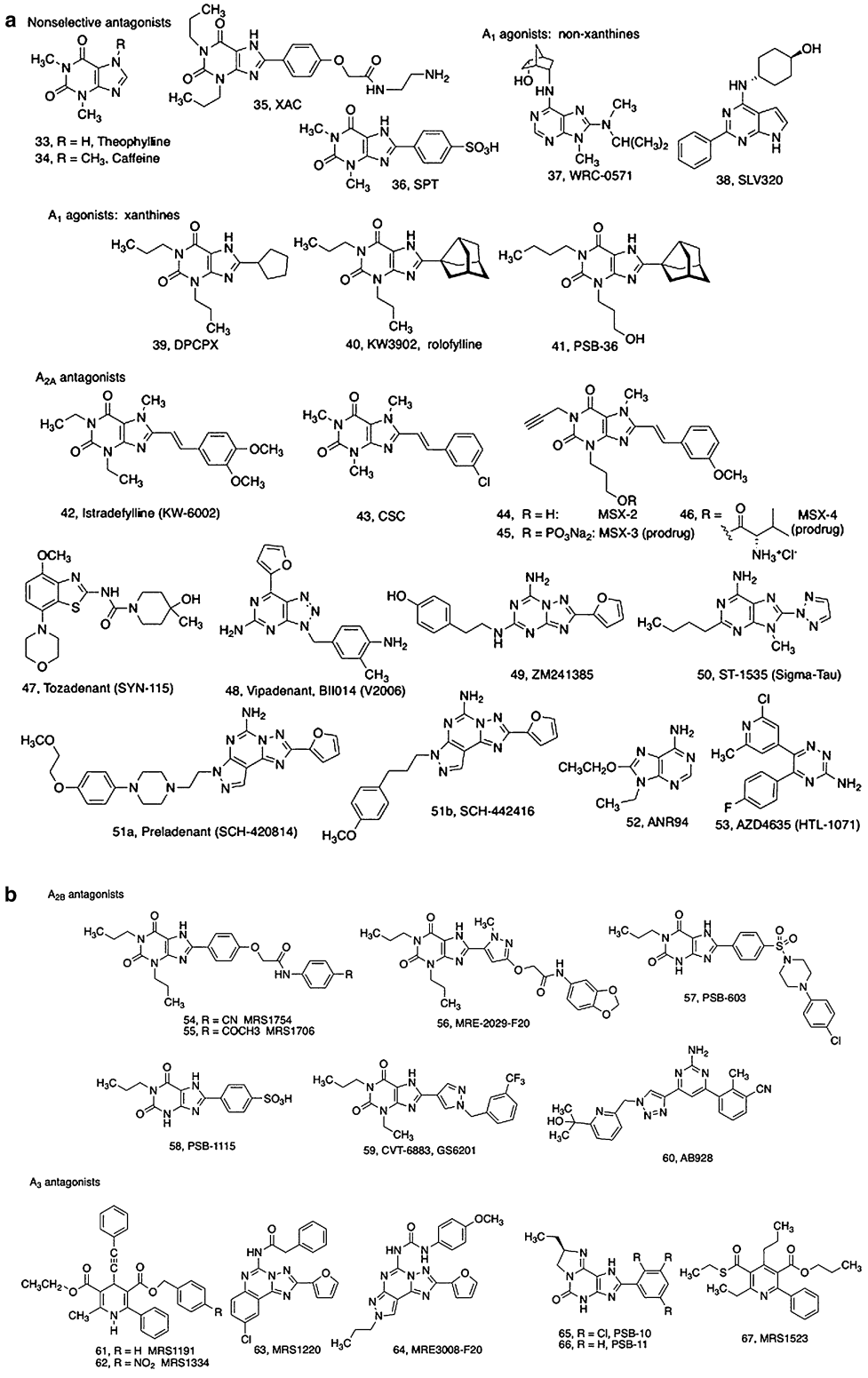
Adenosine production increases upon stress to an organ or tissue, and it acts locally in autocrine or paracrine fashion. It is not a classical neurotransmitter, but it can be formed from ATP, which is released from synaptic vesicles. Most cells in culture and in situ produce and release adenosine and ATP extracellularly. These endogenous purines released tend to influence the outcome of pharmacological studies and can cause misleading results if not properly controlled. Depending on stress factors present, the levels of extracellular adenosine in a given tissue or organ may vary widely, leading to highly variable basal levels of stimulation of the ARs by endogenous adenosine. The level of adenosine may be as low as ~20 nM in the resting brain and as high as 100 μM in severe ischemic conditions. The half-life of adenosine in the blood is short (~1 sec), and the peripheral administration of adenosine has no effect on the extracellular concentration of adenosine in the brain. Both neuronal and glial cell function are regulated by adenosine.

One source of extracellular adenosine may be from inside the cell, where it is present in millimolar concentrations. As a hydrophilic small molecule, adenosine does not diffuse freely through the intact plasma membrane; rather, it may pass through an equilibrative transporter such as the ENT1 nucleoside transporter, for which there are well-characterized inhibitors, e.g., 30–32 (Fig. 1b) (Pastor-Anglada and Pérez-Torras 2018). Approved drug dipyridamole (structure not shown) acts in part by inhibiting adenosine uptake through ENTs. Levels of extracellular adenosine may also rise as a result of the enzymatic hydrolysis of extracellular adenine nucleotides or cell lysis. Nucleotide precursors of adenosine, notably ATP and ADP, have their own extracellular signaling properties that are mediated by P2 receptors. Ectonucleotidases, which are also ubiquitously expressed on the cell surface, but with characteristic distribution patterns, cleave adenine nucleoside 5′-phosphate derivatives (including activators of P2X and P2Y nucleotide receptors) to eventually form adenosine. There are many classes of ectonucleotidases; however, the most relevant species in the family of ectonucleoside triphosphate dihydrolases (E-NTPDases) that act to breakdown P2 agonists are apyrase or NTPDase1 (also known as ENTPD1 and CD39, which converts ATP and ADP to AMP) and NTPDase2 (also known as ENTPD2, which converts ATP to ADP). A separate enzyme, ecto-5′-nucleotidase (CD73), converts AMP to adenosine. CD73 is characteristically found on the surface of astrocytes but not neurons.

Unlike classical neurotransmitters, adenosine does not have a rapid synaptic uptake system (as for the biogenic amines), and its chemical inactivation system is not as rapid as for the transmitter acetylcholine, for example. Adenosine may be metabolized and inactivated with respect to the ARs in a more general fashion by the widespread enzymes adenosine kinase (AK, to produce AMP intracellularly) and adenosine deaminase (AD, to produce inosine both extracellularly and intracellularly). Inosine can weakly activate ARs, depending on the subtype (Xiao et al. 2019).

Inhibition of the metabolism of extracellular adenosine or its uptake proteins is being explored for therapeutic purposes. AK inhibitors have been proposed for the treatment of pain and seizures; however, the promising clinical development of these efficacious compounds was discontinued due to toxicity (Jarvis 2019).

Adenosine Receptors, Fig. 1 (continued) for A_1 receptors (3–11) or A_{2A} receptors (12–17). (**b**) Nucleoside derivatives that are selective for A_{2B}AR alone (18) or mixed with A_{2A}AR (19) or A_3 receptors (20–24). Indirect modulators of AR signaling, either allosteric enhancers (PAMs) or enzyme/transporter inhibitors are shown (25–32). Inhibitors of CD73, which forms adenosine extracellularly, are omitted



Adenosine Receptors, Fig. 2 Structures of widely used AR agonists, both nonselective and selective. Affinities/potencies at the ARs are found in Table 2. (**a**) Nonnucleoside derivatives that are either nonselective or selective for A_1 or A_{2A} receptors. (**b**) Nonnucleoside derivatives that are selective for A_{2B} or A_3 receptors

Signaling Pathways Coupled to ARs and AR Regulation

The four mammalian ARs are members of the rhodopsin-like Class A family of GPCRs, which contain seven transmembrane helical domains (TMs). Characteristics of the four subtypes of the human ARs, length of their primary sequences, their chromosomal localization, and their signaling pathways are given in Table 1. The A_{2A}AR is considerably longer than the other three subtypes, due to its extended carboxy-terminal.

Two AR subtypes, A_1 and A_3, couple through the α-subunit of G_i to inhibit adenylate cyclase, while the other two subtypes, A_{2A} and A_{2B}, stimulate adenylate cyclase through G_s or G_{olf} (for A_{2A}). The A_{2B}AR is also coupled to the activation of PLC through G_q. Furthermore, each of these receptors may couple through the β,γ subunits of the G proteins to other effector systems, including ion channels and phospholipases. Levels of intracellular calcium increase upon stimulation of ARs, which interact with other second messenger systems. ARs have been found to couple to mitogen-activated protein kinases (MAPKs) in a variety of

Adenosine Receptors, Table 1 Characteristics of the four subtypes of adenosine receptors (ARs, human, unless noted). AR structures are available at https://www.rcsb.org/

Receptor subtype	A_1	A_{2A}	A_{2B}	A_3
Genbank accession number	S45235	S46950	X68487	L22607
Amino acids	326	412	332	318
Structures (PBD ID, selected)	5UEN, 5N2S, 6D9H (cryo-EM, with Gi protein)	2YDV, 3PWH, 3RFM and 3UZC (thermostabilized), 3QAK (with agonist), 4EIY (1.8 Å), 5UIG, 6GCG (cryo-EM, with miniGs)	–	–
Chromosomal localization	1q32.1	22q11.2	17p11.2–12	1p13.3
G protein coupling selectivity	Gi/o	Gs; golf	Gs; Gq	Gi/o
Key residues for ligand recognition	H278 (7.43)	E151, E169 (EL2); F182 (5.43); H250 (6.52); N253 (6.55); H278 (7.43); S281 (7.46)	F59 (2.56)	H95 (3.37); N250 (6.55); H272 (7.43)
Mutations specific for reduced agonist potency	E16A (1.39); C85S (3.30); T277A (7.42); L65T (2.60) (rat); I69S (2.64) (rat)	T88A (3.36); S277A (7.42); H278Y (7.43)	–	
Mutations specific for reduced antagonist potency	H251L (6.52) (bovine)	V84L (3.32)	–	K152A (EL2); W243A (6.48)

All of the ARs are found in the CNS and in many peripheral tissues, including immune cells. The A1AR is found in the brain (cortex, cerebellum, hippocampus), dorsal horn of the spinal cord, eye, adrenal gland, heart (atrium), skeletal muscle, liver, kidney, adipose tissue, salivary glands, esophagus, colon, antrum, and testis. The A2AAR is found in spleen, thymus, leukocytes (both lymphocytes and granulocytes), and blood platelets. In the brain it is restricted to the striatum, nucleus accumbens, olfactory tubercle, and large striatal cholinergic interneurons. The A2BAR is found in intestines, brain, spinal cord, lung, epididymis, vas deferens, and pituitary. The message for A3 is found in the lung and liver and to a lesser degree in the brain, aorta, testes, and heart

circumstances, leading to effects on differentiation, proliferation, and cell death.

Cross talk occurs between ARs and other receptors. For example, an otherwise subthreshold concentration of acetylcholine, as might be present in the Alzheimer's brain, still produces a strong calcium signal when the A_1 AR is costimulated. Cross talk occurs with the striatal dopamine receptor system, in which a direct physical association (dimerization) occurs between A_{2A} and D_2 dopamine receptors and between other subtypes (Canas et al. 2018). The A_1AR forms functional heterodimers with the $P2Y_1$ nucleotide receptor, which may be stimulated by $P2Y_1$ receptor agonists but is not blocked by $P2Y_1$ receptor antagonists.

Downregulation of ARs should be considered in pharmacological studies and in the development of agonists for therapeutic purposes. Responses of all four subtypes have been found to desensitize and downregulated the receptors. The most rapid downregulation in cells among the AR subtypes is generally seen with the A_3AR, but the action of A_3AR to reduce chronic neuropathic pain is maintained over days in rodent testing (Jacobson et al. 2019).

Selective Ligands and Indirect Modulators of Adenosine Signaling

The structure activity relationships (SAR) of numerous synthesized analogues of nucleosides, xanthine heterocycles, and nonxanthine heterocycles have been explored at the ARs (Figs. 1 and 2). Potent and selective AR antagonists have been prepared for all four subtypes (Müller and Jacobson 2011), and selective agonists are known for three subtypes (Jacobson et al. 2019). Thus, numerous pharmacological tools are available for in vitro and in vivo use (Table 2). Potent and selective A_{2B}AR agonists are yet to be reported, although several research groups have identified lead compounds.

Agonists

Medicinal chemists have extensively explored the SAR of adenosine derivatives as agonists of the ARs. Except for atypical adenosine agonists that are pyridine-3,5-dicarbonitrile derivatives, such as 10, 11, and 18, nearly all AR agonists have been purine nucleoside derivatives. In general, for the adenine moiety of adenosine, modifications at the N^6 position have led to selectivity for the A_1AR, and modifications at the 2 position, especially with ethers, secondary amines, and alkynes, have led to selectivity for the A_{2A}AR. Some N^6 derivatives are not selective for the A_1AR; for example, N^6-p-(2-aminophenyl)ethyladenosine (APNEA) and metrifudil (structures not shown) are relatively nonselective. 2-Chloroadenosine is a nonselective agonist that is subject to cellular uptake and nonreceptor-mediated effects. Commonly used A_1AR agonists that are N^6-cycloalkyl derivatives are the 2-chloro analogue CCPA 4 and Cl-ENBA 6, and recently reported MRS7469 5 has exceptionally high A_1AR selectivity. When using the human ARs, 5 and 6 are more highly A_1AR-selective than CCPA or its 2-H analogue CPA, although 6 is a mixture of diastereomers. The A_1AR agonist SPA 8 contains a sulfonate group, which tends to prevent passage across the blood–brain barrier. Nitro ester 7 was in unsuccessful clinical trials for glaucoma treatment.

The substitution at the 2 position may also lead to A_{2A}AR enhancing effects, which are further boosted with an uronamido substitution at the 5′ position of adenosine. When the 5′-N-alkyluronamide group alone is present, high affinity [at the A_{2A}AR but not selectivity] is typically observed, similar to the nonselective agonist NECA 2. By combination of this uronamide group with the appropriate 2 position substitution, selectivity may be achieved for the A_{2A}AR. For example, the 5′-N-ethyl derivatives CGS21680 13 and ATL-146e 14 are both selective in binding to the rat A_{2A}AR, but they are less selective at the human subtypes. CGS21680 crosses the blood–brain barrier to only a small degree. UK-432097 16, a drug originally proposed for COPD to be administered by inhalation, was cocrystallized with the human A_{2A}AR to obtain the first agonist-bound AR structure.

Several agonists are selective for the A_{2B}AR, known as the "low affinity" adenosine A_2 receptor. Nonadenosine ligand BAY 60–6583 is

Adenosine Receptors, Table 2 Affinity of commonly used adenosine receptor agonists and antagonists for defining pharmacologically adenosine receptor subtypes

		pKi (nM), unless noted			
Selectivity	Compound	A_1AR[a]	$A_{2A}AR$[a]	$A_{2B}AR$[a]	A_3AR[a]
	Agonists				
None	1 adenosine	7.00	6.51	4.82	6.54
	2 NECA	7.85	7.70	6.85[c]	7.60
A_1	3 CPA	8.64	6.10	n.d.	7.37
	4 CCPA	9.08	5.64	4.73[c]	7.42
	5 MRS7469	8.70	5.45	n.d.	4.97
	6 cl-ENBA	9.29	5.87	n.d.	5.89
	8 SPA	8.10,7.15[b]	5.42	4.28[c]	6.61
	10 capadenoson	8.85	<5	n.d.	<5
A_{2A}	12 regadenoson	<5	6.54	<5	<5
	13 CGS21680	6.54	7.57	<5[c]	7.17
	14 ATL-146e	7.11	9.30	n.d.	7.35
	15 ATL-313	7.24	9.15	<6	6.60
	16 UK-432097	n.d.	8.40	n.d.	n.d.
	17 GW328276X	6.05	8.64	7.29	8.38
A_{2B}	19 MRS3997	–	6.89	6.82	–
A_3	20 IB-MECA	7.29,7.27[b]	5.54,6.02[b]	4.96	8.74,8.96[b]
	21 cl-IB-MECA	6.66	5.27	<4[c]	8.85
	22 MRS5698	<5	<5	n.d.	8.46
	23 MRS5841	<5	<5	n.d.	8.72
	24 MRS5980	<5	<5	n.d.	9.15
	Antagonists				
None	33 theophylline	5.17	5.17	5.04	4.65
	34 caffeine	4.97	4.32	4.98	4.88
	35 XAC	8.17,8.92[b]	7.74,7.20[b]	8.11	7.59
	36 SPT	5.40	5.15	5.88	5.23
A_1	37 WRC-0571	8.77	6.98	n.d.	5.10
	38 SLV320	9.00	6.40	5.40	6.70
	39 DPCPX	8.52	6.89	7.25	6.10
	40 KW-3902	9.14	6.79	6.53	5.36
	41 PSB-36	9.15,9.92[b]	6.01,6.26[b]	6.73	5.64
A_{2A}	42 KW-6002	5.55	7.44	5.74	<5.52
	43 CSC	4.55[b]	7.27[b]	5.09	<5[b]
	44 MSX-2	5.60	8.10	<5	<5
	48 vipadenant	7.19	8.89	7.20	6.00
	49 ZM241,385	6.11	8.80	7.12	6.13
	51a preladenant	5.83	8.96	<5.77	<6
	51b SCH442416	5.95	10.32	<5	<5
	53 HTL-1071	n.d.	8.77	n.d.	n.d.
A_{2B}	54 MRS1754	6.39	6.30	8.70	6.24
	55 MRS1706	6.80	6.95	8.86	6.64
	56 MRE 2029-F20	6.61	<6	8.52	<6
	57 PSB-603	<5	<5	9.26	<5
	58 PSB-1115	<5	4.62[b]	7.27	<5
	59 CVT-6883	5.71	5.48	7.66	5.97

(continued)

Adenosine Receptors, Table 2 (continued)

		pKi (nM), unless noted			
A_3	61 MRS1191	<5	<5	<5[c]	7.50
	62 MRS1334	<4	<4	n.d.	8.57
	63 MRS1220	6.52[b]	7.28[b]	n.d.	9.19
	64 MRE 3008-F20	5.92	6.85	5.68	9.09
	65 PSB-10	5.77	5.57	n.d.	9.37
	66 PSB-11	5.79	5.89	5.68[c]	8.64
	67 MRS1523	4.81[b]	5.69[b]	<5	7.72

n.d., not determined
[a]Affinity at human A1, A2A, A2B, and A3ARs, unless noted, expressed as pKi
[b]Affinity determined at rat ARs
[c]Potency in a functional assay

selective for that subtype, but has variable reports of maximal efficacy, so it can be classified as a partial agonist. An adenosine derivative, the 2-(6-bromotryptophol) ether MRS3997 19, is a full agonist with mixed selectivity at A_{2A} and A_{2B}ARs.

The A_3 agonists Cl-IB-MECA 21 and its corresponding 2-H analogue IB-MECA 20 are widely used as selective agonists of the A_3AR, although even more selective agents are now known, including those in which the ribose-like ring has been conformationally locked in the receptor-preferring north conformation, such as MRS5698 22, MRS5841 23, and MRS5980 24 (Jacobson et al. 2019). MRS5841 contains a sulfonate group that prevents passage across biological barriers and has been used to distinguish peripheral and CNS A_3AR effects.

Antagonists

The classical AR antagonists are xanthines derivatives such as theophylline (33, 1,3- dimethylxanthine) and caffeine 34. The micromolar affinity of the naturally occurring antagonists has been greatly exceeded with the introduction of selective antagonists, even reaching subnanomolar affinity. For example, the A_1AR-selective antagonists DPCPX 39, KW3902 40, and PSB-36 41 are xanthine derivatives. The 8-cyclopentyl derivative DPCPX is highly A_1AR selective in the rat and less A_1AR selective among the human AR subtypes. DPCPX is an inverse agonist at the A_1AR, while the adenine derivatives are neutral antagonists. In general, modifications of the xanthine scaffold at the 8 position with aryl or cycloalkyl groups have led to selectivity for the A_1AR, although the water-soluble 8-sulfophenyl derivative SPT 36 is nonselective and xanthine amine congener (XAC, 35) is potent at all human ARs. Persistent problems in the use of xanthine derivatives as AR antagonists of the A_1AR are their low aqueous solubility and their interaction at the A_{2B}AR. Use of adenine derivatives, such as the inverse agonist WRC-0571 26 37, provides A_1AR-selective antagonists that have low affinity at the A_{2B}AR.

Modifications of xanthines at the 8 position with alkenes (specifically styryl groups) have led to selectivity for the A_{2A}AR. The 8-styrylxanthine derivatives KW6002 42, CSC 43, and MSX-2 44 and its prodrug derivatives 45 and 46 are moderately potent A_{2A}AR antagonists (Müller and Jacobson 2011). Some 8-styrylxanthine derivatives, especially CSC, have been discovered to inhibit monoamine oxidase-B as well. High selectivity of xanthines at the A_1, A_{2A}, and A_{2B} (e.g., MRS1754 54, its p-$COCH_3$ analogue MRS1706 55, and PSB-603 57) ARs has been achieved. Few xanthines are selective for the A_3AR; however, A_3AR antagonist PSB-11 66 contains an elaborated xanthine ring system.

Over several decades, an enormous diversity of heterocyclic structures has been reported as AR antagonists (Table 2) (Müller and Jacobson 2011). For example, the nonselective triazoloquinoline antagonist CGS15943 (structure not shown), first

introduced in the early 1990s, has given rise to numerous derivative compounds, including A_{2A}AR-selective antagonists (a triazolotriazine 49 and pyrazolo-triazolo-pyrimidines 51a and 51b), Preladenant 51a, and several other A_{2A}AR-selective antagonists which have been in clinical trials for Parkinson's disease (Müller and Jacobson 2011). SCH-442416 51b displays >23,000-fold selectivity for the human A_{2A}AR (K_i 0.048 nM) in comparison to human A_1AR and IC_{50} > 10 μM at the A_{2B} and A_3 ARs. Also, elaboration of an adenine scaffold has provided A_1AR-selective antagonists (37, 38) and A_{2A}AR-selective antagonists (48, 50, and 53) (Müller and Jacobson 2011). AB928 60 is an experimental cancer immunotherapy drug that is a mixed antagonist at A_{2A} and A_{2B}ARs.

Although the simple xanthine antagonists have not provided suitable analogues with A_3AR selectivity, a cyclization of the xanthine nucleus, leading to imidazopurinones 65 and 66. For A_3AR, most of the successful leads have come from chemically diverse heterocycles. The dihydropyridine derivatives MRS1191 61, its more potent nitro analogue MRS1334 62, and other heterocyclic scaffolds such as pyridylquinazolines have led to potent, selective A_3AR antagonists. There is a marked species dependence of antagonist affinity at the A_3AR. It is to be noted that many heterocyclic nonnucleoside A_3AR antagonists are potent in the human but are weak at the rat and mouse A_3ARs. Nevertheless, MRS1191 has been used successfully in murine species. MRS1220 42 is very potent and selective at the human A_3AR but not at the rat or the mouse receptor, at which it is A_{2A}AR selective. Commonly used A_3AR antagonists must be treated with caution in non-primate species. In general, one must be cognizant of potential species differences for both AR agonists and antagonists. The pyridine derivative MRS1523 67 is a moderately selective A_3AR antagonist for both the rat and human homologues.

Variations in the relative efficacy of nucleosides, depending on structure, have been noted. This is especially pronounced for the A_3AR, at which changes on the adenine moiety (N^6 and 2 positions) and ribose moiety can either reduce efficacy to the point of pure antagonism (i.e., combination of 2-Cl and N^6-(3-iodobenzyl)) or guarantee a robust, nearly full activation of the A_3AR (i.e., 5′-uronamide) (Canas et al. 2018). Such nucleoside-derived A_3AR antagonists tend to have selectivity that is more general across species.

Radioligands

Radioligands commonly used for the ARs are nonselective agonist [^{3}H]NECA 2, A_1 agonist [^{3}H]CCPA 4, antagonist [^{3}H]DPCPX 39, A_{2A} agonist [^{3}H]CGS21680 13, antagonist [^{3}H]ZM241385 49, A_3 agonist [^{125}I]I-AB-MECA (the N^6-(4-amino-3-iodobenzyl) 2-H analogue of 20), and antagonist [^{3}H]PSB-11 66. Ligands for in vivo positron emission tomographic (PET) imaging of A_1 and A_{2A}ARs have been developed (Müller and Jacobson 2011). Potent fluorescent ligands have been reported for all four AR subtypes and have been used in chemical library screening to discover novel antagonists.

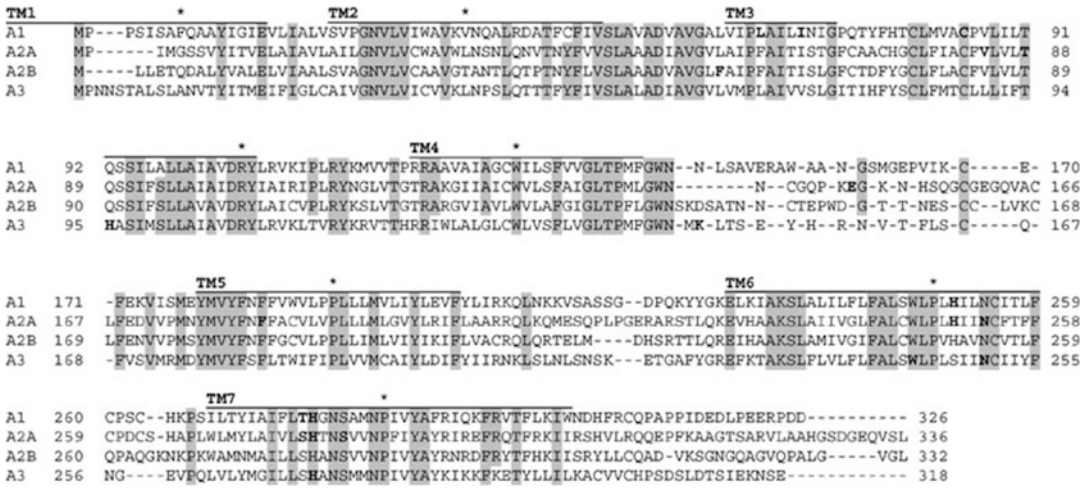
Allosteric modulation: In addition to AR agonists and antagonists that interact directly with the primary (orthosteric) site of the receptor, allosteric modulators of agonist action are also under consideration for disease treatment. Such modulators, either positive allosteric modulators (PAMs) or negative allosteric modulators (NAMs), might have advantages over the directly acting (orthosteric) receptor ligands. The action of the allosteric compounds would depend on the presence of a high local concentration of adenosine, which often occurs in response to a pathological condition (Chen et al. 2013). In some cases (dependent on tissue, receptor subtype, and other conditions), one would wish to boost the adenosine effect, and therefore, an allosteric enhancer would be useful. In other cases, the elevated adenosine may be detrimental, in which instance one would want to apply a negative modulator. Allosteric modulators have been explored and are under development for the A_1 and A_3 AR subtypes (Fig. 1b) (Jacobson et al. 2019).

Receptor Structures

Numerous A_{2A}AR structures and several A_1AR structures have been determined by X-ray crystallography and cryogenic electron microscopy (cryo-EM) (Jespers et al. 2018). Both antagonist and agonist ligands have been cocrystallized with the ARs for structure determination. Some structures include a G protein or G protein segment to simulate the active state of the receptor. The A_{2A}AR has become one of the most studied GPCRs, and it was used for the development of the technique by which stabilizing mutations are introduced strategically, in order to study the receptor using biophysical methods. Much of the current discovery of AR ligands uses structure-based approaches.

Receptor structures and computational modeling, including molecular dynamics (MD) simulation, have aided in the drug discovery process for ARs. Each of the ARs has been modeled effectively based on homology to the already reported A_{2A}AR or A_1AR structures (Table 1), and numerous theoretical models are now available. Molecular modeling of the ARs and ligand docking have provided insights into the putative binding sites of all of the subtypes, which has aided in the rational design of ligands (Müller and Jacobson 2011). For example, AZD4635 53 was discovered through an iterative process of biophysical mapping of the A_{2A}AR binding site determined in multiple X-ray structures. It is currently in clinical trials for treating solid tumors, either as a monotherapy or as a cotherapy with other agents for cancer immunotherapy.

Essential residues for the binding of ligand and activation of all four ARs have been defined through structure determination, site-directed mutagenesis, and modeling. Similar to other GPCRs having small molecular ligands, TMs 3, 5, 6, and 7 of the ARs are thought to be most closely associated with bound agonists and antagonists. An alignment of the primary sequences of the four human AR subtypes (Fig. 3) indicates a high degree of homology within the TMs. Key functional residues for ligand binding and activation have been identified. Extensive mutagenesis of the ARs had predicted, even before the X-ray structures were available, a putative, hydrophilic ribose-binding region spanning TMs 3 and 7. The putative adenine-binding region contains more hydrophobic residues, particularly in the vicinity



Adenosine Receptors, Fig. 3 An alignment of the primary sequences of the four human AR subtypes. Regions of conservation are highlighted. ∗ indicates the most conserved (X.50) residue in each TM region. Bold residues correspond to those which have a role in ligand recognition. The A_{2A} receptor is truncated in the carboxy-terminal region

of the N^6 substituent. The binding and activation steps of receptor action have been dissected using inactive and active-like structures, including strategic mutagenesis, computational methods, and other biophysical techniques such as NMR. The conformational dynamics of the activation of the A_3AR and other subtypes have been studied. The second extracellular loop (EL2) generally contacts the receptor ligands and is highly variable in sequence and contains a relatively high content of charged residues, in addition to sterically constraining disulfide bridges.

Drugs

Presently, only agonists adenosine and regadenoson and antagonist istradefylline are potent AR ligands that are approved for clinical use. Adenosine is used widely in the treatment of supraventricular tachycardia and in cardiac stress imaging to assess coronary artery disease (Jacobson et al. 2019). Other agonists and antagonists, and an allosteric modulator of the A_1 receptor T-62 25, have been in clinical trials for a variety of indications.

Selective AR agonists have been in or are being considered for clinical trials for cardiac arrhythmias and pain (A_1), cardiac imaging and inflammation (A_{2A}), hepatocellular carcinoma (HCC), non-alcoholic steatohepatitis (NASH), rheumatoid arthritis, psoriasis, and chronic neuropathic pain (A_3). Selective AR antagonists have been in or under consideration for clinical trials for kidney disorders (A_1); Parkinson's disease and cancer (A_{2A}); cancer, diabetes, and asthma (A_{2B}); and glaucoma (A_3). CD73 inhibitors, both small molecules and monoclonal antibodies, are in clinical trials for cancer immunotherapy.

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Adenyl Cyclase (Original)

▶ Adenylyl Cyclases

Adenyl Cyclase (Preferred)

▶ Adenylyl Cyclases

Adenylate Cyclase (Not Chemically Correct)

▶ Adenylyl Cyclases

Adenylyl Cyclases

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Abbreviations

2′,3′-dd-Ado	2′,3′-Dideoxyadenosine
2′,5′-dd-2,5′-di-F-Ado	2′,5′-Dideoxy-5′-fluoro-2-fluoro-adenosine
2′,5′-dd-2-F-Ado	2′,5′-Dideoxy-2-fluoro-adenosine
2′,5′-dd-2F-Ado-3′-P-bis(Me-SATE)	2′,5′-dd-2-Fluoro-adenosine-3′-(acetyl-2-thioethyl)-phosphate
2′,5′-dd-3′-A4P	2′,5′-Dideoxyadenosine-phosphate-3′-tetraphosphate
2′,5′-dd-3′-ADP	2′,5′-Dideoxyadenosine-phosphate-3′-diphosphate
2′,5′-dd-3′-AMP	2′,5′-Dideoxyadenosine-3′-monophosphate
2′,5′-dd-3′-AMP-bis (Me-SATE)	2′,5′-Dideoxyadenosine-3′-(acetyl-2-thioethyl)-phosphate
2′,5′-dd-3′-AMP-bis (Ph-SATE)	2′,5′-Dideoxyadenosine-3′-(phenyl-2-thioethyl)-phosphate
2′,5′-dd-3′-AMP-bis(t-Bu-SATE)	2′,5′-Dideoxyadenosine-3′-(pivaloyl-2-thioethyl)-phosphate
2′,5′-dd-3′-ATP	2′,5′-Dideoxyadenosine-3′-triphosphate
2′,5′-dd-Ado	2′,5′-Dideoxyadenosine
2′-d-3′-ADP	2′-Deoxyadenosine-3′-diphosphate
2′-d-3′-AMP	2′-Deoxyadenosine-3′-monophosphate
2′-d-3′-AMPS	3′-(Thiophosphoryl)-2′-deoxyadenosine
2′-d-3′-ATP	2′-Deoxyadenosine-3′-triphosphate
3′-MANT-2′-d-5′-ATP	3′-O-*N*-Methylanthraniloyl-2′-deoxy-5′-ATP
5′-APP(CH2)P	Adenosine 5′-(β(−methylene)-triphosphate
9-Ara-Ade (Ara-A; Vidarabine)	9-(Arabinofuranosyl)-adenine
9-THF-Ade (SQ22,536; THFA)	9-(Tetrahydrofuryl)-adenine
9-Xyl-Ade	9-(Xylofuranosyl)-adenine
Ado	Adenosine
cAMP	Adenosine-3′:5′ monophosphate
KH7	2-(1H-benzimidazol-2-ylthio)-N′-[(1E)-(5-bromo-2-hydroxyphenyl) methylene]propanohydrazide
LRE1 (RU-0204277)	6-Chloro-N4-cyclopropyl-N4-(2-thienylmethyl)-2,4-pyrimidinediamine
MANT-5′ATP	3′-(2′)-O-*N*-Methylanthraniloyl-5′-ATP
MANT-5′GTP	3′-(2′)-O-*N*-Methylanthraniloyl-5′-GTP
MANT-5′-GTPγS	3′-(2′)-O-*N*-Methylanthraniloyl-guanosine-5′-[γ-thio] triphosphate
MANT-5′-ITPγS	3′-(2′)-O-*N*-Methylanthraniloyl-inosine-5′[γ-thio] triphosphate
NKY 80	2-Amino-7-(2-furanyl)-7,8-dihydro-5(6H)-quinazolinone
PMEApp (active metabolite of bis (POM)PMEA, adfovir)	9-(2-Phosphonylmethoxyethyl) adenine diphosphate
SKF-83566	8-Bromo-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepin-7-ol hydrobromide

ST034307	6-Chloro-2-(trichloromethyl)-4H-1-benzopyran-4-one
β-L-2′,3′-dd-5′-ATP	β-L-2′,3′-Dideoxyadenosine-5′-triphosphate
β-L-5′-ATP	β-L-Adenosine-5′-triphosphate

Synonyms

Adenylate cyclase (not chemically correct); Adenyl cyclase (original); Adenyl cyclase (preferred); ATP pyrophosphate-lyase; Cyclizing (E.C.4.6.1.1.)

Definition

Adenylyl cyclases (AC) comprise a family of enzymes that catalyze the synthesis of adenosine 3′:5′-monophosphate (cyclic AMP, cAMP) from adenosine 5′-triphosphate (ATP). The second messenger cAMP regulates effects in all eukaryotic cells, through the activation of cAMP-dependent protein kinase (PKA), cAMP-gated ion channels (CNGs), hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, exchange proteins activated by cAMP (Epacs), and Popeye domain containing (Popdc) proteins, in addition to the regulation of a subset of cAMP- and cGMP-degrading enzymes (PDEs, phosphodiesterases) (Fig. 1). Cellular levels of cAMP levels reflect the balance of activities of adenylyl cyclases (ACs: 5′-ATP to cAMP + PPi) and cAMP phosphodiesterases (PDEs: cAMP to 5′-AMP). Adenylyl cyclases occur throughout the animal kingdom and play diverse roles in cell regulation. Mammalian cells express nine membrane-spanning AC isoform and a tenth soluble AC (Dessauer et al. 2017).

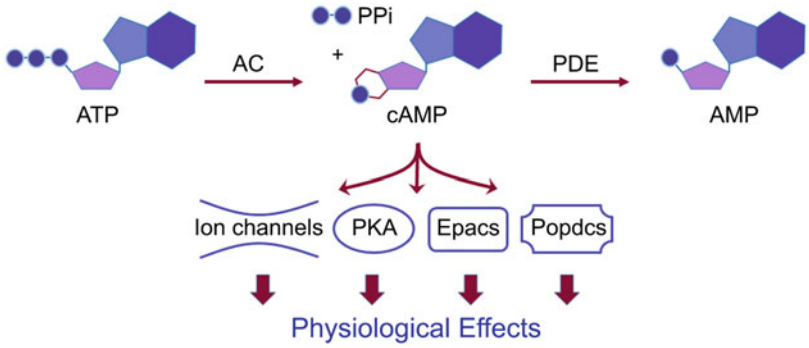
Basic Characteristics and Mechanisms

Classes of Adenylyl Cyclases

Adenylyl cyclases belong to the larger class of purine nucleotide cyclases. These have been divided into classes I–VI (Linder and Schultz 2003). Classes I, II, and IV–VI consist of cyclases from gram-negative bacteria, the extracellular soluble toxins of certain pathogens (e.g. *Bacillus anthracis*, *Bordetella pertussis*, and *Pseudomonas aeruginosa*), and additional bacteria classes, respectively. Class III cyclases include most adenylyl and guanylyl cyclases (catalyzing the formation of cyclic GMP). These enzymes respond to changes in the extracellular environment. In mammals (subclass IIIa) this occurs via hormones, neurotransmitters, odorants, or tastes for transmembrane ACs (AC1–AC9). For the mammalian soluble AC (subclass IIIb) and lower organisms, enzymatic activity responds to changes in ionic factors, bicarbonate, glucose, or serum factors and through osmoregulation, chemotaxis, phototaxis, or pH, in various bacteria.

Mammalian Adenylyl Cyclases

Of the ten known isozymes of mammalian adenylyl cyclase (AC1–AC10; Table 1), all but one are membrane-bound and are regulated via cell surface receptors linked to heterotrimeric ($\alpha\beta\gamma$) stimulatory (G_s) and inhibitory (G_i) guanine nucleotide-dependent regulatory proteins (G-proteins) (Fig. 2, Table 2) (Dessauer et al. 2017). These receptors are referred to as G-protein-coupled receptors, or GPCRs, and mediate effects of stimulatory and inhibitory hormones and neurotransmitters. $G\alpha_s$ stimulates all of the mammalian AC isozymes, except the soluble AC10 (regulation of AC10 is G-protein-independent and differs significantly from the membranous ACs; discussed below). The plant-derived diterpene, forskolin, binds at the interface of the two cytosolic domains (**C1 • C2**; see below) of AC1–AC9 to stimulate enzymatic activity, although AC9 is only weakly stimulated by forskolin and requires the presence of $G\alpha_s$ (Baldwin et al. 2019); forskolin does not activate AC10. The AC isozymes differ more significantly in their responses to $G\alpha_i$ and $G\beta\gamma$ and in the physiological responses they control (Baldwin and Dessauer 2018; Dessauer et al. 2017) (Table 2). For example, $G\alpha_i$ inhibits some but not all isozymes and $G\beta\gamma$ inhibits AC1 and AC8, but significantly stimulates AC2, AC4, and AC7,



Adenylyl Cyclases, Fig. 1 Synthesis and degradation of the second messenger cAMP and regulation of downstream effectors. *AC* adenylyl cyclase, *PDE* phosphodiesterase

Adenylyl Cyclases, Table 1 Accession numbers, size, and gene loci for human adenylyl cyclase isoforms

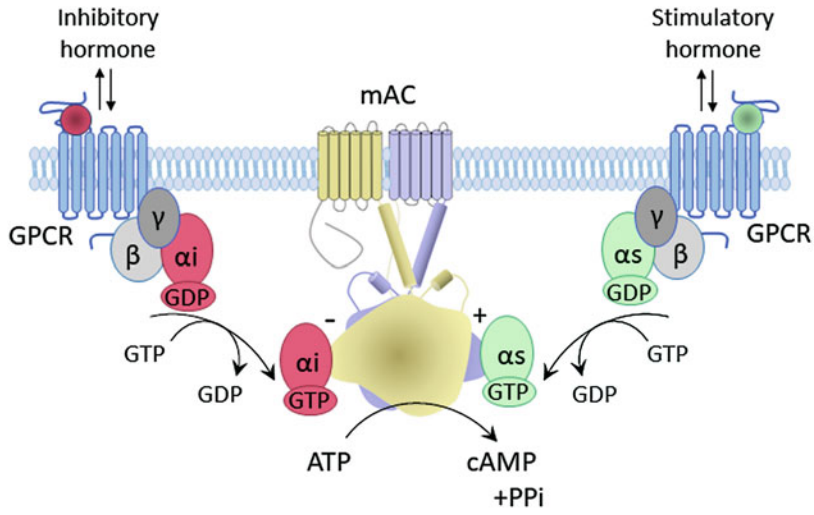
ADCY[a]	Reference accession numbers[b]	Base pairs (mRNA)	Amino acids	Chromosome
1	NM_021116.4	12,657	1119	7p12.3
2	NM_020546.3	6645	1091	5p15.31
3	NM_004036.5	4950	1144	2p23.3
4	NM_139247.4	3405	1077	14q12
5	NM_183357.2	6098	1261	3q21.1
6	NM_015270.5	6092	1168	12q13.12
7	NM_001114.5	6271	1080	16q12.1
8	NM_001115.3	4421	1251	8q24.22
9	NM_001116.4	7980	1353	16p13.3
10	NM_018417.6	5342	1610	1q24.2

[a]Adenylyl cyclases have been numbered in the order in which they were cloned and sequenced. In databases they are referred to as adcy#; soluble AC10 is sometimes referred to as sAC
[b]Accession numbers are for the Reference Sequence (*RefSeq*) collection of data from the National Center for Biotechnology Information (NCBI). Values pertinent to the human isozymes of adenylyl cyclases are compiled here. The link is http://www.ncbi.nlm.nih.gov/

with weaker stimulatory effects on AC5 and AC6 (Table 2).

Stimulation and inhibition of the enzyme by the GPCR-G-protein cycle occur by analogous mechanisms. Agonists induce hormone receptors to increase nucleotide exchange on Gα (GDP to GTP) which activates the alpha subunit and promotes Gαβγ dissociation (Fig. 2). Consequently, agents that affect either the dissociation of either G_i or G_s or the association of their respective α_s, α_i, or βγ subunits with adenylyl cyclase could affect rates of cAMP formation in enzyme preparations or in intact cells and tissues. There are several important examples. $G\alpha_s$ is stably activated by poorly hydrolyzable analogs of GTP, e.g., GTPγS or GPP(NH)P, and activation is hindered by GDPβS. A less obvious example is fluoride. It activates most mammalian adenylyl cyclases, indirectly by promoting an active form of $G\alpha_s$ ($G\alpha_s$•GDP•AlF_4^-). Another example includes the ADP-ribosyltransferase activities of bacterial toxins. The toxin of *Vibrio cholerae* catalyzes the ADP-ribosylation from NAD of GTP•$G\alpha_s$, and that of *Bordetella pertussis* similarly ADP-ribosylates α_i of GDP•$\alpha_i\beta\gamma$, preventing its dissociation. In both cases the effect is elevated adenylyl cyclase activity and contributes to the pathophysiology of these bacteria. Of therapeutic relevance, of course, are agents acting as agonist or antagonist on GPCRs coupled to adenylyl cyclase, with the prominent example being antagonists of β-adrenergic receptors (i.e., β-blockers).

Activities of all isozymes are affected by Ca^{2+}. At higher concentrations (mM), Ca^{2+} is inhibitory



Adenylyl Cyclases, Fig. 2 Regulation of membrane adenylyl cyclase isoforms (AC1–9) by G-proteins. Hormones bind to G-protein-coupled receptors to activate $G\alpha_s$ or $G\alpha_i$, which leads to stimulation or inhibition, respectively, of adenylyl cyclases (AC1–9). G_s and G_i are GTP-dependent regulatory proteins comprising βγ subunits and their respective α_s and α_i subunits

through competition with divalent cation required for catalysis (see below). At lower concentrations (<μM), Ca^{2+} regulates activity physiologically. This can be (i) through a direct effect at the catalytic active site, increasing activity of AC10 or decreasing activity of AC5 and AC6; (ii) as a Ca^{2+}/calmodulin complex, activating ACs 1, 3, and 8; (iii) with calcineurin to inhibit AC9 or with calmodulin kinase (CaMK) to inhibit AC1 and AC3; or (iv) indirectly through activation of PKC (Table 2; Dessauer et al. 2017). Phosphorylation of adenylyl cyclase varies among the isozymes and is determined by differences in their primary sequences. Phosphorylation catalyzed by protein kinase C (PKC) can be stimulatory or inhibitory depending on the AC isoform (see Table 2), while phosphorylation by cAMP-dependent protein kinase (PKA) or calmodulin kinase (CaMK) is inhibitory and often works in a feedback form of regulation. Raf can also activate ACs 2, 5, and 6. Activity of adenylyl cyclases can be indirectly influenced by the specific phosphorylation of hormone receptors or of G-proteins.

Membrane-bound forms of mammalian adenylyl cyclases exhibit a putative topology with 12 membrane-spanning regions and 2 largely homologous ~40 kDa cytosolic domains (**C_1** and **C_2**) (Fig. 3). Differences in N-terminal and other domains are significant and influence regulation by a variety of agents as noted above (Table 2). AC5 (**C_1**) and AC2 (**C2**) domains have been separately expressed and recombined, and the resulting structure was solved in complex with GTP•$G\alpha_s$ (Fig. 4) (Tesmer et al. 1997). α_s•GTP activates the enzyme through interaction with **C_2**, yielding the active enzyme. More recently, a cryo-EM structure of full-length AC9 bound to GTP•$G\alpha_s$ was solved (Fig. 3) (Qi et al. 2019). There is remarkable similarity with the previous x-ray structures of **C_1•C_2•$G\alpha_s$** within the catalytic core. One notable surprise was the auto-inhibition of AC9 by its C_{2b} region; this appears to be a feature unique to AC9. Inhibition of adenylyl cyclase by $G\alpha_i$ occurs through the interaction with the analogous $G\alpha_s$ site in the **C_1** domain of adenylyl cyclase. The structure obtained with β-L-2′,3′-dd-5′-ATP (a competitive inhibitor of ATP binding) allowed the demonstration that the pseudosymmetric cleft formed by the interface of the **C_1•C_2** domains binds 5′-ATP, forskolin, and two metal cations (Tesmer et al. 1999). The active site shares topology and reaction mechanism with guanylyl cyclases (which share homology within the active site) and with oligonucleotide polymerases.

AC10 (sAC, Adcy10) was initially described as adenylyl cyclase activity in the soluble fraction of rat testis homogenate, but the nature of this enzyme remained elusive for several decades. The unique properties of this "soluble adenylyl

Adenylyl Cyclases, Table 2 Regulatory characteristics of mammalian adenylyl cyclases[a]

AC	$G\alpha_i$[b]	$G\beta\gamma$[b]	$Ca^{2+}$and/or calmodulin[c]	Protein kinases	Major tissue distribution[g]	Physiological functions[f]
1	↓	↓	↑↑	No Δ PKA ↓ CaMK ↑ PKC	Brain (neuron), blood leukocytes	LTP, learning/memory, circadian rhythm, opioid withdrawal, brain development
2	No Δ	↑↑	No Δ	No Δ PKA ↓ PKC ↑ Raf	Brain, skeletal muscle, testis	?
3		↓	~↑ (in vitro)	↓ CaMK ↑ PKC	Olfactory epithelium, brain, placenta, testis, ovary, colon	Olfactory and pheromone response, sperm functions, maternal and aggressive behavior, diet-induced obesity
4	No Δ	↑↑	No Δ	↓ PKC	Ubiquitous, not brain	?
5	↓	↑	↓ Ca^{2+}	↓ PKA ↑ PKC ↑ Raf	Heart, brain (striatum), testis, prostate, ovary, small intestine, colon	Cardiac function, motor coordination, striatum-dependent learning, opioid dependency, pain and stress responses, renin secretion
6	↓	↑	↓ Ca^{2+}	↓ PKA ↓ PKC ↑ Raf	Heart, kidney, brain, widespread	Cardiac function, renal function, sympathetic tone, bone adaptations, pancreatic fluid secretion
7	No Δ	↑	No Δ	↑ PKC	Brain, platelets, blood leukocytes, spleen, lung	Ethanol dependency, immune responses, depression
8		↓	↑↑	↓ PKA	Brain	LTP, learning and memory, opioid withdrawal, mood disorders, anxiety, glucose homeostasis
9	No Δ	No Δ	↓ (Ca^{2+}/ calcineurin)[d]	No Δ CaMK, PKC	Skeletal muscle, heart, brain, widespread	Cardiac function, immune responses
10[e]	No Δ	No Δ	↑ (no CaM)		Testes, widespread	Sperm motility, $HC0_3^-$ sensor; defect associated with absorptive hypercalciuria

[a]Empty cells imply that no information was available. *Direct* effects on adenylyl cyclase activity are as indicated: up (↑) arrow, increase; down (↓) arrow, decrease; or "no Δ" (tested, but no effect on activity seen)

[b]Effects of $G\alpha_i$ or $G\beta\gamma$ are on enzyme stimulated by either $G\alpha_s$ or forskolin. In some instances differences were noted in the effects of different isoforms of β or γ. These are not distinguished here

[c]For effects of Ca^{2+} and/or calmodulin, stimulation of adenylyl cyclase by Ca^{2+} usually requires calmodulin, except in the case of AC10 (sAC). All adenylyl cyclases are inhibited by high (mM) concentrations of Ca^{2+}, through competition with divalent cation required for catalysis (cf. Figures 4 and 5). The inhibition indicated here occurs with low (<μM) concentrations of Ca^{2+}, without calmodulin; inhibition of AC9 by Ca^{2+} is with calcineurin

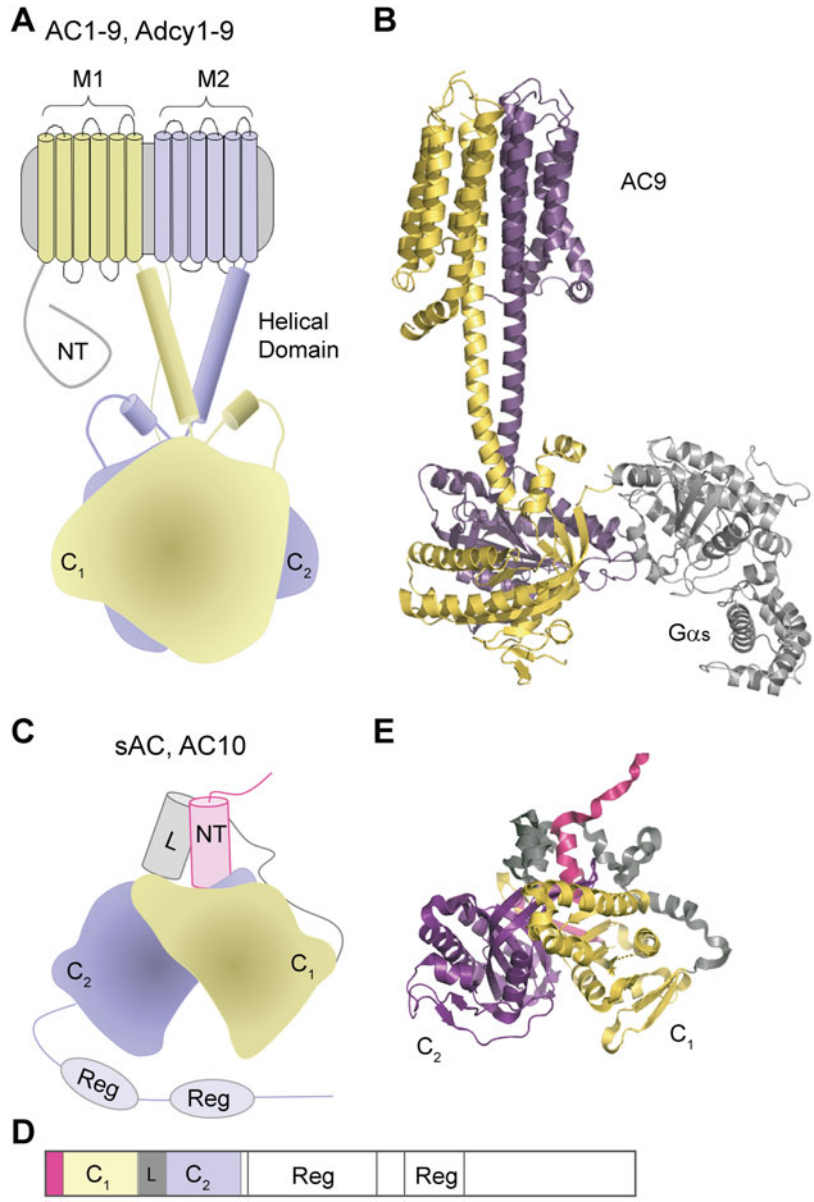
[d]Ca^{2+}/calcineurin has been observed to inhibit mouse AC9 but not human AC9

[e]AC10 (sAC), a soluble adenylyl cyclase discovered in the testes, is widely distributed and functions as a $HC0_3^-$ ion sensor. It is also stimulated by Ca^{2+}, independently of calmodulin

[f]LTP, long-term potentiation in neuronal function; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; CaM, calmodulin

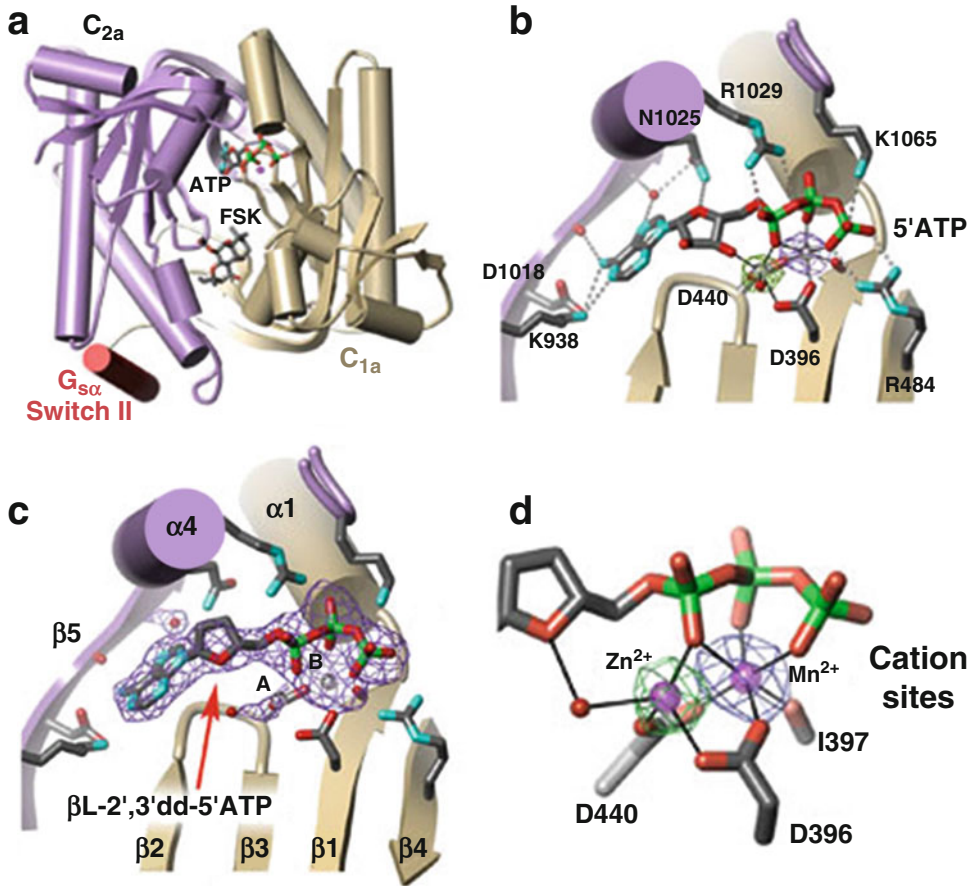
[g]Tissue distribution is based on RNA expression (RT-PCR) from 16 human tissues; physiological functions are based on overexpression or knockout studies

cyclase" were conclusively elucidated with the cloning of testis-derived cDNAs, determination of its regulation, and the subsequent solution of its bicarbonate-bound structure (Kleinboelting et al. 2014). AC10 exhibits an amino acid sequence and domain organization more closely related to cyanobacteria than to the other mammalian AC isoforms (Fig. 3). Domain composition



Adenylyl Cyclases, Fig. 3 Topology and structure of mammalian adenylyl cyclases. (**a**, **b**) Topology and structure of AC1–9 transmembrane AC isoforms. The first set of six transmembrane helices and the C_1 cytoplasmic domain are shown in gold; this structure is repeated (purple) to create the holoenzyme. (**b**) cryo-EM structure of AC9. $G\alpha_s$ (gray) binds to the C_2 domain. (**c**, **d**) Topology and schematic of AC10 (sAC). (**e**) AC10 crystal structure: the C_1 domain is shown in gold, C_2 domain in purple, N-terminus (NT) in pink, and linker domain (L) in gray. (Adapted from Baldwin and Dessauer 2018, Qi et al. 2019, and Kleinboelting et al. 2014)

includes a conserved C_1–C_2 catalytic domain and a carboxyl terminal domain of variable length, with a putative auto-inhibitory domain and other regulatory sites, but no transmembrane motifs. The short splice variant initially found in the testis contains the C_1–C_2 catalytic domains but lacks the extended C-terminus. AC10 activity is insensitive to forskolin and G-protein regulation; it is instead regulated by HCO_3^- and by Ca^{2+} (Table 2). The crystal structure of human AC10 indicates that binding of HCO_3^- disrupts a salt bridge involving an aspartate residue which is involved in the substrate ATP binding site (Kleinboelting et al. 2014). AC10 activity is sensitive to ATP levels in the cell due to its relatively low affinity for ATP, compared to AC1–9. AC10 is also more promiscuous in terms of substrate, as it can also use UTP and CTP to generate the corresponding cyclic nucleotides. Loss of HCO_3^--stimulated adenylyl cyclase activity in the testis leads to complete infertility

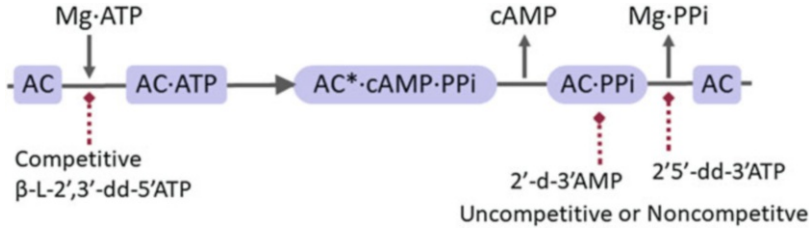


Adenylyl Cyclases, Fig. 4 Catalytic cleft and active site of a chimeric mammalian adenylyl cyclase, AC5-C_1•AC2-C_2•Gα_s. The catalytic cleft is formed by the pseudo-symmetric interaction of the cytosolic domains, C_1 and C_2. Panel (**a**): crystal structure of AC5-C_1•AC2-C_2, indicating binding sites for substrate (5′ATP) and forskolin (FSK) (only switch II domain of Gα_s that interacts with the C_2 domain of AC is shown). Panel (**b**): the catalytic active site modeled with 5′ATP; based on structure with β-L-2′,3′-dd-5′-ATP (see panel **c**). Amino acids involved in substrate binding and catalysis are indicated. Panel (**c**): structure with bound β-L-2′,3′-dd-5′-ATP and loci for two metal sites, A and B. Panel (**d**): enlargement of panel **c** with Zn^{2+} (metal A) and Mn^{2+} (metal B) used in forming the crystal. Catalysis occurs with the metal-catalyzed attack of the ribosyl 3′-OH group of the substrate α-phosphate. (Adapted from Tesmer et al. 1999)

due to defective sperm motility. Localization of sAC in the mitochondria of other cell types is important for PKA-mediated regulation of respiratory chain enzymes and ATP production, while localization in cilia may control ciliary beat frequency (Levin and Buck 2015).

Catalytic Mechanism

Catalysis by adenylyl cyclases involves cation-mediated attack of the 3′-OH on the α-phosphate of 5′-ATP, with PP_i as the leaving group. It is a reversible bireactant sequential mechanism with metal•5′-ATP as substrates and cAMP and metal•PP_i as products (Fig. 5; transition state is depicted as **AC**∗; (Dessauer and Gilman 1997)). Two metal binding sites exist in the catalytic site and are required for catalysis, similar to nucleotide polymerases (Fig. 4) (Tesmer et al. 1999). These are generally occupied by Mg^{2+}, but site A can be occupied by Zn^{2+} (an inhibitor of AC), while Mn^{2+} (an activator of AC) can occupy site B (Fig. 4). Typically, reaction velocities are



Adenylyl Cyclases, Fig. 5 Adenylyl cyclase catalytic cycle. Points during the catalytic cycle at which inhibition by competitive and uncompetitive or noncompetitive nucleotides occur are shown; AC∗ represents the catalytic transition state. (Adapted from Dessauer and Gilman 1997)

considerably greater with Mn^{2+} as cation than with Mg^{2+} due to effects on both K_mATP and V_{max}. K_m values for rat brain cyclase are K_{MnATP}, ~9 μM, and K_{MgATP}, ~60 μM. Notably, activation of adenylyl cyclases by hormones or by $G\alpha_s$, via the active enzyme configuration GTP•α_s•**AC**, causes a reduction in $K_{Mg}{}^{2+}$ of more than an order of magnitude from to $K_{Mg}{}^{2+}$ ~ 860 μM to ~50 μM, without a change in K_{MgATP}.

Miscellaneous Observations

Typical enzyme preparations, whether from native or recombinant sources, are of membranes or membrane extracts that contain enzyme activities that can alter substrate or product concentrations for adenylyl cyclases. These include activities of cyclic nucleotide phosphodiesterases (PDEs) and ATPases, among others, which must be taken into consideration in assays of adenylyl cyclase activities. In addition, the enzyme is protected by thiols, with β-mercaptoethanol and dithiothreitol being the most commonly used. Conversely, adenylyl cyclases are generally susceptible to oxidants, e.g., H_2O_2 (IC_{50} ~ 3 μM) or benzoquinone (IC_{50} ~ 3 μM), and alkylating agents, e.g., *N*-ethylmaleimide (IC_{50} ~ 100 μM). Not surprisingly, the membrane-bound enzyme is susceptible to thermal inactivation (e.g., 50% inactivation at 35° in 10 min). Finally, proteases can alter adenylyl cyclase activity for some isoforms. For example, trypsin and thrombin can cause 5–10-fold activation (AC2 > AC3 > > AC5). The basis for this activation in each case is not clear, though serine proteases are known to cleave $G\alpha_i$, and this could lead to indirect effects on adenylyl cyclase activity. Cleavage of the C-terminal auto-inhibition region of AC9 can also lead to enhanced activity due to a lowered K_m for ATP (Qi et al. 2019).

Bacterial and Other Adenylyl Cyclases

Adenylyl cyclases are found throughout the animal kingdom and serve a variety of roles. Available evidence indicates that there is little sequence homology between these adenylyl cyclases and the membrane-bound mammalian form. They are regulated quite differently from mammalian isoforms. This lack of homology between mammalian and bacterial forms could serve as an advantage, as several class II forms of adenylyl cyclase constitute a toxic factor in mammals, including enzymes from *Bordetella pertussis*, *Bacillus anthracis*, *Pseudomonas aeruginosa*, and *Yersinia pestis*. These enzymes constitute the "toxin class" of adenylyl cyclases. The well-studied adenylyl cyclases of *Bordetella pertussis* and *Bacillus anthracis* are both soluble, Ca^{2+}/calmodulin-dependent, but G-protein-independent enzymes that are exported from the respective bacteria (Tang and Guo 2009). Because these enzymes are then transported into infected cells, adenylyl cyclase actually constitutes a virulence and toxic factor in mammals. Much like the toxin from *Vibrio cholera* that ADP-ribosylates $G\alpha_s$ to increase cAMP, these toxic adenylyl cyclases increase cAMP. For example, the massive cAMP production by CyA from *Bordetella pertussis* results in inhibition of immune cell

function and contributes to pathogenesis of whooping cough. The *B. anthracis* adenylyl cyclase (800 amino acids), also known as "edema factor" (EF), and the *B. pertussis* adenylyl cyclase (1706 amino acids) are large enzymes with multifunctional domains. Some of the toxic effects of these adenylyl cyclases may not be due to cAMP production, as ExoY from *Pseudomonas aeruginosa* is actually much more effective at producing cGMP and cUMP, than cAMP. The fact that the mammalian adenylyl cyclases differ so substantially from these pathogenic virulence and toxic factors gives motive to the idea that small molecule inhibitors of the pathogen adenylyl cyclases may be discovered that do not interact with mammalian forms of the enzyme and can be used clinically to treat these devastating diseases. Derivatives of the antiviral drug PMEA (adefovir) may hold promise, as some inhibit CyA and edema factor with good selectivity over mammalian adenylyl cyclase isoforms (Cesnek et al. 2018).

Drugs

Drugs which indirectly activate or inhibit mammalian adenylyl cyclases are common, especially drugs targeting G-protein-coupled receptors. However agents that act directly on the adenylyl cyclase enzyme have been less well explored. Importantly, for most activators and inhibitors of adenylyl cyclase, high selectivity for specific isozymes has not been demonstrated. The main classes of such agents are derivatives of forskolin and of adenine nucleosides. Forskolin analogs show some selectivity for activation of select isoforms, particularly AC1, while BODIPY-modified forskolin can stimulate AC1 and AC5 but serve as an inhibitor of AC2 (reviewed in Dessauer et al. 2017). Adenosine and its derivatives have long been known to inhibit adenylyl cyclases, with modifications substantially increasing inhibitory potency. Notable are the approximately threefold increase in potency seen with the 2-fluorine substitution on adenine and the increases in potency seen with various modifications to the ribose moiety. The orientation of the ribose (α vs β) and the presence, orientation, or absence of hydroxyl groups clearly contribute to inhibitory potency (Table 3). For example, arabinose and xylose differ from ribose only in the orientation of the 2′- and 3′-OH groups yet exhibit markedly different potencies. The antiviral drug 9-(arabinose)-Ade (also known as Ara-A or vidarabine) is clinically used to treat herpes simplex and varicella zoster viruses, but exhibits some selectivity for inhibition of isoforms AC5 and AC6 (Brand et al. 2013). Of note, acyclic nucleoside phosphonate antiviral drugs (e.g., PMEApp, also known as adefovir) can inhibit mammalian adenylyl cyclases (IC_{50} = 170 nM), the *Bacillus anthracis* toxin edema factor (K_i = 27 nM), and the *Bordetella pertussis* toxin CyaA (K_i = 25 nM), while 7-halo-7-deazapurine analogs of PMEA are potent and selective inhibitors of AC1 (Cesnek et al. 2018).

SQ 22,536 (9-(tetrahydrofuryl)-Ade) lacks the hydroxyl groups present in Ara-A or 9-(xylose)-Ade and is less potent, but offers metabolic and biochemical stability useful for many types of

Adenylyl Cyclases, Table 3 ***Nucleoside inhibitors of adenylyl cyclase***. Assays were with a detergent-dispersed adenylyl cyclase from rat brain and were with 100 μM 5′ATP and 5 mM $MnCl_2$ as substrates. Common name for frequently used inhibitors is indicated in parenthesis

Nucleoside	IC_{50} (μM)
β-Adenosine	82
α-Adenosine	>300
9-(arabinose)-Ade (Ara-A; vidarabine)	30
9-(xylose)-Ade	3.2
9-(Tetrahydrofuryl)-Ade (SQ 22,536; THFA)	20
β-2′,3′-dd-ado	9
β-2′,5′-dd-ado	2.8
β-2′,5′-dd-2-F-ado	0.89
β-2′,5′-dd-2,5′-di-F-ado	0.98
Non-nucleoside derivatives	
NKY80	~8–1000

studies. NKY 80 is a non-nucleoside derivative of SQ 22,536. Both SQ 22,536 and NKY 80 show selectivity among the adenylyl cyclase isoforms, inhibiting types 5, 6 > 1, 3 > 2, 4, 7 > 8, 9 (Brand et al. 2013). It is, however, the removal of two of the hydroxyl groups that elicits the largest improvement in inhibitory potency, in particular the 2′,5′-dideoxy modification (Table 3). With these improvements in potency, these cell-permeable compounds, in particular 2′,5′-dd-Ado, Ara-A, SQ 22,536, and NKY 80, have become useful research tools to inhibit transmembrane adenylyl cyclases and lower cAMP levels in isolated cells or intact tissues; these three inhibitors poorly inhibit AC10, with IC_{50} values greater than 1 mM (Bitterman et al. 2013).

An early observation that 2′-d-3′-AMP was a more potent inhibitor of adenylyl cyclases than 2′-d-Ado suggested that the enzyme would accept substitutions at the 3′-ribose position and that phosphate was particularly well tolerated. This led to the generation of a family of 3′-phosphoryl derivatives of 2′,5′-dideoxyadenosine exhibiting ever greater inhibition with the addition of an increasing number of 3′-phosphoryl groups, the most potent of which is 2′,5′-dideoxyadenosine-3′-tetraphosphate (2′,5′-dd-3′-A4P; Table 4) (Desaubry et al. 1996). These constitute a class of inhibitors historically referred to as P-site ligands that caused inhibition of adenylyl cyclase that was kinetically either noncompetitive or uncompetitive (cf. Figure 5; Dessauer and Gilman 1997). Uncompetitive inhibition was due to P-site binding to the product-like transition state (AC-PPi); the polyphosphate inhibitors (2′,5′-dd-3′ATP and 2′,5′-dd-3′-A4P) are non-competitive, binding either AC or AC-PPi state (PPi may simply promote the high-affinity product-like transition state) (Tesmer et al. 2000). Since a round of catalysis is required to generate the AC-PPi state, this mode of inhibition means that potency is dependent on the activity state of the enzyme. The highest potency by P-site inhibitors is observed when adenylyl cyclase is most active. All tested membrane-bound forms of mammalian adenylyl cyclase are inhibited by adenine nucleosides and their 3′-polyphosphates derivatives; bacterial adenylyl cyclases are largely unaffected. Inhibition of AC10 has not been extensively tested but shows inhibition by 2′,5′-dd-3′-ATP (IC_{50} = 0.69 μM) (Bitterman et al. 2013).

All adenylyl cyclases are inhibited competitively by substrate analogs, which bind to the enzyme active site (cf. Figure 4). One of the best competitive inhibitors is β-L-2′,3′-dideoxyadenosine-5′-triphosphate (β-L-2′,3′-dd-5′-ATP; Table 4) (Shoshani et al. 1999). The two inhibitors, 2′,5′-dd-3′-ATP and β-L-2′,3′-dd-5′-ATP (Fig. 6), are comparably potent (Table 4), but inhibit adenylyl cyclase by conformationally distinct mechanisms (cf. Figure 5) by binding within the catalytic cleft in unique structures (Fig. 4).

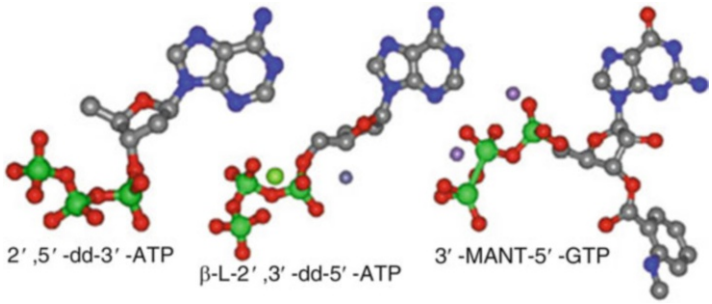
Development of 2′(3′)-*O*-MANT-derivatives of nucleoside 5′-triphosphates took advantage of the tolerance to large substitutions at the 3′-ribose position (Gille and Seifert 2003) (Table 4). It was surprising, though, that potent inhibition was seen with bases other than adenine, implying that base specificity is less stringent than had been generally assumed. Subsequently, fluorescent derivatives have been made with different fluorophores at 2′- and 3′-positions (see Seifert et al. 2012 and Dessauer et al. 2017). 3′-Substitutions showed advantage over corresponding 2′-substitutions, and 2′(3′)- *O*-MANT-substitutions were clearly preferable to coumarin and dansyl derivatives, but followed the order of guanosine ≥ inosine > adenosine (Table 4). Unfortunately, the MANT-derivatives also inhibit soluble guanylyl cyclase and interact with numerous nucleotide-binding proteins (Seifert et al. 2012).

Inhibitors of the soluble adenylyl cyclase AC10 have not been as well explored as their other mammalian counterparts. AC10 is relatively insensitive to the adenosine analogs (Table 3), but the polyphosphate 2′,5′-dd-3’-ATP will inhibit AC10 (IC_{50} = 0.69 μM), with only a ~15-fold reduction in potency (Bitterman et al. 2013). The catechol derivative of estrogen (2 CE) was the first known inhibitor of AC10, but it shows weak to no selectivity over AC1–9. LRE1 and KH7 are significantly more potent for AC10 compared to the transmembrane isoforms AC1–9 (Table 5). However, KH7 displays off-target and toxic cellular

Adenylyl Cyclases, Table 4 ***Nucleotide inhibitors of adenylyl cyclase***. Enzyme source and assay conditions were as for Table 3. Values obtained for 3′-ATP are overestimations due to the formation of 2′:3′-cAMP from 3′-ATP that occurs nonenzymatically in the presence of divalent cation

Adenine nucleoside 3′-phosphates (IC_{50}, μM)			
3′-Phosphate	Ado	2′-d-Ado	2′,5′-dd-Ado
None	82	15	2.7
3′ ∼ P	8.9	1.2	0.46
3′ ∼ PP	3.9	0.14	0.1
3′ ∼ PPP	2	0.09	0.04
3′ ∼ PPPP	–	0.011	0.0074
3′ ∼ PS	–	3.1	0.6

Substrate analogs (IC_{50}, μM)	
β-D-5′-AP(CH_2)PP	30
β-L-5′-ATP	3.2
β-D-2′,3′-dd-5′-ATP	0.76
β-L-2′,3′-dd-5′-ATP	0.024
2′- and 3′-Substituted-5′-NTPs (IC_{50}, μM)	
2′(3′)-MANT-5′-GTPγS	0.02
2′(3′)-MANT-5′-ITPγS	0.039
2′(3′)-MANT-5′-ATP	0.064
3′-MANT-2′-d-5′-ATP	0.14
Additional selective inhibitors (IC_{50}, μM)	
SKF-83566 (AC2)	5.0
ST034307 (AC1)	2.3



Adenylyl Cyclases, Fig. 6 Structures of potent inhibitors of adenylyl cyclase. Structures for 2′,5′-dd-3′-ATP (IC_{50} ∼ 40 nM; noncompetitive inhibitor), β-2′,3′-dd-5′-ATP with Mg^{2+} and Zn^{2+} (IC_{50} ∼ 24 nM; competitive inhibitor), and 3′-MANT-GTP with Mn^{2+} (IC_{50} ∼ 90 nM; competitive inhibitor) are from coordinates obtained for these compounds in respective crystal structures with AC5-$\mathbf{C_1}$•AC2-$\mathbf{C_2}$. The 3′-MANT-group fits into a hydrophobic pocket of the enzyme. Note the difference in contortion of the phosphate chains in these structures relative to positions for divalent cation (shown as small spheres: Mn^{2+}, purple; Mg^{2+}, green; Zn^{2+}, blue)

effects, and its mechanism of inhibition is poorly understood (Bitterman et al. 2013). LRE1 is a more recently developed inhibitor that binds to the bicarbonate allosteric site of AC10 and displays less toxicity than KH7 (Ramos-Espiritu et al. 2016).

Cellular Inhibition of AC

Although the 3′- and 5′-polyphosphate derivatives of the MANT- and related derivatives mentioned above exhibit exquisite inhibitory potency, these compounds are not cell permeable. To take advantage of the potency of such derivatives for studies

Adenylyl Cyclases, Table 5 ***Inhibitors of AC10***. IC_{50} is based on inhibition of purified AC10, while determination of AC1–9 (mAC) used Mn^{2+}/forskolin-stimulation (KH7) or $G\alpha_s$-stimulation (LRE1) of cell lysates (Bitterman et al. 2013)

AC10 (sAC) inhibitors	AC10 (IC_{50}, μM)	Inhibition of mAC
KH7	3.0	None at 500 μM
LRE1 (RU-0204277)	~10	None at 50 μM

Adenylyl Cyclases, Table 6 ***Prodrug inhibition of cAMP formation in intact cells***. The indicated cells were treated for 15 min with 50 μM forskolin and prodrug nucleotides

	OB-1771 preadipocytes	THP1 monocytes
Pronucleotide	IC_{50} (nM)	
2′,5′-dd-3′-AMP-bis(me-SATE)	6.7	260
2′,5′-dd-2F-Ado-3′-P-bis(me-SATE)	9.8	110

with intact cells and tissues, prodrugs have been developed. Prodrugs are precursor molecules that are cell permeable and are then metabolized into an inhibitor by intracellular enzymes. Families of protected monophosphate derivatives have been generated that exhibit all the hallmarks of prodrugs. They are taken up, deprotected, and converted to extremely potent inhibitors of adenylyl cyclase, *but only by intact cells and tissues* (Laux et al. 2004). These prodrugs have been used to block cAMP formation in isolated cells and intact tissue and elicit functional effects (Table 6); however, most MANT-nucleotide derivatives bind other nucleotide-binding proteins.

Cell-permeable AC inhibitors include the nucleoside-based inhibitors and their analogs, particularly 2′,5′-dd-Ado, Ara-A, SQ 22,536, and NKY 80; although not particularly potent, the latter three are widely used but display some isoform selectivity for AC5 and AC6 which limits general use (Brand et al. 2013). One should note that SQ 22,536 and NKY80, but not Ara-A, also serve as antagonists for adenosine A1 and A2a receptors. Other commercially advertised adenylyl cyclase inhibitors should be used with considerable caution. MDL 12330A is a widely used inhibitor for membrane isoforms in intact studies but only shows modest inhibition of AC2 and AC3 (IC_{50} ~100 μM) and not AC5 or AC10 (Seifert et al. 2012; Bitterman et al. 2013); it is not recommended for cellular studies due to its low potency and pleotropic effects. Similarly NB001 is sold as an AC1-selective inhibitor, but it does not inhibit AC1 directly, and its mechanism of action is unknown (Seifert et al. 2012; Brand et al. 2013). Several isoform-selective adenylyl cyclase inhibitors have been developed for cellular-based assays, including SKF83566 (~20-fold selectivity for AC2 over AC1, AC5) (Conley et al. 2013) and ST034307 (selective for AC1 at 30 μM over AC1–9 in cell-based assays) (Brust et al. 2017). Note SKF83566 is also a potent antagonist of dopamine D1 and 5HT-2 receptors (K_i ~0.5 and 11 nM, respectively). The 7-halo-7-deazapurine analogs of PMEA also hold promise for AC1 (IC_{50} = 4–5 μM; tenfold selectivity over AC1, AC5), but also inhibit *B. pertussis* adenylyl cyclase (Cesnek et al. 2018). Currently no drugs on the market exert their main therapeutic effects via direct inhibition of adenylyl cyclase. Clearly additional work is needed to identify not only general inhibitors of mammalian adenylyl cyclase isoforms but also isoform-selective agents that are cell permeable.

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Adipocyte-Derived Factors

► Adipokines

Adipocytokines

► Adipokines

Adipokines

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Synonyms

Adipocyte-derived factors; Adipocytokines

Definition

Adipose tissue (AT) expresses and secretes a variety of bioactive peptides, termed adipokines, which affect important biological processes in organs such as the liver, brain, pancreas, muscle, intestine, or vascular system. Adipokines play an important role in the regulation of appetite and satiety, energy metabolism, insulin sensitivity and secretion, and inflammatory response (Fasshauer and Blüher 2015). Adipokine secretion reflects the functional state of AT and is an important mechanistic link between obesity and metabolic (e.g., type 2 diabetes (T2D), nonalcoholic fatty liver disease (NAFLD)), cardiovascular (e.g., hypertension, coronary artery disease, stroke), and other diseases (e.g., certain types of cancer, asthma, gout, osteoarthritis) (Fig. 1). Some adipokines are already used as pharmacological treatment or targets or are in early stages of clinical development. In the future, adipokines could gain clinical importance as circulating markers or predictors for metabolic and cardiovascular diseases, for the treatment success of weight-reducing therapies, and also for pharmacological therapy approaches (Blüher 2014).

Basic Characteristics

Adipose Tissue as an Endocrine Organ

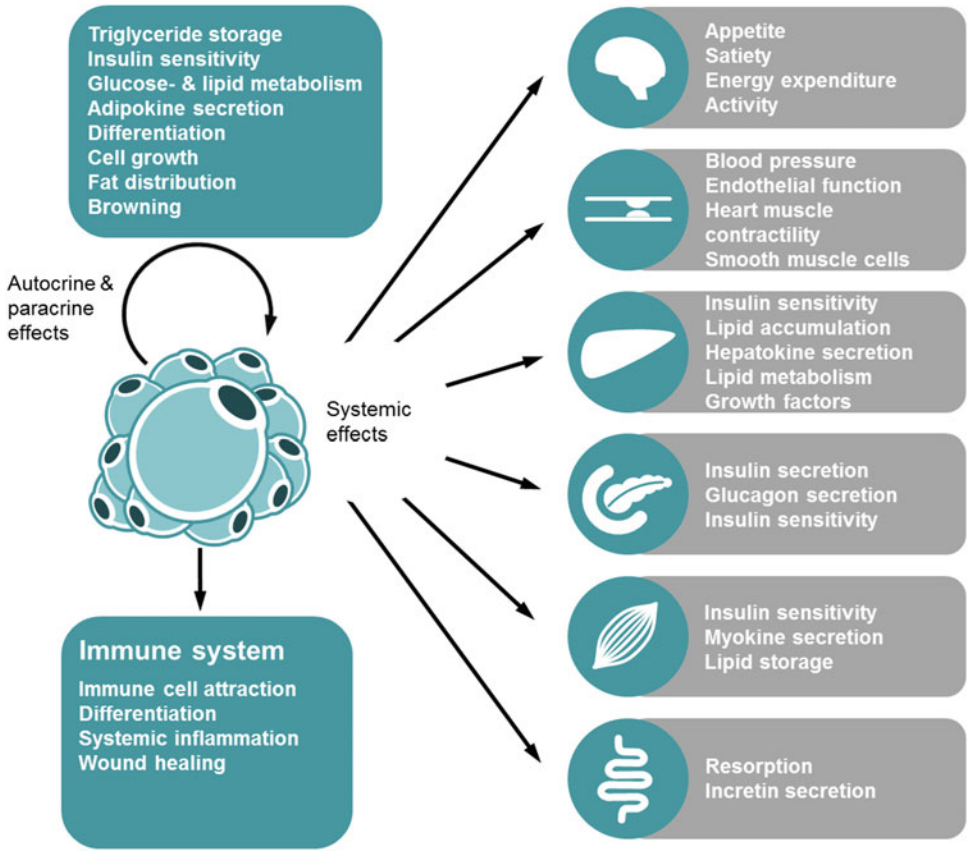
In addition to its important functions as an energy store, mechanical organ protection, and thermoregulation, AT is an active endocrine organ that produces hundreds of peptide hormones known as adipokines (Table 1). Genetic and environmental factors as well as energy overload affect AT size, cell composition in AT, and function. Thus, in obesity, the function of AT is often disturbed. The resulting changes in circulating adipokine levels increase the risk of developing a variety of diseases or predisposing states such as insulin resistance (IR) (Fig. 2) (Blüher 2014).

Therefore, adipokines are considered as valuable tools or targets for the treatment of metabolic and cardiovascular diseases (Table 2).

Leptin: An Adipokine Dysregulated in Obesity Which Influences Appetite, Body Weight, and Vascular Function

Since the discovery of leptin by the group of Jeffrey Friedman in 1994, quickly followed by the cloning of the leptin receptor in 1995, much progress has been made in our understanding of the neuroendocrine control of feeding, energy expenditure, and ultimately whole-body energy homeostasis. Although controversial at the time it was proposed four decades ago, Douglas Coleman's early hypothesis that a circulating factor released from white adipocytes communicates to the brain to regulate body weight (BW) proved to be true with the discovery of leptin (Fève and Bastard 2012).

Leptin signaling is regulated from its release by adipocytes to its transport into the brain where it activates the leptin receptor in key neuro-circuits of feeding control. Plasma leptin concentration is directly proportional to body fat mass, but high leptin levels in obesity are not sufficient to suppress appetite and enhance satiety most likely due to central leptin resistance.



Adipokines, Fig. 1 Different para- and endocrine functions of adipose tissue-derived adipokines. (Modified from Fasshauer and Blüher (2015))

In accordance with an appetite-suppressive effect, leptin-deficient *ob/ob* mice, as well as rare cases of leptin loss of function mutations in humans, develop severe hyperphagia and excessive obesity associated with IR, hyperinsulinemia, and T2D. Moreover, leptin possesses its anti-atherogenic effects indirectly through reduction of hypercholesterolemia and liver steatosis, as well as upregulation of insulin-sensitizing and atheroprotective adiponectin (Hoffmann et al. 2016). Administration of recombinant leptin in *ob/ob* mice and leptin-deficient humans reduces food intake, body weight, and fat mass suggesting that the absence of functional leptin is responsible for the obese phenotype in these states. For the rare cases of monogenetically inherited leptin deficiency in humans, recombinant leptin is now available for compassionate use and leads to significant weight loss. Leptin (metreleptin) is also an approved medication for patients with lipodystrophy (Oral et al. 2002), and further indications such as hypothalamic amenorrhea or Rabson-Mendenhall syndrome are under consideration (Brown et al. 2013).

Adiponectin: Insulin Sensitizer and Potential Endothelial Protector

Adiponectin was cloned in 1995 and 1996 by four independent groups using different experimental approaches (Fasshauer and Blüher 2015). However, only since 2001, it has become clear that adiponectin, unlike all other known adipokines, has a profound insulin-sensitizing effect. Some studies show that adiponectin increases insulin-

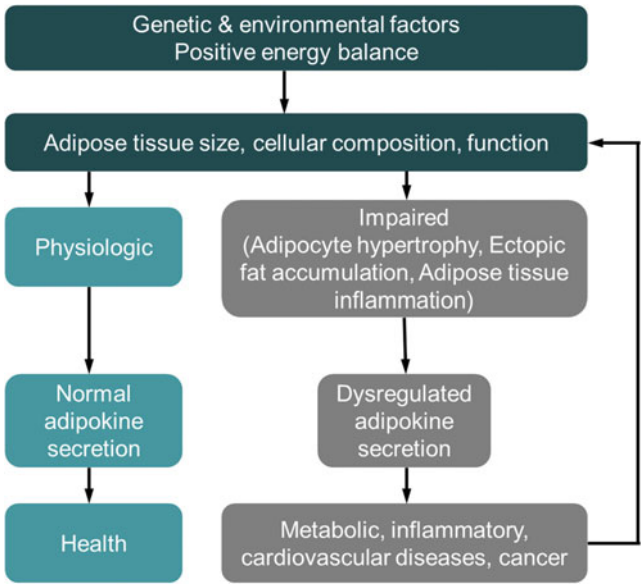
Adipokines, Table 1 Overview about selected adipokines. AdipoQ, adiponectin; C1Q, and collagen domain containing; AFABP, adipose fatty acid-binding protein; ANGPLT8, angiopoietin-like protein 8; AT, adipose tissue; BM, bone marrow; BMP, bone morphogenetic protein; CCL2, CC-chemokine ligand 2; CFD, complement factor D; DDP4, dipeptidyl peptidase 4; FABP4, fatty acid-binding protein 4; FGF21, fibroblast growth factor 21; FIZZ3, cysteine-rich secreted protein; GDF15, growth differentiation factor 15; GLP-1, glucagon-like peptide 1; GIP, glucose-dependent insulinotropic polypeptide; IL-1β, interleukin-1β; IL-1R, interleukin-1β receptor; IR, insulin resistance; MIC-1, macrophage inhibitory cytokine 1; NAG-1, nonsteroidal anti-inflammatory drug-activated gene-1; NGAL, neutrophil gelatinase-associated lipocalin; MCP1, monocyte chemotactic protein 1; NAMPT, nicotinamide phosphoribosyltransferase; OP-1, osteogenic protein-1; PDF, prostate-derived factor; PLAB, placental bone morphogenetic protein; PTGFB, anti-placental TGF-β; Rarres2, retinoic acid receptor responder protein 2; RBP4, retinol-binding protein 4; RIFL, refeeding induced in fat and liver; SP-40, seminal clusterin 40; TGFβ, transforming growth factor β; TIG2, tazarotene-induced gene 2; TIMP-1, tissue inhibitor of metalloproteinase 1; TNF-α, tumor necrosis factor α; VEGF, vascular endothelial growth factor

Adipokine	Synonyms	Mainly expressed and secreted by	Main functions
Adiponectin	AdipoQ	AT	Improvement of insulin sensitivity, antidiabetic, anti-atherogenic, and anti-inflammatory
Adipsin	CFD	AT	Activation of the complement pathway, beneficial role in maintaining β-cell function
ANGPLT8	RIFL	Liver, AT	Improvement of glucose tolerance, promotion of β-cell proliferation
Apelin		Heart, AT, placenta	Inhibition of insulin secretion
Asprosin	Fibrillin 1	AT, placenta	Induction of hepatic glucose production
BMP4		Small intestine, liver, AT	Regulation of adipogenic precursor cell commitment and differentiation, bone and joint development
BMP7	OP-1	Thyroid, placenta, AT	Brown adipogenesis stimulation, food intake reduction
Cathepsins S,L,K		Placenta, AT	Regulation of glucose metabolism and adipose tissue mass
Chemerin	Rarres2, TIG2	AT, liver	Regulation of adipogenesis, chemoattractant protein
Clusterin	SP-40, Apolipoprotein J	Brain, liver, testis, AT	Activation of tumor progression and angiogenesis
DPP4	Cd-26	Small intestine, duodenum, AT, liver	Degradation of GIP and GLP-1
FABP4	AFABP; ap2	AT	Fatty acid uptake, transport, and metabolism
Fetuin-A	Alpha 2-Heremans-Schmid glycoprotein	Liver, AT	Bone metabolism regulation, vascular calcification, IR
FGF21		Liver, AT	Stimulation of adipocyte glucose uptake, activation of thermogenesis, energy expenditure, improvement of glucose, and lipid metabolism
GDF15	PDF; MIC1; PLAB; NAG-1; PTGFB	AT, liver	Biomarker of various pathological states, such as certain tumors, inflammatory diseases, cardiovascular disease, obesity
Gremlin-1		Liver, AT	Inhibition of BMP4 and BMP7
IL-1β		Macrophages	Inflammation
IL-6		Macrophages	Inflammation
Leptin		AT, placenta	Regulation of energy homeostasis, satiety, immune and inflammatory responses, hematopoiesis, angiogenesis, reproduction

(continued)

Adipokines, Table 1 (continued)

Adipokine	Synonyms	Mainly expressed and secreted by	Main functions
Lipocalin 2	NGAL	AT, liver, immune cells	Regulation of IR and inflammation
MCP-1	CCL2	Macrophages	Inflammation, chemoattraction
Nesfatin-1	Nucleobindin derivative 2	AT, testis	Modulation of energy metabolism
Omentin	Intelectin-1	AT	Insulin sensitizer, anti-inflammatory
Progranulin		AT, lung, BM, immune cells	Inflammation, chemoattraction
RBP4		Liver, adipose tissue	Hepatic steatosis, lipid and glucose metabolism, IR
Resistin	FIZZ3	BM, AT, duodenum	Aging, IR, osteoarthritis
TGFβ		Immune cells	Regulation of proliferation, differentiation, and apoptosis
TIMP-1		Placenta, AT, gall bladder	Glucose metabolism
TNF-α		Macrophages	Inflammation
Vaspin		AT, skin	Regulation of food intake and glycemia
VEGF		Endothelial cells	Stimulation of angiogenesis
Visfatin	Nampt	BM, AT	β-Cell function
Wnt1-inducible signaling pathway protein 1		AT	Regulation of adipogenesis and inflammation in AT



Adipokines, Fig. 2 Adipose tissue as an endocrine organ with impact on severe disease entities. (Modified from Fasshauer and Blüher (2015))

Adipokines, Table 2 Adipokine-based pharmacological treatment strategies. BMP7, bone morphogenetic protein 7; DDP4, dipeptidyl peptidase 4; FGF21, fibroblast growth factor 21; GDF15, growth differentiation factor 15; GLP-1, glucagon-like peptide 1; GIP, glucose-dependent insulinotropic polypeptide; IL-1β, interleukin-1β; IL-1R, interleukin-1β receptor; OP-1, osteogenic protein-1; TNF-α, tumor necrosis factor-α

Adipokine	Drug name (agonist/ antagonist)	Mechanism of action (therapeutic potential)	Stage of drug development	Reference
Adiponectin	AdipoRon	Orally active, synthetic small-molecule, adiponectin receptor agonist	Preclinical	(Okada-Iwabu et al. 2013)
BMP7	OP-1	Human recombinant BMP7	Approved for local treatment of bone fractures	(Vaccaro et al. 2008)
DDP4	For example, alogliptin, sitagliptin, linagliptin, saxagliptin, vildagliptin	Delayed degradation of GLP-1 and GIP hormones	DPP4 inhibitors widely used for treatment of type 2 diabetes	(Nauck 2011)
FGF21	LY2405319	Synthetic FGF21 variant	Phase II clinical trial	(Gaich et al. 2013)
GDF15	Recombinant GDF15 (HAS-GDF15)	Half-life extended GDF15	Preclinical (rodent and nonhuman primate studies)	(Mullican et al. 2017)
IL-1β	For example, Anakinra	Human recombinant IL-1R antagonist	Approved for the treatment of rheumatoid arthritis	(Larsen et al. 2007)
Leptin	Metreleptin	Synthetic leptin analogue	Approved for compassionate use for patients with congenital leptin deficiency	(Chou and Perry 2013)
TNF-α	For example, infliximab, etanercept	TNF-a neutralization	Approved for the treatment of rheumatoid arthritis	(Stanley et al. 2011)

induced inhibition of glucose secretion from hepatocytes. Adiponectin also has an insulin-sensitive effect on skeletal muscle, with a C-terminal 16 kDa fragment in particular very potent in stimulating fatty acid oxidation and thus lowering plasma glucose levels (Fasshauer and Blüher 2015). Adiponectin signals to target cells through its receptors AdipoR1, AdipoR2, and T-cadherin. AdipoR1/AdipoR2 double-knockout mice exhibit a phenotype with IR and impaired glucose tolerance, increased inflammation, and oxidative stress in the skeletal muscle, liver, and AT, leading to increased gluconeogenesis and decreased glucose uptake in these mice (Iwabu et al. 2019; Yamauchi et al. 2007). Thus, while adiponectin may indirectly inhibit atherosclerosis through effects on glucose and lipid metabolism, it also directly acts on vascular endothelial and inflammatory cells, thus directly inhibiting atherosclerosis in humans (Iwabu et al. 2019). Studies in mice lacking the adiponectin gene also show that the intima media of vessels was significantly thicker compared to control animals, suggesting a function of adiponectin as an endothelial protective factor (Fasshauer and Blüher 2015). In line with the role of adiponectin as an endogenous insulin sensitizer, in several clinical studies, low adiponectin levels are associated with an increased risk of developing T2D (Fasshauer and Blüher 2015). In obesity, adiponectin serum levels are decreased. Moreover, high circulating adiponectin levels are associated with a significantly lower risk of myocardial infarction in humans (Iwabu et al. 2019). Adiponectin/AdipoR-enhancing and AdipoR-activating agents are potential pharmacological tools for the treatment of obesity and the metabolic syndrome. Indeed, the small-molecule AdipoR-activating compound (adiponectin receptor agonist,

AdipoRon) does not only improve metabolism at the organ level (e.g., skeletal muscle, liver, and AT) but provides antidiabetic properties at the organism level, while it normalizes a shortened life span due to obesity (Okada-Iwabu et al. 2013). In more detail, diminished adiponectin-associated metabolic effect in AdipoR1-/AdipoR2-knockout mice and restoration of obesity-induced metabolic alterations in high-fat-diet-fed mice by AdipoRon administration with implication on fatty acid combustion suggest that AdipoRon is a promising agent for treating T2D (Holland et al. 2017).

Thus, orally active small-molecule adiponectin receptor agonists, such as AdipoRon, could potentially be an important therapeutic agent for patients with NAFLD, T2D, or other comorbidities of obesity. Until today, there are no clinical studies available that directly test the effects of adiponectin or adiponectin analogues on human insulin sensitivity and arteriosclerosis.

FABP4: Lipid Transport Protein Implicated in Several Aspects of the Metabolic Syndrome

Fatty acid-binding protein (FABP) 4 has been identified in a cDNA library from differentiated 3 T3-L1 adipocytes in 1984 and encodes a polypeptide with a molecular mass of 14.6 kDa (Furuhashi 2019). FABP4 mRNA and protein production are significantly induced during adipocyte differentiation. Results from in vitro and in vivo studies suggest that FABP4 expression in adipocytes induces IR, triglyceride (TG) release, and pro-inflammatory gene expression. Furthermore, expression of FABP4 in macrophages appears to be crucial for its effects on atherosclerosis. Thus, FABP4 induces foam cell development by increasing cholesterol and TG accumulation, as well as by inducing expression of pro-inflammatory genes (Hotamisligil and Bernlohr 2015). Macrophage infiltration into AT is stimulated by FABP4. These data indicate that FABP4 might be an interesting therapeutic target for metabolic and vascular disease even if a FABP4 receptor has not yet been discovered. Since 2006, data have accumulated that circulating FABP4 concentrations are positively correlated with markers of the metabolic syndrome and vascular disease in various cross-sectional (Kralisch and Fasshauer 2013).

Several drugs, including statins, eicosapentaenoic acid (EPA)/docosahexaenoic acid (DHA) agents, angiotensin II receptor blockers, and dipeptidyl peptidase-4 inhibitors (DPP4i), can decrease FABP4 levels (Furuhashi 2019). The specific FABP4 inhibitor BMS309403 is an orally active small molecule that interacts with the fatty acid-binding pocket within the interior of FABP4 to inhibit binding of endogenous fatty acids. Treatment with BMS309403 has beneficial effects by improving IR, T2D, NAFLD, and atherosclerosis in experimental models, indicating a therapeutic strategy against several aspects of metabolic syndrome due to chemical inhibition of FABP4. Furthermore, treatment with antagonists of receptors for FABP4, especially PA-bound FABP4, would be a novel therapeutic strategy but require the identification of a FABP4 receptor. In 2015, targeting high serum FABP4 levels using a monoclonal antibody (mAb, CA33) to inhibit FABP4 was introduced (Burak et al. 2015). The antidiabetic effect of CA33 is predominantly linked to the regulation of hepatic glucose output and peripheral glucose utilization. Although no clinical trials have been reported, these results suggest FABP4 mAb-mediated treatment of T2D.

FGF21: Beneficial Effects on Glucose and Lipid Metabolism in Multiple Target Organs

The fibroblast growth factor (FGF) family is a group of multifunctional signaling molecules that have a wide variety of functions. FGF21 was first identified as a 23 kDa protein that increases insulin-independent glucose uptake in murine adipocytes (Sonoda et al. 2017). FGF21 is expressed in several metabolically active organs such as the AT, liver, and muscle and interacts with different tissues (Itoh 2014). FGF21 signaling is mediated by activation of FGF receptor 1 (FGFR1) c with β-Klotho as a cofactor.

FGF21 has thermogenic effects mediated through activation of FGF21 expression in white AT and brown AT expressing Fgfr1c and β-Klotho after cold exposure. In humans, FGF21 activates the thermogenic machinery against hypothermia.

Adiponectin acts as a downstream effector of FGF21 in WAT and mediates the effects of FGF21 on energy metabolism and insulin sensitivity in the liver and skeletal muscle.

Fgf21 single polymorphisms are possibly related with metabolic diseases. In obesity, there are elevated levels of FGF21 and exogenous FGF21 does not work as usual. This fact suggests that obesity is an FGF21-resistant condition (Fisher et al. 2010).

FGF21 treatment leads to beneficial metabolic effects in animal models (Itoh 2014). Therefore, great efforts are being made to identify a pharmacological target. Over the last decade, FGF21 analoga and other forms of agonists that directly activate the FGFR1c/ß-receptor complex have been tested in nonhuman primates and humans, revealing their potential to ameliorate obesity and obesity-related comorbidities. At least nine FGF21-class molecules have been tested in humans, and several compounds are still in different stages of clinical development for the treatment of T2D and NAFLD. To date, approaches targeting FGF21 for anti-obesity and antidiabetic treatment include modified FGF21 as well as agonistic antibodies against the FGF21 receptor – FGF receptor 1 (FGFR1) and the obligatory co-receptor βKlotho. In addition, a novel approach to increase endogenous FGF21 activity by inhibiting the FGF21-degrading protease fibroblast activation protein (FAP) is discussed.

In proof of principle clinical trials, LY2405319, a variant of human FGF21, and PF05231023, a bivalent fusion of human FGF21 to human IgG have been tested (Sonoda et al. 2017). LY2405319 has beneficial effects in these phase 1, placebo-controlled, blinded studies on body weight reduction and fasting insulin and caused significant improvements in dyslipidemia (Sonoda et al. 2017). Noteworthy, the compound did not significantly improve glucose concentrations. The mechanisms contributing to weight loss and other metabolic effects were not clear as food intake or energy expenditure was not measured nor reported in either study. Based on preclinical data, it is likely that FGF21 analogues will affect both energy intake and expenditure; however, the relative contribution of these pathways to weight loss in human subjects is still unknown (Sonoda et al. 2017).

Long-acting FAP-resistant FGF21 analogues and FGFR1/β-Klotho agonist antibodies with a superior pharmacokinetic profile represent an exciting opportunity to expand the therapeutic potential of FGF21-class molecules. In addition, a combination therapy utilizing fibrate and a FAP inhibitor may offer an oral therapy to augment endogenous FGF21 action. Further preclinical and clinical studies should determine whether any of these approaches will be viable to approach the global obesity and T2DM epidemic (Sonoda et al. 2017).

BMP7: An Adipokine that Induces Brown Adipogenesis and Reduces Food Intake and Weight Gain

Bone morphogenetic protein (BMP) 7 is a member of the transforming growth factor (TGF)-β superfamily promoting brown adipocyte differentiation and thermogenic function in adipose progenitor cells and increases brown AT-mediated energy expenditure in vivo (Tseng et al. 2008). Recently, several members of the BMP family of ligands and receptors have been found to associate with obesity-related traits in humans (Blüher 2014); however, a detailed mechanism for how the BMPs affect energy balance and obesity remains to be elucidated. Clinical trials studying the effects of BMP and growth factors in the treatment of pseudoarthrosis and bone defects as well as in coated implants will shed further light on the significance of these factors in inducing bone growth as well as on BMP7 inducing brown adipogenesis, reducing food intake, increasing energy expenditure, and reducing weight gain (Townsend et al. 2012).

Recombinant BMP, marketed under the brand name OP-1 (osteogenic protein 1), is already available for 10 years, but there is little data on standardized clinical implementation in humans. The results of 120 applications are currently assembled, and approval of BMP-7 by the Food and Drug Administration (FDA) is expected in the next few years. Whether the encouraging results of experimental animal studies can be applied to treat human obesity

and metabolic disease will be revealed in the future.

Chemerin: Multifaceted Adipokine Displays Various Roles in the Pathogenesis of Inflammation and Metabolic Disease

Chemerin encoded by the gene retinoic acid receptor responder 2 (Rarres2), also known as tazarotene-induced gene 2 (TIG2), is an adipokine with autocrine, paracrine, and even endocrine roles in vivo (Helfer and Wu 2018). Chemerin is a chemokine highly expressed in the white AT, liver, and lung, while its receptor chemerin chemokine-like receptor 1 (CMKLR1) is predominantly expressed in adipocytes and immune cells. Interestingly, chemerin increases food intake in seasonal animals by acting on hypothalamic stem cells, the tanycytes. In peripheral tissues, chemerin increases cell expansion, inflammation, and angiogenesis in AT, collectively resulting in adiposity (Helfer and Wu 2018).

High chemerin levels are associated with a significant risk for developing metabolic syndrome. It is hypothesized that chemerin is involved in the pathogenesis of metabolic diseases by regulating meta-inflammation, adipocyte plasticity, and glucose metabolism.

With regard to therapeutic options, the novel chemerin (ChemR23) antagonist CCX832 has been shown to protect against chemerin-related arterial contraction, thus linking higher chemerin concentrations in obesity to impaired vascular function (Watts et al. 2013). Sequence analysis revealed a PPARγ response element within the chemerin promoter (Muruganandan et al. 2011); thus, insulin-sensitizing drugs that activate PPARγ might be beneficial for the treatment of obesity and T2D. In support, in normal and leptin-deficient *ob/ob* mice, the PPARγ agonist rosiglitazone increased the expression of chemerin transcript in AT and raised plasma chemerin levels (Helfer and Wu 2018). However, pioglitazone and metformin decreased plasma chemerin levels in patients with T2D (Helfer and Wu 2018). It is therefore not clear whether an agonist or antagonist of chemerin action might be useful for the treatment of T2D and the contribution of chemerin to the pathology of obesity and T2D remains elusive.

Specifically, the pharmacology and signaling properties of chemerin receptors in the hypothalamus have not been characterized. Given the importance of the hypothalamus in sensing and integrating peripheral signals, studies into hypothalamic chemerin signaling might help explaining some of the contradictory findings on the mode of chemerin's action. Furthermore, it is important to understand whether chemerin exerts a pro- or anti-inflammatory response or if the effect is indeed bimodal in different biological systems. Chemerin remains a promising target candidate for the pharmacological treatment of obesity.

Fetuin-A: Circulating Protein Linked to Insulin Resistance and Obesity

Fetuin-A, also termed alpha2-Heremans-Schmid glycoprotein, is a 46 kDa adipokine with multifaceted functions mainly due to its potential interaction with the insulin receptor. In diabetes models, it has been shown that fetuin-A binds the β-subunit of the insulin receptor to attenuate insulin signaling, thereby contributing to IR in T2D. Fetuin-A may also, together with free fatty acids, induce apoptotic signals in pancreatic beta cells, reducing the secretion of insulin and further exacerbating T2D. Associations of fetuin-A levels with the metabolic syndrome have been described (Jirak et al. 2019). Fetuin-A may also serve as a protective agent in severe systemic inflammation. A novel aspect is the putative role of fetuin-A in AT inflammation.

From the clinical perspective, higher circulating fetuin-A levels were associated with increased risk of T2D. However, the causality deserves further analysis (Guo et al. 2018). At the present, there is no indication of a possible therapy strategy using fetuin-A.

GDF15: Stress Response Cytokine with Impact on Energy Homeostasis

GDF15 is a stress response cytokine and a distant member of the TGF-β superfamily, with no close relatives. It acts via a recently identified

receptor called glial-derived neurotrophic factor (GDNF) receptor alpha-like. GDF15 expression and serum levels rise in response to many stimuli that initiate cell stress and as part of a wide variety of disease processes, most prominently cancer and cardiovascular disease. The best documented actions of GDF15 are on the regulation of energy homeostasis. When GDF15 serum levels are substantially elevated in diseases like cancer, it subverts a physiological pathway of appetite regulation to induce an anorexia/cachexia syndrome initiated by its actions on hindbrain neurons. These effects make it a potential target for the treatment of both obesity and anorexia/cachexia syndromes, disorders lacking any highly effective, readily accessible therapies (Tsai et al. 2018).

Recombinant GDF15 and half-life-extended variants of GDF15 demonstrated strong efficacy in lowering body weight and improved metabolic parameters in obese mice, rats, and monkeys (Xiong et al. 2017). However, the knowledge of its pathophysiological function at the molecular level is still limited and requires more investigation. Recent identification of the endogenous receptor for GDF15 may provide additional insight into its molecular mechanisms and relationship to disease states and potential therapy strategies (Emmerson et al. 2018).

IL-1β: Inflammatory Marker of Obesity

Interleukin (IL)-1β – a 17 kDa cytokine – is mainly secreted from immune cells but also by AT. IL-1β is a pro-inflammatory cytokine that has been proven to be effective in the treatment of T2D.

The expression of the IL-1-receptor antagonist is reduced in pancreatic islets of patients with T2D, and high glucose concentrations induce the production of IL-1 β in human pancreatic beta cells, leading to impaired insulin secretion, decreased cell proliferation, and apoptosis.

IL-1β inhibits the function and promotes apoptosis of beta cells. The blockade of IL-1 with the recombinant human IL-1 receptor antagonist (anakinra) has been shown to improve glycemia and beta cell function and reduce markers of systemic inflammation in a double-blind, parallel-group trial involving 70 patients with T2D (Larsen et al. 2007). Therefore, IL-1β, in addition to the direct use of adipokines as a therapeutic strategy, can be indirectly used as target molecules for the treatment of obesity.

TNF-α: Inflammatory Marker of Obesity

TNF-α was first described in animal studies in 1993 as a 25 kDa adipokine that significantly reduces insulin sensitivity in animal models. The IR-inducing effect is mainly caused by an inhibition of insulin signaling molecules. In line with the negative effect of TNF-α on insulin sensitivity, various mouse models of obesity and IR show increased serum concentrations of this protein. TNF-α can bind to two different receptors (Fasshauer and Blüher 2015).

Neutralization of elevated TNF-α levels by antibodies also increases insulin sensitivity in animal models (14). However, these promising data could not be translated into a clinically relevant treatment option in humans. TNF-α protein production in AT is very low, and studies suggest that the serum concentrations of this adipokine do not differ between insulin-resistant and insulin-sensitive volunteers with similar body weights. Of note, in patients with spondyloarthritis, anti-TNF-α therapy contributed to an early significant increase in both visceral and subcutaneous AT after 1–2 years of treatment (Fasshauer and Blüher 2015). Moreover, in a prospective, randomized, double-blind, placebo-controlled pilot study of insulin-resistant obese men, chronic TNF-α neutralization by infliximab did not improve IR and questioned the suggested causative link between adiposity and IR, at least in human disease (Wascher et al. 2011).

A first study on the clinical therapeutic application of TNF-α inhibition was disappointing. In contrast to the animal model, insulin sensitivity was not increased by neutralizing TNF-α. Also in view of the numerous side effects of such a treatment described, it seems unlikely that future therapies of IR, T2D, and obesity will be based on inhibition of this adipokine.

Omentin: A Biomarker of Metabolic Disorders

Omentin-1, also known as intelectin-1, is a recent adipokine of 31 kDa, which is expressed in visceral AT as well as in vascular cells, small intestine, colon, and ovary. The concentration of omentin-1 expression in (pre-)adipocytes is reduced by glucose or insulin and enhanced by FGF21 and corticosteroid (dexamethasone). Multiple experimental data have shown that omentin-1 may play a crucial role in supporting the body's energy metabolism and insulin sensitivity and has anti-inflammatory, anti-atherosclerotic, and cardiovascular protective effects. Clinical studies have shown the use of circulating low omentin-1 levels as a biomarker for obesity, IR, T2D, and atherosclerotic cardiovascular diseases. However, omentin-1 increases to counteract the acute phase after onset of cardiometabolic disorders. These findings indicate that omentin-1 may be a risk factor for cardiometabolic diseases and may also act as an acute-phase reactant by its anti-inflammatory and atheroprotective effects. A therapeutic approach to restoring omentin-1 levels can be beneficial for the prevention or treatment of these diseases. Losing weight, eating a diet rich in olive oil, doing aerobic exercise, and treatment with drugs to lower blood cholesterol (atorvastatin) and antidiabetics (e.g.. metformin and pioglitazone) are effective tools for increasing blood circulation of it. These data support the possible use of omentin-1 as a biomarker and therapeutic target for these pathologies (Watanabe et al. 2017).

In summary, adipokines are signal molecules that are released in relation to the status of adipose tissue function. Adipokines communicate information about energy reserves with the body – especially the brain. In the future, adipokines could gain clinical importance as circulating markers or predictors for metabolic and cardiovascular diseases, for the success of weight-reducing therapies, and also for pharmacological therapy approaches.

Drugs

For examples, see Table 2 (list of relevant drugs summarized in Table 2).

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Ado Receptors

► Adenosine Receptors

α-Adrenergic Receptors

► α-Adrenergic System

α-Adrenergic System

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Synonyms

α-Adrenergic receptors; α-Adrenoceptors

Definition

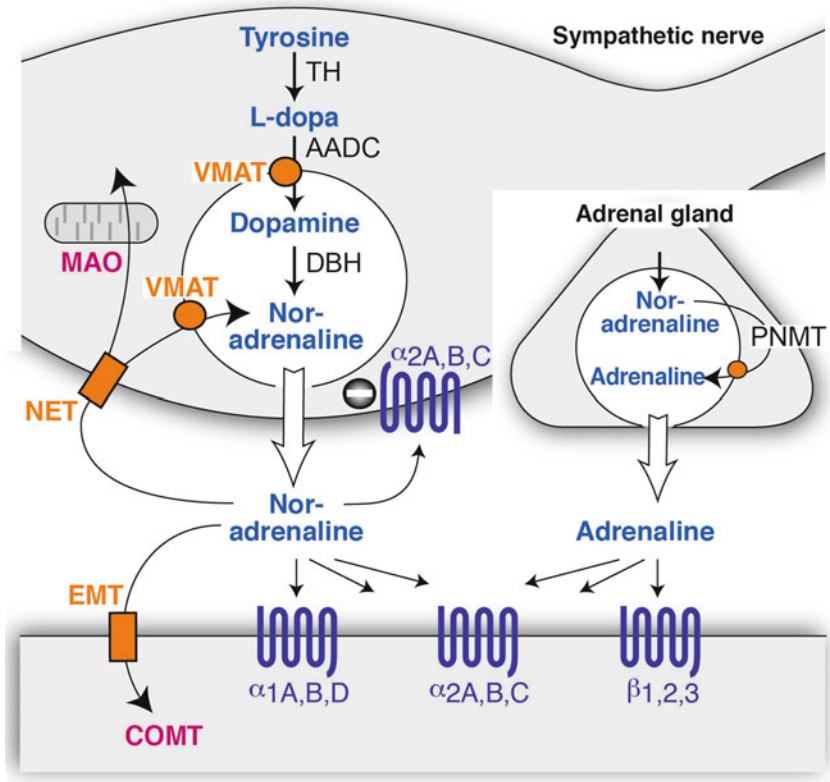
The α-adrenergic system consists of six subtypes of G-protein-coupled receptors which mediate part of the biological actions of the catecholamines adrenaline and noradrenaline. α-Adrenergic receptors (adrenoceptors) are important regulators of smooth muscle cell contraction (α_1-adrenergic receptors) (Akinaga et al. 2019) and presynaptic neurotransmitter release (α_2-adrenergic receptors) and several other functions in the peripheral and central nervous systems (Hein 2006; Starke 2001). In addition, adrenaline and noradrenaline activate β-adrenergic receptors, which stimulate cardiac contractility and rhythm and inhibit bronchial, vascular, and uterine smooth muscle contraction.

Basic Characteristics

The adrenergic system is an essential regulator that increases cardiovascular and metabolic capacity during situations of stress, exercise, and disease. Nerve cells in the central and peripheral nervous system synthesize and secrete the neurotransmitters noradrenaline and adrenaline. In the peripheral nervous system, noradrenaline (norepinephrine) and adrenaline (epinephrine) are released from two different sites: noradrenaline is the principal neurotransmitter of sympathetic neurons that innervate many organs and tissues. In contrast, adrenaline, and to a lesser degree noradrenaline, is produced and secreted from chromaffin cells in the adrenal gland into the circulation (Fig. 1). Thus, the actions of noradrenaline are mostly restricted to the sites of release from sympathetic nerves, whereas adrenaline acts as a hormone to stimulate many different cells via the bloodstream.

Together with dopamine, adrenaline and noradrenaline belong to the endogenous catecholamines that are synthesized from the precursor amino acid tyrosine (Fig. 1). In the first biosynthetic step, tyrosine hydroxylase generates ► l-DOPA which is further converted to dopamine by the aromatic l-amino acid decarboxylase (dopa decarboxylase). Dopamine is transported from the cytosol into synaptic vesicles by a vesicular monoamine transporter (VMAT). In sympathetic nerves, vesicular dopamine β-hydroxylase generates the neurotransmitter noradrenaline. In chromaffin cells of the adrenal medulla, approximately 80% of the noradrenaline is further converted into adrenaline by the enzyme phenylethanolamine-*N*-methyltransferase (PNMT).

Several mechanisms serve to terminate the biological actions of noradrenaline and adrenaline. From the synaptic cleft, most of the released noradrenaline is recycled by reuptake into the nerve terminals via a specific noradrenaline transporter (NET, gene symbol *SLC6A2*). Alternatively, (nor)



α-Adrenergic System, Fig. 1 Synthesis and release of noradrenaline and adrenaline from sympathetic nerve endings (left) and from the adrenal gland (right). Noradrenaline and adrenaline are synthesized from the precursor amino acid tyrosine and are stored at high concentrations in synaptic vesicles. Upon activation of sympathetic nerves or adrenal chromaffin cells, noradrenaline and adrenaline are secreted and can activate adrenergic receptors on surrounding cells (sympathetic nerve), or they enter the blood circulation (adrenaline released from the adrenal gland). Release of noradrenaline from nerve terminals is controlled by presynaptic inhibitory α_2- and activating β_2-adrenergic receptors. Actions of noradrenaline are terminated by uptake into nerve terminals (NET) and synaptic vesicles (VMAT) and by uptake into neighboring cells (EMT). Abbreviations: *AADC*, aromatic l-amino acid decarboxylase; *COMT*, catechol *O*-methyltransferase; *DBH*, dopamine β-hydroxylase; *EMT*, extraneuronal noradrenaline transporter (organic cation transporter); *MAO*, monoamine oxidase; *NET*, noradrenaline (norepinephrine) transporter; *PNMT*, phenylethanolamine-*N*-methyltransferase; *TH*, tyrosine hydroxylase; *VMAT*, vesicular monoamine transporter

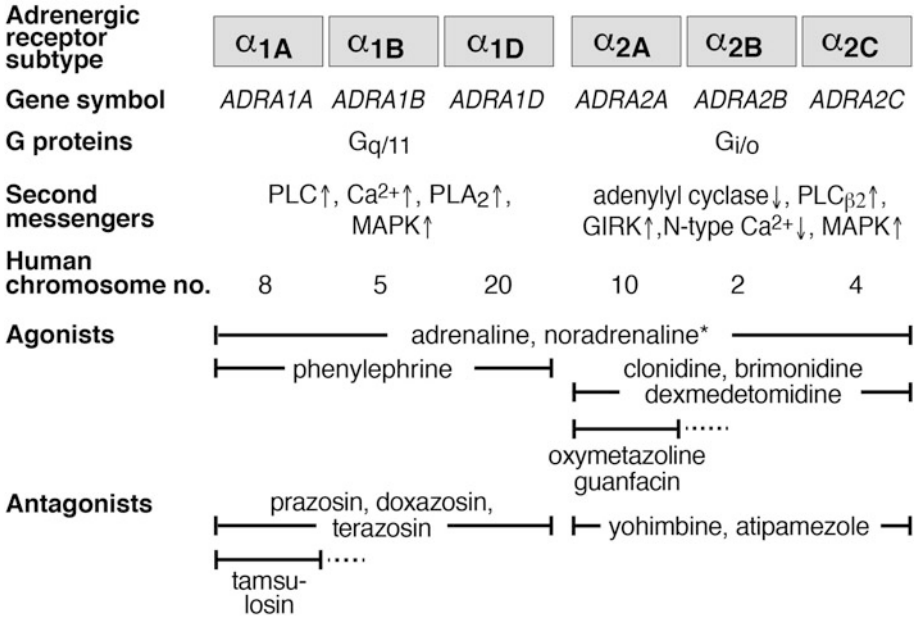
adrenaline can be transported into nonneuronal cells by extraneuronal monoamine transporters, which belong to the family of organic cation transporters (e.g., OCT1-3). The noradrenaline transporter (NET) is blocked by cocaine, tricyclic antidepressants, or selective noradrenaline reuptake inhibitors (SNRIs). After reuptake into the nerve, most of the noradrenaline is transferred into synaptic vesicles. A smaller fraction is destined for degradation by the enzymes monoamine oxidase (MAO, in sympathetic nerves) or ▶ catechol-*O*-methyltransferase (COMT, in neighboring cells). COMT plays a major role in the metabolism of circulating catecholamines. MAO and COMT are widely distributed, and inhibitors of these enzymes are used for the treatment of mental depression (MAO-A inhibitor, moclobemide) or Parkinson's disease (MAO-B inhibitor, selegiline).

The biological actions of adrenaline and noradrenaline are mediated via nine different ▶ G-protein-coupled receptors, which are located in the plasma membrane of neuronal and

nonneuronal target cells. These receptors are divided into two different groups, α-adrenergic receptors and β-adrenergic receptors (see β-adrenergic system). The distinction between α- and β-adrenergic receptors was first proposed by Ahlquist in 1948 based on experiments with various catecholamine derivatives to produce excitatory (α) or inhibitory (β) responses in isolated smooth muscle systems. Initially, a further subdivision into presynaptic α_2- and postsynaptic α_1-receptors was proposed. However, this anatomical classification of α-adrenergic receptor subtypes was later abandoned. Six α-adrenergic receptors have been identified by molecular cloning: three α_1-adrenergic receptors (α_{1A}, α_{1B}, α_{1D}) and three α_2-subtypes (α_{2A}, α_{2B}, α_{2C}) (Fig. 2). Due to the lack of sufficiently subtype-selective ligands, the unique physiological and pathophysiological roles of these α-receptor subtypes, for the most part, have been identified by studies in mice that carry deletions in the genes encoding for individual α-receptor subtypes.

The structure of the α_{2B}-adrenergic receptor in complex with the G protein G_o was recently identified by cryo-electron microscopy (Yuan et al. 2020). The α_2-agonist dexmedetomidine interacts with the α_{2B}-receptor mostly via aromatic and van der Waals interactions, which is different from adrenaline's interaction with the β_2-adrenergic receptor via ionic or hydrogen bonds (Yuan et al. 2020).

α_1-Adrenergic receptors mediate contraction and hypertrophic growth of vascular smooth muscle cells and cardiac myocytes (Akinaga et al. 2019). The three α_1-receptor subtypes share 75% identity in their transmembrane domains, whereas the degree of homology between α_1- and α_2-receptors is significantly smaller (35–40%). Due to initial discrepancies between the pharmacological subtype classification, mRNA and protein expression data, and experiments with cloned α_1-receptor subtypes, some confusion exists in the early literature with respect to the assignment of α_1-receptor subtype nomenclature. In the present terminology, α_{1A} (gene symbol *ADRA1A*), α_{1B} (gene symbol *ADRA1B*), and α_{1D}-receptors (*ADRA1D*) can be distinguished (Altosaar et al.



α-Adrenergic System, Fig. 2 Subtypes of α-adrenergic receptors, their signaling pathways, and agonist and antagonist binding profiles. *Adrenaline and noradrenaline can also activate β-adrenergic receptors (see β-adrenergic system). PLA_2, *PLC*: phospholipases A, C; *GIRK*: G-protein-activated inwardly rectifying potassium channel, *MAPK*: mitogen-activated protein kinase

2019). All three subtypes seem to be involved in the regulation of vascular tone, with the α_{1A}-receptor maintaining basal vascular tone and the α_{1B}-receptor mediating the constrictory effects of exogenous α_1-agonists. Cardiac α_1-receptors increase contractile force and mediate anti-apoptotic and hypertrophic effects (O'Connell et al. 2014). All α_1-receptor subtypes can activate Gq-proteins, resulting in intracellular stimulation of phospholipases C, A_2, and D, mobilization of Ca^{2+} from intracellular stores and activation of mitogen-activated protein kinase and PI3 kinase pathways. Mutagenesis of receptor subtypes has led to the identification of a number of amino acids involved in agonist binding and receptor activation as well as binding sites for antagonists within the receptor's binding crevice (Cotecchia et al. 2004).

Three genes encoding for α_2-adrenergic receptor subtypes have been identified from several species, termed α_{2A}, α_{2B}, and α_{2C}, respectively (Fig. 2). The pharmacological profile of the α_{2A}-subtype differs significantly between species, thus giving rise to the pharmacological subtypes α_{2A} in humans, rabbits, and pigs and α_{2D} in rats, mice, and guinea pigs. Part of the pharmacological difference between α_{2A}- and α_{2D}-receptors can be explained by a Ser-Ala mutation in the fifth transmembrane helix of the α_{2A}-receptor rendering this receptor less sensitive to the antagonists, rauwolscine and yohimbine. α_2-Adrenergic receptors regulate a wide range of signalling pathways via interaction with multiple heterotrimeric $G_{i/o}$ proteins including inhibition of adenylyl cyclase, stimulation of phospholipase D, stimulation of mitogen-activated protein kinases, stimulation of K^+ currents, and inhibition of Ca^{2+} currents. The three α_2-receptor subtypes have unique patterns of tissue distribution in the central nervous system and in peripheral tissues. The α_{2A}-receptor is expressed widely throughout the central nervous system including the locus coeruleus, brain stem nuclei, cerebral cortex, septum, hypothalamus, and hippocampus. In the periphery, α_{2A}-receptors are expressed in the kidney, spleen, thymus, lung, and salivary gland. The α_{2B}-receptor primarily shows peripheral expression (the kidney, liver, lung, and heart) and only low-level expression in thalamic nuclei of the central nervous system. The α_{2C}-receptor appears to be expressed primarily in the central nervous system (striatum, olfactory tubercle, hippocampus, and cerebral cortex), although very low levels of its mRNA are present in the kidney.

α_{2A}-, α_{2B}-, and α_{2C}-receptors are located presynaptically and function to inhibit noradrenaline release from sympathetic nerves as part of a negative feedback loop (Schlicker and Feuerstein 2017; Starke 2001). Activation of these receptors leads to decreased sympathetic tone and decreased blood pressure and heart rate (Hein et al. 1999). Central α_{2A}-receptors mediate sedation and analgesia (Hein 2006). Sedation induced by α_2-receptors follows a similar hypothalamic circuit as recovery sleep in response to sleep deprivation (Zhang et al. 2015).

α_{2B}-Receptors mediate contraction of vascular smooth muscle (Link et al. 1996; MacMillan et al. 1996), and they are required for placenta development (Philipp et al. 2002), and in the spinal cord they are essential components of the analgesic effect of nitrous oxide. Upon stimulation by agonists, α_1- and α_2-receptor signalling pathways are attenuated by several mechanisms at the receptor and postreceptor levels (see β-adrenergic system).

Drugs

Therapeutically, α_1-receptor-mediated vasoconstriction contributes to the beneficial actions of adrenaline applied as an emergency medicine during hypotensive or anaphylactic shock. Addition of adrenaline or noradrenaline to local anaesthetics retards diffusion of the local anaesthetic from the site of injection and thereby prolongs its action. α_1-Receptor antagonists including prazosin, doxazosin, and terazosin are used to treat patients with hypertension. However, α_1-receptor antagonists are no longer first-line

antihypertensive agents (Williams et al. 2018) since the ALLHAT clinical trial revealed that hypertensive patients taking doxazosin had a higher risk of developing congestive heart failure than patients with diuretic treatment. Tamsulosin is the first α_1-receptor antagonist with selectivity for the α_{1A}-receptor over α_{1B}- and α_{1D}-subtypes. The α_{1A}-selectivity is thought to contribute to the beneficial actions of tamsulosin in the treatment of benign prostate hypertrophy without strong hypotensive effects.

At present, no drugs have been approved for clinical use that can activate α_2-receptor subtypes with high selectivity. However, oxymetazoline and guanfacine have some selectivity for α_{2A} versus α_{2B} and α_{2C} (Altosaar et al. 2019). Clonidine stimulates all three α_2-subtypes with similar potency. Clonidine lowers blood pressure in patients with hypertension, and it decreases sympathetic overactivity during opioid withdrawal. In intensive and perioperative care, clonidine and dexmedetomidine are potent sedative and analgesic α_2-agonists and can prevent postoperative shivering. Guanfacine has been approved for treatment of attention deficient hyperactivity disorder (ADHS) in children and adolescents. Clonidine and its derivative brimonidine lower intraocular pressure of glaucoma patients when applied locally. Moxonidine may have less sedative side effects than clonidine when used as an antihypertensive. It has been suggested that moxonidine activates "imidazoline receptors" instead of α_2-receptors. However, the molecular identity of "imidazoline receptors" has not been revealed, yet (Bousquet et al. 2020). The α_2-receptor agonists oxymetazoline and xylometazoline are being used as nasal decongestants. At present, selective α_2-receptor antagonists are not used in human medicine. However, in veterinary practice the α_2-receptor antagonist atipamezole can rapidly reverse anaesthesia mediated by the α_2-agonist medetomidine. In the future, subtype-selective drugs may greatly improve the therapy of diseases involving α_1- or α_2-adrenergic receptor systems.

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α-Adrenoceptors

► α-Adrenergic System

Adrenomedullin Receptor

► Calcitonin Family Receptors

Adrenomedullin/Intermedin

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Synonyms/Abbreviations

Adrenomedullin: AM, ADM

Adrenomedullin 2: AM2, ADM2, Intermedin, IMD

Definition

Adrenomedullin (AM) and Adrenomedullin 2 (AM2) or Intermedin (from here on referred to solely as Adrenomedullin 2) are members of the calcitonin gene-related peptide (CGRP) superfamily of peptides. These peptide hormones are agonists at, and exert their physiological effects through the calcitonin receptor-like receptor (CLR). The peptides have a multitude of different biological activities; foremost among them is AM's potent activity as a vasodilator. It also plays a key regulatory role in the cardiovascular and lymphatic system, as well as in angiogenesis, and vascular homeostasis. AM2 has effects on the cardiovascular system, adipose tissue, macrophages, and the kidney; it can also activate the sympathetic nervous system.

Basic Characteristics

AM was first discovered in 1993 (Kitamura et al. 1993) and AM2 by two groups in 2004 (Roh et al. 2004; Takei et al. 2004). They are from a peptide family also containing calcitonin (CT), calcitonin gene-related peptide (CGRP) – α and β form in humans, and amylin (Hay et al. 2018). Within this superfamily there are a few residues which are highly conserved and found in all members: these are two N-terminal cysteines forming a disulfide-bonded ring structure and a threonine within this structure (Schönauer et al. 2017). Compared to the other members of the superfamily AM and AM2 are most closely related to each other, despite having only limited sequence homology (28%) (Zhang et al. 2018).

AM and AM2 are 52 and 53 amino acids in length, respectively. The human AM gene is located on chromosome 11 and contains 4 exons and 3 introns. It is translated into the 185 amino acid long precursor prepro-AM which is eventually converted into mature AM (Schönauer et al. 2017). The human AM2 gene is found on chromosome 22 and codes for prepro-AM2 which is 148 amino acid residues in length before it is converted into mature AM2 (Zhang et al. 2018) Mature $AM2_{1–53}$ can also be cleaved into $AM2_{1–47}$ and $AM2_{1–40}$.

Currently antibodies cannot distinguish between them, and the prominence of each in vivo remains uncertain. As does the physiological and pharmacological relevance of these cleavage fragments (Zhang et al. 2018).

Pharmacology

Both AM and AM2 act at a Class B GPCR – the CLR. It is suggested that they also have some activity at the calcitonin and amylin receptors. The CLR is unique in that it needs to associate with a receptor activity-modifying protein (RAMP) to form a functional receptor. There are three known RAMPs (RAMP1, 2, and 3), and each has an extracellular N-terminus, one intracellular domain, and an intracellular C-terminus. AM and AM2 bind all three functional receptor-RAMP complexes with varying affinities (Hay et al. 2018).

While the predominant CLR signaling pathway is through G_s and cAMP, there is also evidence for G_i coupling and cAMP inhibition, as well as G_q coupling and intracellular Ca^{2+} release. The extent to which pathways are activated is determined by both the receptor/RAMP combination and the bound peptide (AM or AM2).

CLR/RAMP1 forms the CGRP receptor, CLR/RAMP2 forms the AM_1 receptor, and CLR/RAMP3 forms the AM_2 receptor. When comparing the pharmacology of the human peptides at the human receptors, AM2 has activity at all three receptors (measured in terms of cAMP) and appears to be most potent at activating the AM2 receptor. AM also activates all three receptors with its activity at AM_1 and AM_2 significantly higher than at the CGRP receptor. Comparing the peptides with each other they have similar potencies at the CGRP and AM_2 receptor, with the main difference being AM's significantly greater potency at the AM_1 receptor (Hay et al. 2018), although further research is needed to elucidate the precise differences in signaling between the peptide-receptor combinations in cellular systems where the receptors are endogenously expressed.

The signaling bias of AM and AM2 through pathways beyond purely cAMP has recently been documented. There is evidence of this at the CGRP receptor where AM elicits a comparatively weak cAMP response (G_s). However, it has been shown that AM in fact also signals through G_i proteins at the CGRP receptor (Hay et al. 2018). Providing an explanation for the weak cAMP signal induced. Thus far these results have only been shown in overexpression systems and need demonstrating in the native system in order to determine the physiological relevance of AM and AM2's biased signaling.

In terms of internalization and receptor recycling, research suggests AM can drive the internalization of CGRP, AM_1, and AM_2 receptors. Furthermore, when AM internalizes with AM_1 it likely stays bound throughout the internalization and degradation process (Schönauer et al. 2017); little is known at present about AM2-driven internalization.

Physiology, Pathophysiology, and Therapeutic Potential

AM is mainly synthesized and secreted by endothelial cells and vascular smooth muscle cells, and has been detected in many tissues and organs including the heart, lung, liver, kidney, placenta, spleen, thyroid, adrenal medulla, and central nervous system (Schönauer et al. 2017). In comparison AM2 is widely expressed throughout the body, and particularly high expression levels are seen in the hypothalamus, pituitary, heart, GI tract, kidney, and circulation. It is believed to play a protective role in the renal and cardiovascular systems, inhibiting ER stress, inflammation, and oxidative stress (Takei et al. 2004).

AM is reported to be cardioprotective through regulating cell proliferation and antiapoptotic pathways in the vasculature. AM can also increase cardiac output by causing vasodilation of coronary vessels and decreasing vascular resistance (Schönauer et al. 2017). The potent vasodilatory effects of AM are important in the lung circulation and can decrease vascular resistance in pulmonary hypertension as well as protecting against hypoxic pulmonary damage. AM2 acts similarly; having been demonstrated to increase blood flow and decrease vascular resistance in many organs

including the heart, lungs, liver, and kidneys (Zhang et al. 2018).

AM is involved in lymphangiogenesis and the control of lymphatic vascular function through stabilizing the lymphatic endothelial barrier and sustaining vascular permeability (Schönauer et al. 2017). AM is also expressed alongside CLR and RAMP2 in osteoblasts and promotes bone growth and mineralization (Schönauer et al. 2017). There is a large increase in plasma AM in healthy pregnant women and it plays a role in decidualization, implantation, and placentation. Equally AM2 is elevated in pregnancy and has a hypotensive effect, while low AM2 levels are associated with preeclampsia (Zhang et al. 2018).

Several clinical trials have been performed using AM in patients, due to the potential protective effects AM might provide in multiple disease states. Two examples include trails using AM in patients with pulmonary hypertension and myocardial infarction. The possible use of AM as a therapy option comes with the advantages and disadvantages often associated with peptide drugs, such as high potency and low toxicity, but also low bioavailability and stability (Schönauer et al. 2017).

While the circulating plasma concentration of AM and AM2 is generally low, it can be greatly increased in the disease state. One of these diseases is diabetes where plasma levels of AM in patients are significantly high. It is not yet completely clear whether AM contributes to the disease state or acts as a protective factor.

AM can, however, act as a tumor survival factor through its angiogenic, proliferative, and antiapoptotic effects. It is produced by cancer cells and endothelial cells, and its expression has been shown to be upregulated in multiple tumor types. It can be involved in multiple stages of cancer development including tumor progression and invasion (Schönauer et al. 2017).

In the case of AM2, levels are increased during myocardial infarction and acute coronary syndrome (Zhang et al. 2018), and multiple studies have suggested that it plays a protective role in these conditions, particularly during ischemic cardiac injury (Zhang et al. 2018).

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Advanced Glycation End-Proteins

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List of Abbreviations

ADAM10	A disintegrin and metalloproteinase domain-containing protein 10
AF	Atrial fibrillation
AGE	Advanced glycation end-product

ARDS	Acute respiratory distress syndrome
CPOD	Chronic obstructive pulmonary diseases
DIAPH1 or mDia1	Protein diaphanous homolog 1
GOL1	Glyoxalase 1
HMBG1	High-mobility group box 1
LPA	Lysophosphatidic acid
LPS	Lipopolysaccharide
MAC-1	Macrophage adhesion ligand-1
NF-κB	nuclear factor κ-light-chain-enhancer of activated B cells
PTH	Parathyroid hormone
RAGE	Receptor for AGE
TLR	Toll-like receptors

Definition

The role of advanced glycation end-products (AGEs) in initiating, promoting, and worsening of metabolic derangement has been highlighted during recent years. There are well-reported connections between AGEs production and not only the development of metabolic syndrome and diabetes, but also micro- and macrovascular complications of diabetes (Asadipooya and Uy 2019; Ruiz et al. 2020).

AGEs are the products of irreversible non-enzymatic reactions between a reducing sugar and macromolecules (proteins, lipids, or nucleic acids). The condensation and oxidation process between reducing sugar and proteins, lipids, or nucleic acid leads to the production of AGEs. The process of AGEs production usually occurs in the background of hyperglycemia, but can also happen in other situations such as consumption of high fat diet, excessive alcohol consumption, smoking, renal failure, inflammation, and aging (Asadipooya et al. 2019; Asadipooya and Uy 2019). AGEs not only have direct toxic effects but also interact with other receptors, which leads to inflammation and oxidant injury. Generally, there are contributing factors that result AGEs production and ended up developing diseases or affecting the course of diseases with worsening outcomes.

Basic Mechanisms

General

The chemistry of glycation is complex and AGEs are highly heterogeneous molecules that can accumulate in various tissues and plasma. The process of AGEs accumulation is associated with oxidative stress and inflammation, which lead to changes in metabolic activities and even more AGEs production. However, scavenger receptors, such as CD36, can block AGEs mediated injury. AGEs enter into the cells through CD36, undergo degradation, and are eventually eliminated by kidneys. In addition, detoxification by AGE receptor complex is another defense mechanism against AGEs. Moreover, the enzyme glyoxalase 1 (GOL1) plays a key role in the detoxification of AGEs by regulating the production and function of pre-AGEs species, such as methylglyoxal and its derivatives (Asadipooya et al. 2019; Ott et al. 2014; Senatus and Schmidt 2017).

AGEs can cause oxidant injury and inflammation directly or through interaction with various receptors, including AGE-receptor complexes, scavenger receptors, and the multiligand receptor for AGEs (RAGE) (Asadipooya and Uy 2019; Ruiz et al. 2020). The multiligand receptor for AGEs (RAGE) on cell membrane is a well-studied receptor since the 1990s (Schmidt et al. 1992). RAGE is structurally like an immunoglobulin with an extracellular domain, transmembrane part, and intracellular portion. The extracellular portion contains one variable and two constant parts (figure). It interacts with not only AGEs, but also with other ligands, such as high-mobility group box 1 (HMGB1), S100/calgranulins, amyloid-b peptide, and other forms of amyloid, complement proteins, macrophage adhesion ligand-1 (MAC-1), lysophosphatidic acid (LPA), and lipopolysaccharide (LPS).

AGE-RAGE interaction can lead to signal transduction and initiation of different intrinsic signaling pathways, including Ras–mitogen-activated protein kinase, PI3K/Akt, Src/RhoA, JAK/STAT, and NADPH oxidase. This leads to an increase in the levels of transcription factors, such as Egr-1 and NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells), and then

their translocation into nucleus. Finally, these processes result in cellular and tissue consequences besides helping as a feed forward loop to increase AGEs production (Asadipooya et al. 2019; Asadipooya and Uy 2019; Ott et al. 2014; Ruiz et al. 2020).

RAGE has multiple isoforms, including truncated or soluble RAGE (sRAGE) and endogenous secretory RAGE (esRAGE). Soluble RAGE (sRAGE) is the product of cleavage of the extracellular part of the RAGE, which lacks transmembrane and intracellular parts. The alternatively spliced RNA variant of RAGE leads to the production of esRAGE. Both sRAGE and esRAGE lack intracellular portions and are not stable on cell membrane. They act as decoy receptors that block the interaction between RAGE and AGEs or other RAGE ligands (Asadipooya et al. 2019; Asadipooya and Uy 2019; Ruiz et al. 2020) **(Figure)**.

AGEs and Diseases

AGEs form endogenously or derive from exogenous sources such as diet, alcohol, smoking, and air pollutants (Asadipooya et al. 2019; Ruiz et al. 2020). In addition to exogenous sources of AGEs, the other conditions, such as aging and inflammation that increase AGEs production, are associated with disease conditions such as obesity, insulin resistance, diabetes, cardiovascular diseases, and osteoporosis. The path of obesity, insulin resistance, diabetes, and cardiovascular diseases moves through complex metabolic and inflammatory events. Increase in AGEs production and their interaction with RAGE are the important parts of the metabolic and inflammatory events. Circulatory AGEs accumulate in tissues, which leads to the production of damaged proteins and reactive oxygen species, induction of inflammation, and finally, organ damage. AGEs, especially high fat and processed food derived AGEs, contribute to the adipocyte hypertrophy, obesity, and its consequences such as metabolic disturbances and insulin resistance (Asadipooya et al. 2019; Asadipooya and Uy 2019; Ruiz et al. 2020).

Notably, the role of AGE-RAGE interaction and its implications in diseases development and complications are relatively well known. RAGE is able to bind to different ligands. RAGE and its ligands not only contribute to diabetes development, but also have crucial roles in diabetic macrovascular and microvascular complications (Asadipooya and Uy 2019; Ruiz et al. 2020). Glucose or lipid oxidation may lead to the AGEs production. Conversely, RAGE and its ligand could be the potential cause of glucose and lipid metabolic abnormalities (Wang et al. 2020). In addition, RAGE and its ligands have fundamental roles in the immunometabolic system, thermogenesis, and energy metabolism (Hurtado Del Pozo et al. 2019), which can lead to obesity, insulin resistance, and diabetes. In addition to its role in obesity and diabetes, the metabolic conditions, such as PCOS, that are associated with insulin resistance have correlation with AGEs (Ruiz et al. 2020). Furthermore, RAGE and its ligands participate in aging, atherosclerosis, vascular calcification, and peripheral arterial disease in diabetic and nondiabetic settings. They contribute to thrombosis and thrombotic disorders (Egaña-Gorroño et al. 2020; Reynaert et al. 2016). AGEs have correlation with cardiac rhythm abnormalities, such as atrial fibrillation (AF) (Egaña-Gorroño et al. 2020). The role of AGE-RAGE signaling pathway in the development of AF by affecting atrial structure and electrical remodeling (Prasad 2020) highlights the significance of AGE-mediated tissue damages. AGE-RAGE axis is also involved in osteoporosis and chronic obstructive pulmonary diseases (COPD) development and progression (Reynaert et al. 2016). RAGE in plasma has positive correlation with acute respiratory distress syndrome (ARDS) development (Van Der Zee et al. 2020) and its inhibition can reduce inflammation and tissue injury due to ARDS (Audard et al. 2019). Furthermore, RAGE activation triggers a positive feedback loop, collaborates with immune pathways, such as Toll-like receptors (TLR)-related pathways, and leads to strengthening of RAGE signaling and cell death. The cross talk between RAGE and TLR is potentially the important mechanism and treatment target for neuroinflammatory diseases (Gąsiorowski et al. 2018). HMGB1 and its receptors (RAGE and TLR4) contribute to amyotrophic lateral sclerosis

pathogenesis (Paudel et al. 2020). Moreover, RAGE can affect apoptotic proteins. It participates in cancer cell progression by upregulating antiapoptotic proteins and downregulating proapoptotic proteins (El-Far et al. 2020).

In summary, AGEs production and accumulation, AGE-RAGE interaction, RAGE interaction with other ligands, and AGEs interaction with other receptors (such as TLRs) result in regulation of inflammatory response and cell proliferation, motility, survival and differentiation besides affecting apoptosis, autophagy, energy metabolism, and thermogenesis (Asadipooya et al. 2019; Hurtado Del Pozo et al. 2019; Sorci et al. 2013). As a result, they are the cause of or correlate with aging and noncommunicable diseases.

It is worth mentioning that RAGE and its ligands have critical roles in initiation and regulation of inflammation. RAGE inhibition reduces inflammation, but may affect macrophage phagocytosis and tissue homeostasis (Asadipooya et al. 2019). RAGE could be the potential cause of antibody-mediated autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis, development (Eichhorst et al. 2019). It has a potential role in maintaining chronic inflammation and sustainability of inflammatory bowel disease (Ciccocioppo et al. 2019). Moreover, inhibition of RAGE has protective roles in infectious diseases and sepsis (Zhao et al. 2018).

Nevertheless, AGE-RAGE signaling pathway seems to have some protective roles. It is evident the hyperlipidemic RAGE deficient mouse does not have higher risk of developing fatty liver and atherosclerosis on western diet compared to hyperlipidemic wild type mouse (Bijnen et al. 2018). In addition, RAGE deficiency attenuates PTH-mediated bone formation and causes loss of lean mass (Asadipooya and Uy 2019). Furthermore, RAGE plays an important role in the regulation of immune response, inflammation, fibrosis, tissue regeneration, and repair (Asadipooya et al. 2019; Sorci et al. 2013).

Diagnostic Opportunities

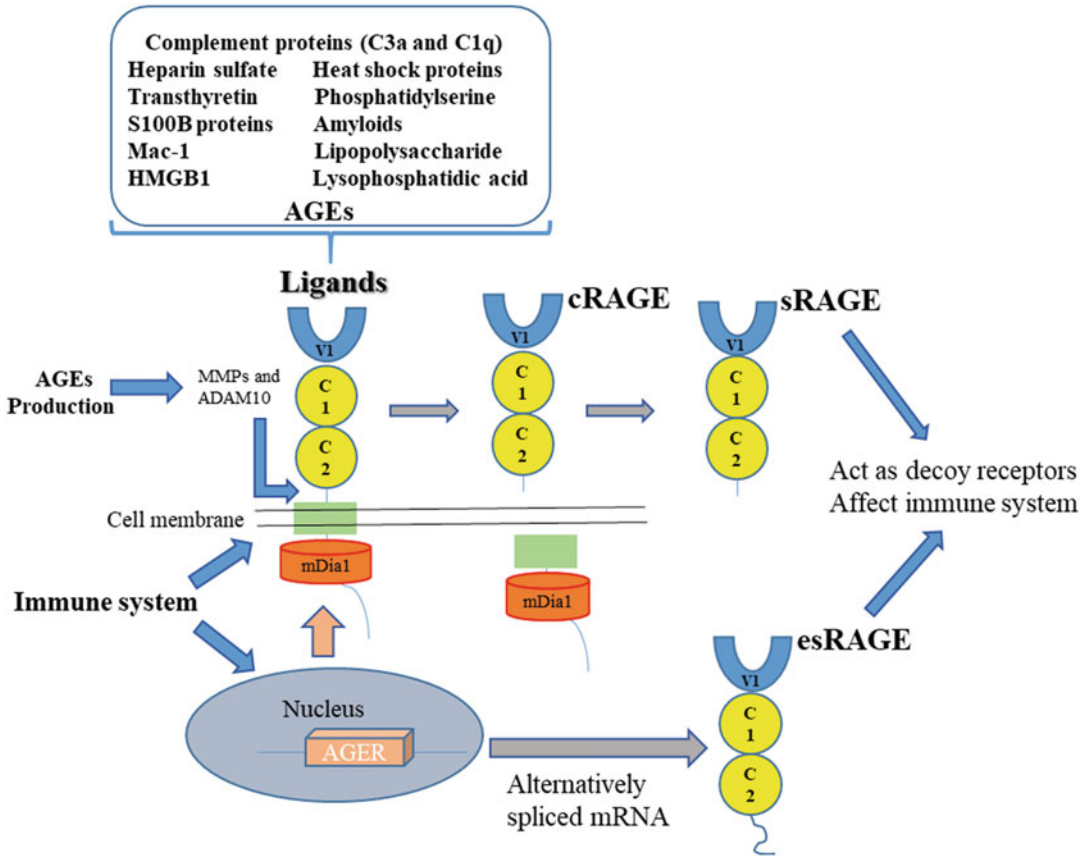
Although the concept of sRAGE application looks interesting in terms of its correlation with different clinical outcomes and potential therapeutic utilities, it faces some limitations. The correlation between sRAGE or esRAGE and different clinical outcomes seems to be bidirectional. Increase in AGE creation can induce ADAM10 (matrix metalloproteinases or a disintegrin and metalloproteinase domain-containing protein 10), which leads to RAGE cleavage and sRAGE production. In addition, contribution of the immune system in esRAGE production and its positive correlation with AGEs (Asadipooya et al. 2019; Asadipooya and Uy 2019; Yamagishi and Imaizumi 2007) can explain the bidirectional correlation of soluble RAGE with different clinical conditions. In other words, elevated sRAGE could be due to an increase in AGE production or RAGE destruction (Asadipooya 2019).

Pharmacological Intervention

There are tons of therapeutic interventions to mitigate or block the AGE-RAGE signaling pathway. Current approaches target AGEs, RAGE, or post-receptor signaling pathway. In addition, they may ameliorate the AGE-RAGE related complications. These include sRAGE (a ligand decoy), Anti-RAGE antibodies, small-molecule RAGE antagonists, or inhibitor of cytoplasmic domain of RAGE (ctRAGE)-DIAPH1 interaction, Aptamers or genetic suppression of RAGE by siRNA (Asadipooya and Uy 2019). In addition, there are medications or agents that reduce AGEs induced damages, such as antidiabetic medications (metformin, GLP-1 agonists), antihypertensive medications (Angiotensin II receptor blocker and angiotensin converting enzyme inhibitor), antioxidants (statins), medications with anti-inflammatory properties (bisphosphonates), and natural substances (vitamin D and K) (Asadipooya and Uy 2019; Byun et al. 2017).

Furthermore, sRAGE technically blocks AGE-RAGE interaction and can protect the high fat fed mouse from weight gain and insulin resistance (Song et al. 2014) or reduce angiotensin II mediated endothelial damage (Jeong et al. 2019), but may increase or decrease other RAGE ligands such as HMGB1 (Jeong et al. 2019; Song et al.

2014). Moreover, AGEs are highly heterogeneous and recombinant sRAGE cannot bind to all of them. In addition, AGE-RAGE signaling is associated with some beneficial effects (Asadipooya et al. 2019; Asadipooya and Uy 2019) as lack of RAGE could not reduce fat deposition in liver (Bijnen et al. 2018). As a result, although sRAGE has protective roles against AGE-RAGE associated pathologies, its use in clinical practice is facing barriers, including heterogeneity of AGEs, accessibility of sRAGE to its ligands, and loss of the beneficial effects of RAGE signaling pathway. However, transfecting immune cells (macrophage) with a lentivirus that carries sRAGE may improve the clinical outcomes of the sRAGE, as macrophage can increase accessibility of sRAGE to RAGE ligands (Asadipooya et al. 2019; Asadipooya and Uy 2019). Additionally, DIAPH1 (mDia1) has an important role in cytoskeleton and cell organelle kinetics. It interacts with cytoplasmic domain of RAGE and is responsible for stabilizing RAGE on cell membranes. DIAPH1 in humans is associated with sensorineural hearing loss and platelets disorders (Asadipooya et al. 2019; Egaña-Gorroño et al. 2020). Technically, targeting DIAPH1 can reduce stability of RAGE on cell membrane, which leads to reduction of harmful effects of RAGE without



Advanced Glycation End-Proteins, Fig. 1 Schematic representation of RAGE and the process of soluble RAGE (sRAGE and esRAGE) production. DIAPH1 (mDia1) interacts with cytoplasmic domain of RAGE and stabilizes the RAGE on cell membrane. Absence of DIAPH1 affects intracellular response of AGE-RAGE signaling pathway. The RAGE (gene name is AGER) interacts with its known ligands, such as AGEs, HMGB1, S100 family proteins, etc. The RAGE ligands bind to the extracellular domains of RAGE. The AGEs, matrix metalloproteinases (MMPs), or ADAM10 and immune system contribute to sRAGE production. sRAGE can bind to RAGE ligands without activating intracellular signaling. Additionally, sRAGE affects immune function and has protective roles. The extracellular domain of RAGE includes V1 (variable part) and C1 and C2 (constant parts)

losing beneficial effects. Hence, efforts to target RAGE or reduce RAGE stability might improve the clinical outcomes in metabolic syndrome, obesity, diabetes, and diabetic complications (Fig. 1).

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AGTRL1 Ligand

▶ Apelin

A-Kinase Anchoring Proteins (AKAPs)

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Synonyms

Cyclic AMP-dependent protein kinase anchoring proteins; Protein kinase A anchoring proteins

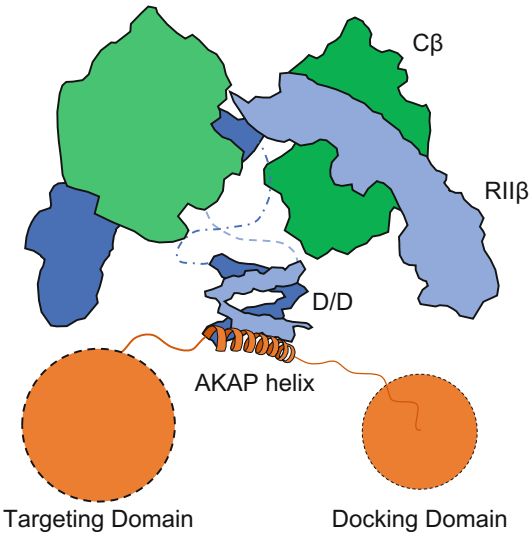
Definition

AKAPs are a diverse family of about 50 scaffolding proteins, which are defined by their ability to bind the regulatory subunits of cyclic AMP-dependent protein kinase (PKA; Fig. 1) [reviewed in (Welch et al. 2010)]. PKA is an abundant and ubiquitously expressed protein kinase. However, despite its ubiquity in signaling processes, there are only four isoforms of the PKA regulatory subunit with which to impart specificity in localization. Instead, the diversity of AKAPs that tether PKA along with other signaling proteins, including G protein-coupled receptors, adenylyl cyclases, kinases, phosphodiesterases, and phosphatases, among others, to create multivalent signaling complexes, dictates their localization and integrates cellular signaling processes at specific sites and times. The compartmentalization of signaling by AKAPs contributes to the specificity of cellular responses to extracellular cues.

Basic Mechanisms

AKAP-Dependent Control of cAMP/PKA Signaling

A large variety of extracellular stimuli including hormones and neurotransmitters stimulate the generation of the second messenger molecule 3′,5′-cyclic adenosine monophosphate (cAMP) by the activation of adenylyl cyclase, which catalyzes the conversion of ATP to cAMP. While cAMP binds to several effector proteins including ion channels, cAMP-dependent guanine-nucleotide-exchange



A-Kinase Anchoring Proteins (AKAPs), Fig. 1 A-kinase anchoring protein (AKAP) interaction with cAMP-dependent protein kinase (PKA). The unifying characteristic of canonical AKAPs is the presence of a structurally conserved amphipathic binding helix (orange) which binds in a hydrophobic cleft created by the docking and dimerization (D/D) domains of the regulatory (R) subunits of PKA. The N-terminal D/D domain is connected to the rest of the protein by an intrinsically disordered linker region (blue dotted lines). In the inactive state, PKA forms a tetramer consisting of a dimer of two R subunits (blue) each bound to one catalytic subunit (C, green). The binding of two molecules of cAMP to each R subunit takes places in the cyclic nucleotide-binding (CNB) domains A and B, causing a conformational change that results in the release of the C subunits, which can then phosphorylate downstream substrate proteins. In addition to the amphipathic binding helix, AKAPs contain unique docking domains, which bind further signaling proteins to create larger signaling complexes, which among other components can include phosphodiesterases, phosphatases or other kinases, and a targeting domain, which tethers the AKAP complex to specific cellular compartments. AKAPs without a targeting domain such as GSK3β interaction protein (GSKIP) are located in the cytosol. A few AKAPs possess catalytic activity such as the RhoGEF activity in AKAP-Lbc conferred by a DH domain. The proteins within the AKAP family range in size from 81 amino acids (AKAP18α) to more than 3000 amino acids (AKAP-Lbc or AKAP450) and are without obvious sequence homology

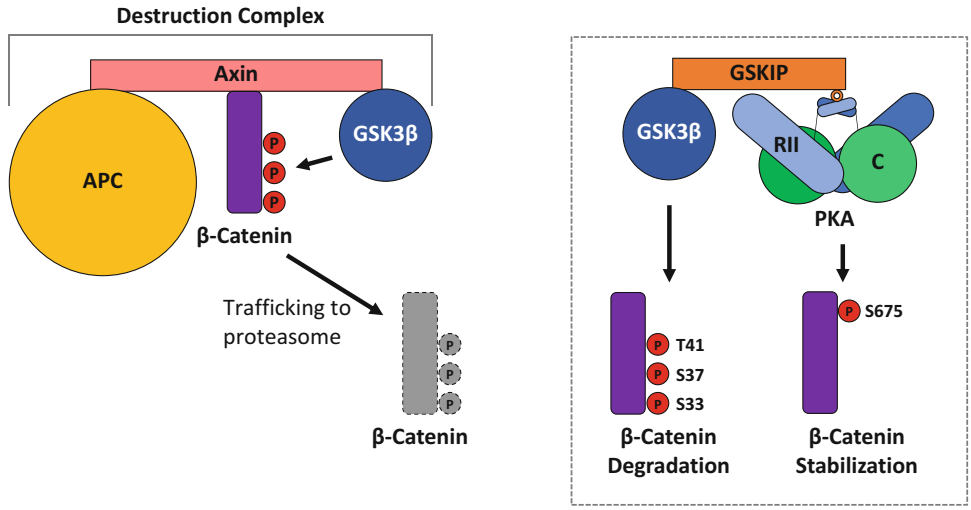
factors (Epacs), and the more recently discovered Popeye proteins [reviewed in (Swan et al. 2019)], PKA is its main intracellular receptor.

The classical PKA holoenzyme consists of two regulatory subunits (RIα, RIβ, RIIα, RIIβ) which form homodimers. Each R subunit is bound to a single catalytic subunit (Cα, Cβ, Cγ), creating a tetramer with two regulatory and two catalytic subunits. However, it is likely, based on the evidence that the copy number of R subunits greatly exceeds that of C subunits, that most regulatory subunits are not bound to catalytic subunits at any given time (Walker-Gray et al. 2017). Each of the regulatory subunits contains two cyclic nucleotide-binding domains (CNBs), which exhibit sequential binding to cAMP, meaning it takes four molecules of cAMP to fully activate a PKA holoenzyme (Taylor et al. 2013; Herberg et al. 1994; Zhang et al. 2012). The binding of the second cAMP molecule to each regulatory subunit elicits a conformational change that displaces the catalytic subunit from the regulatory subunit (Taylor et al. 2012). The free catalytic subunit requires no further activation to phosphorylate downstream targets (Keshwani et al. 2012). The PKA catalytic subunit transfers the γ-phosphate from ATP to consensus sites on many different substrate proteins and thereby modulates their activity through reversible phosphorylation reactions [reviewed in (Taylor and Kornev 2011)].

Different external stimuli mediate the activation of specific pools of PKA located at defined sites within subcellular compartments including mitochondria, nuclei, exocytic vesicles, sarcoplasmic reticulum, or the cytosol (Welch et al. 2010; Zaccolo and Pozzan 2002). The diversity of AKAPs that tether PKA to such cellular compartments allows for its local activation, and, acting in a coordinated manner with other signaling molecules, the consequent phosphorylation of particular substrates in close proximity, creating specificity in its activation (Langeberg and Scott 2005). Spatial and temporal coordination of PKA signaling through compartmentalization by AKAPs is considered essential for the specificity of PKA-dependent cellular responses to a particular external stimulus (Wong and Scott 2004). It remains somewhat unclear what molecular mechanisms drive the inactivating reassociation of catalytic subunits with regulatory subunits. However, there is an emerging understanding that the interplay of free and bound C subunits in conjunction with the localization, affinity, and stoichiometry of free regulatory subunits may be essential to the commencement and cessation of signaling cascades, and there are indications that localization and membrane association driven by AKAPs may play a role (Walker-Gray et al. 2017). There is also some conjecture that the PKA catalytic subunit might not be released from regulatory subunits at all and may instead be active while still bound to the regulatory subunit in a manner that would no longer require the interaction of the C subunit ATP-binding cleft with the substrate or pseudosubstrate sequences of the R subunits and would be independent of the binding of cAMP (Smith et al. 2017). In this model, the N-terminal disordered region would act as a tether between the AKAP bound D/D and the CBDs, which are in turn associated with the C subunit, thereby imparting an active radius of about 125 Å from the anchoring location in which substrates could be phosphorylated. Fig. 2 illustrates the local control of PKA by the AKAP GSKIP, which is involved in Wnt signaling.

AKAP-PKA interactions play a role in a variety of cellular processes including β-adrenoceptor-dependent regulation of cardiac myocyte contraction or vasopressin-mediated water reabsorption.

The tethering of PKA through AKAPs by itself is not sufficient to compartmentalize and control a cAMP/PKA-dependent pathway. Cyclic AMP readily diffuses throughout the cell. Therefore, discrete cAMP/PKA signaling compartments are only conceivable if this diffusion is limited. Phosphodiesterases (PDEs) establish gradients of cAMP by local hydrolysis of the second messenger and thereby regulate PKA activity locally [reviewed in (Musheshe et al. 2018; Bers et al. 2019). Several AKAPs interact with PDEs and thus play a role at this level of control [reviewed in (Maurice et al. 2014; Ercu and Klussmann 2018). For example, the interaction of muscle-specific mAKAP with cAMP-specific PDE4D3 and the ryanodine receptor (RyR) facilitates hydrolysis of cAMP



A-Kinase Anchoring Proteins (AKAPs), Fig. 2 The AKAP-GSK3β interaction protein (GSKIP) colocalizes PKA and GSK3β for control of β-catenin in the canonical Wnt signaling cascade. Though the spatial and temporal picture is still evolving, our current understanding suggests that in the absence of Wnt signaling, a relatively large complex of proteins known as the "destruction complex" forms in the cytosol. The destruction complex consists of axin (pink), a largely disordered protein which plays an important scaffolding role in the destruction complex and which binds to the kinases glycogen synthase kinase 3β (GSK3β, dark blue) and casein kinase (CK1, not shown), as well as adenomatous polyposis coli (APC, yellow), a tumor suppressor which in turn binds to the transcription activator protein β-catenin (Song et al. 2014; Dema et al. 2016). This complex directs the phosphorylation of β-catenin by GSK3β at threonine-41 and serines-33 and 37, which in turn leads to its targeting for ubiquitination and ultimately degradation in the proteasome. When Wnt signaling is initiated, the ligand binds to the single-pass low-density lipoprotein receptor-related protein 5/6 (LRP5/6) transmembrane proteins and G protein-like receptors from the Frizzled family, which in turn recruit the destruction complex, preventing the degradation of β-catenin. Under these conditions, GSKIP (orange), which binds GSK3β as well as PKA, coordinates the inactivation of GSK3β by PKA-mediated phosphorylation of GSK3β serine-9 and helps coordinate the stabilizing phosphorylation of β-catenin at serine 675. The inactivation of GSK3β prevents the phosphorylation and subsequent degradation of β-catenin, resulting in its increased concentration in the cell and ultimately its passage into the nucleus and downstream expression of Wnt signaling-induced genes

in the vicinity of RyR at the sarcoplasmic reticulum of cardiac myocytes. Local cAMP hydrolysis maintains mAKAP-associated PKA activity low. An increase in the cAMP level exceeding the PDE4D3 hydrolyzing capacity activates PKA, which phosphorylates RyR and increases the open probability of this Ca^{2+} channel. PKA also phosphorylates PDE4D3 and thereby enhances PDE4D3 activity. This again increases local cAMP hydrolysis, switches off PKA, and eventually reduces RyR phosphorylation. This negative feedback loop regulating RyR phosphorylation is completed by association of mAKAP with protein phosphatase 2A (PP2A), dephosphorylating RyR. Dephosphorylation decreases the channel open probability of RyR.

AKAP-PKA Binding

Canonical AKAPs contain an alpha helix in which one side presents a hydrophobic surface while the other side presents a hydrophilic surface, which is known as an amphipathic helix (Newlon et al. 2001). The hydrophobic surface of the amphipathic helix binds to a corresponding hydrophobic region formed by regulatory subunits of PKA. As the name suggests, the association of the AKAP with the regulatory subunit and the regulatory subunits with each other takes place in an ordered region of the N-terminus known as the docking and dimerization (D/D) domain. The D/D domains of two R subunits come together to form a four helical-X-type bundle in which a hydrophobic cleft is created that binds to the hydrophobic face of the AKAP amphipathic

helix (Banky et al. 2003; Sarma et al. 2010; Gold et al. 2006; Götz et al. 2016). Differences in the structure and affinity between type I and type II regulatory subunit D/Ds underlie the differences in RI- and RII-specific AKAPs. There is also an emerging understanding that additional residues outside of this cleft-helix interaction may play an important part in AKAP recognition and binding affinity (Götz et al. 2016).

AKAP-Dependent Integration of Cellular Signaling

In addition to PKA, PDEs, and protein phosphatases involved in cAMP signaling, AKAPs interact with other signaling proteins the activation of which depends on other second messenger molecules, including Ca^{2+}. AKAPs may bind additional kinases such as protein kinases C (PKC) and D (PKD) and protein phosphatases like calcium/calmodulin-dependent phosphatase (calcineurin, protein phosphatase 2B, PP2B). This scaffolding function allows AKAPs to integrate cellular signaling processes. For example, rat AKAP150, and its human ortholog AKAP79, bind PKA, PKC, and calcineurin (Patel et al. 2017). In neurons, AKAP150-bound PKC is activated through an M1 muscarinic receptor-induced pathway that depends on the G protein Gq and leads to elevation of cytosolic Ca^{2+} and diacylglycerol. AKAP150 interacts directly with M channels, which are K^+ channels that negatively regulate neuronal excitability, and facilitates PKC phosphorylation which in turn inhibits the channel. AKAP79 coordinates the phosphorylation of AMPA channels. Cyclic AMP-activated, AKAP79-bound PKA phosphorylates and thereby activates the channels. A rise in cytosolic Ca^{2+} activates AKAP79-bound calcineurin, which in turn dephosphorylates the channels. The dephosphorylation mediates the rundown of AMPA channel currents. Another example is AKAP-Lbc.

AKAP-Lbc binds PKA, PKC, and PKD and possesses intrinsic catalytic activity (Rho guanine-nucleotide-exchange factor (RhoGEF) activity). Through its RhoGEF activity, it catalyzes the exchange of GDP for GTP on the small GTPase Rho. The GTP form of Rho is active and induces the formation of F-actin-containing stress fibers. Agonists stimulating receptors coupled to the G protein G_s may mediate activation of AKAP-Lbc-bound PKA, which in turn phosphorylates AKAP-Lbc. Subsequently, a protein of the 14-3-3 family binds to the phosphorylated site and inhibits the RhoGEF activity. In contrast, agonists stimulating receptors coupled to the G protein G_{12} increase the RhoGEF activity.

AKAPs Optimize the Limited Repertoire of Cellular Signaling Proteins

Intriguingly, the same AKAP may coordinate regulation of different target proteins. In hippocampal neurons, AKAP150 positions PKA and calcineurin to modulate AMPA channels and maintains PKC inactivity. In superior ganglial neurons, AKAP150 facilitates PKC phosphorylation of M channels while keeping PKA and calcineurin inactive. The difference is due to the interaction of AKAP150 with the scaffolding protein SAP97, which occurs in hippocampal neurons but not in superior ganglial neurons. SAP97 positions AKAP150 such that PKA and calcineurin are in close proximity to AMPA channels. Thus, by variation of a single interacting partner, an AKAP optimizes the usage of the limited set of cellular signaling proteins. In summary, the function of AKAPs goes far beyond controlling cAMP/PKA signaling by simply tethering PKA to cellular compartments and confining the access of PKA to a limited set of local substrates. AKAPs are scaffolds forming multiprotein signal transduction modules that coordinate and integrate cellular signaling processes.

Pharmacological Intervention

Disturbances of compartmentalized cAMP signaling in processes such as the ones mentioned above cause or are associated with major diseases including heart failure, diabetes insipidus, diabetes mellitus, obesity, diseases of the immune system including AIDS, cancer, and neurological disorders including schizophrenia. However, AKAPs participating in compartmentalized cAMP signaling networks are not targeted by

drugs which are currently applied for the treatment of such diseases. Recently, clinically relevant intracellular protein-protein interactions have gained much interest as potential drug targets. The cell-type specificity of such interactions and the finding that mostly only selected isoforms of proteins interact with each other offers great opportunities for highly selective pharmacological intervention. For targeting AKAP-dependent protein-protein interactions, initially peptides non-selectively displacing PKA from all AKAPs have been developed.

In renal collecting duct principal cells, arginine vasopressin (AVP) regulates water reabsorption from primary urine by triggering the PKA activation and subsequent redistribution of aquaporin-2 (AQP2) from intracellular vesicles into the plasma membrane [reviewed in (Vukicevic et al. 2016). The redistribution depends on the compartmentalization of PKA by AKAPs including AKAP18δ. The PKA-anchoring disruptor peptides displace PKA and inhibit the AVP-induced redistribution of AQP2 and thereby water reabsorption. In heart failure, an elevated level of AVP causes a predominant localization of AQP2 in the plasma membranes of the renal principal cells and through this excessive water retention contributing to edema. Considering this, the disruption of AKAP-PKA interactions may be a viable treatment option. This and other examples suggest that cell-type-specific pharmacological intervention at selected AKAP-PKA interactions is a feasible concept for the treatment of human diseases and have further spurred the search for small molecule inhibitors of this interaction [reviewed in (Baltzer and Klussmann 2019). Peptidomimetics as well as the first small molecule that non-selectively target AKAP-PKA interactions in a similar manner to the disruptor peptide are available (Schäfer et al. 2013; Christian et al. 2011). More selective small molecules targeting the interaction of AKAP-Lbc and RhoA have recently been discovered (Schrade et al. 2018; Diviani et al. 2016). The examples illustrate that finding modulators of AKAP-dependent protein-protein interactions is a feasible concept.

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Alcohol Dependence

▶ Ghrelin, Physiological Roles and Clinical Relevance of

Alkali Cation/Proton Antiporter

▶ Na^+/H^+ Exchangers

Alkali Cation/Proton Exchanger

▶ Na^+/H^+ Exchangers

Allergy

▶ Ca^{2+}-Binding Proteins
▶ S100 Proteins

Alzheimer's Dementia

▶ Alzheimer's Disease

Alzheimer's Disease

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Synonyms

Alzheimer's dementia; Dementia due to Alzheimer's disease; Morbus Alzheimer

Definition

Alzheimer's disease (AD), named after the psychiatrist and neuropathologist Alois Alzheimer, is an age-associated, chronic, and progressive neurodegenerative illness and the most common cause of neurocognitive disorder (dementia) in the elderly. AD is characterized by a disease duration of two to four decades, starting with 10–20 years of biochemical, cellular, and neurodegenerative changes in the brain (the preclinical or pre-symptomatic phase). In this initial stage, neuropathological changes appear in the brain, such as extracellular amyloid plaque deposits, intraneuronal neurofibrillary tangles, and brain atrophy. This stage is followed by at first subtle but not debilitating cognitive symptoms, finally leading into a progressive worsening and impairment of executive functions (the clinical or symptomatic phase), which makes an unassisted life impossible (Aisen et al. 2017). Age is the most important risk factor for AD with approximately every fifth 85-year-old suffering from dementia (in large part due to AD). It is projected that 100 million individuals will be affected by the year 2050 worldwide, if no progress is made in prevention and treatment (Collaborators GBDD 2019).

Basic Mechanisms

General

The key pathological hallmarks of AD are extracellular amyloid plaque deposits, intracellular neurofibrillary tangles (NFTs), and brain atrophy. In the past, these pathological, AD-specific alterations could only be confirmed via autopsy. However, modern diagnostic tests, using positron emission tomography (PET) scans (to detect plaques and tangles) and magnetic resonance imaging (to estimate brain atrophy), can reliably reveal these abnormalities in patients (Masters et al. 2015).

Amyloid plaques are mainly comprised of the protein fragment amyloid-β (Aβ). These plaques can be detected in patients with a PET amyloid tracer, such as ^{11}C-Pittsburgh compound B. Based on pre-established thresholds, i.e., the global cortical standard uptake value ratio, individuals will be categorized as being amyloid positive or

negative. In the surrounding of Aβ plaques, various abnormalities can be observed, such as the loss of synapses and neurons, dystrophic neurites, as well as indicators of inflammation.

NFTs occur inside neurons and consist of the hyperphosphorylated form of the microtubule-associated protein tau (MAPT). Similar to amyloid plaques, NFTs can be detected in patients using PET tau tracers (Leuzy et al. 2019). The occurrence of NFTs in AD dementia is often followed by signs of neurodegeneration, which can be visualized in patients with structural magnetic resonance imaging. These three major AD pathologies start appearing in the preclinical phase of the disease, and once brain atrophy is well advanced, cognitive symptoms manifest, and individuals transition from the preclinical into the clinical phase (Fig. 1). The exact molecular mechanism that is responsible for the progressive neurodegeneration and ultimately dementia is unknown.

Sporadic Alzheimer's Disease

The vast majority of symptomatic AD cases manifest without a clear inheritance pattern after age 65 and are therefore termed late-onset or sporadic AD. It has been hypothesized that sporadic AD is caused by a combination of lifestyle and genetic risk factors and likely involves a complex cellular phase involving various cell types and signaling pathways (De Strooper and Karran 2016). A major genetic factor known to increase the risk for AD is the ε4-allele of APOE, which codes for the apolipoprotein E, a glycoprotein that transports lipids. Three alleles of APOE exist: ε2, ε3, and ε4. While the ε4 allele is associated with an increased risk, ε2 is associated with a decreased risk. Carriers of the ε4 allele show more pronounced cerebral Aβ plaque depositions.

In genome-wide association studies, other genetic loci were identified, which contribute to a person's risk of developing dementia. These loci affect gene products that have roles, among others, in endosomal vesicle trafficking, inflammatory/immune response, and lipid metabolism. Such processes potentially affect production, aggregation, and/or clearance of Aβ (Van Cauwenberghe et al. 2016). In addition to genetic factors, lifestyle factors, e.g., cardiovascular risk (midlife hypertension, obesity, physical inactivity, diabetes mellitus, and smoking), as well as depression and low educational attainment, may contribute to the overall risk for sporadic AD (Livingston et al. 2017).

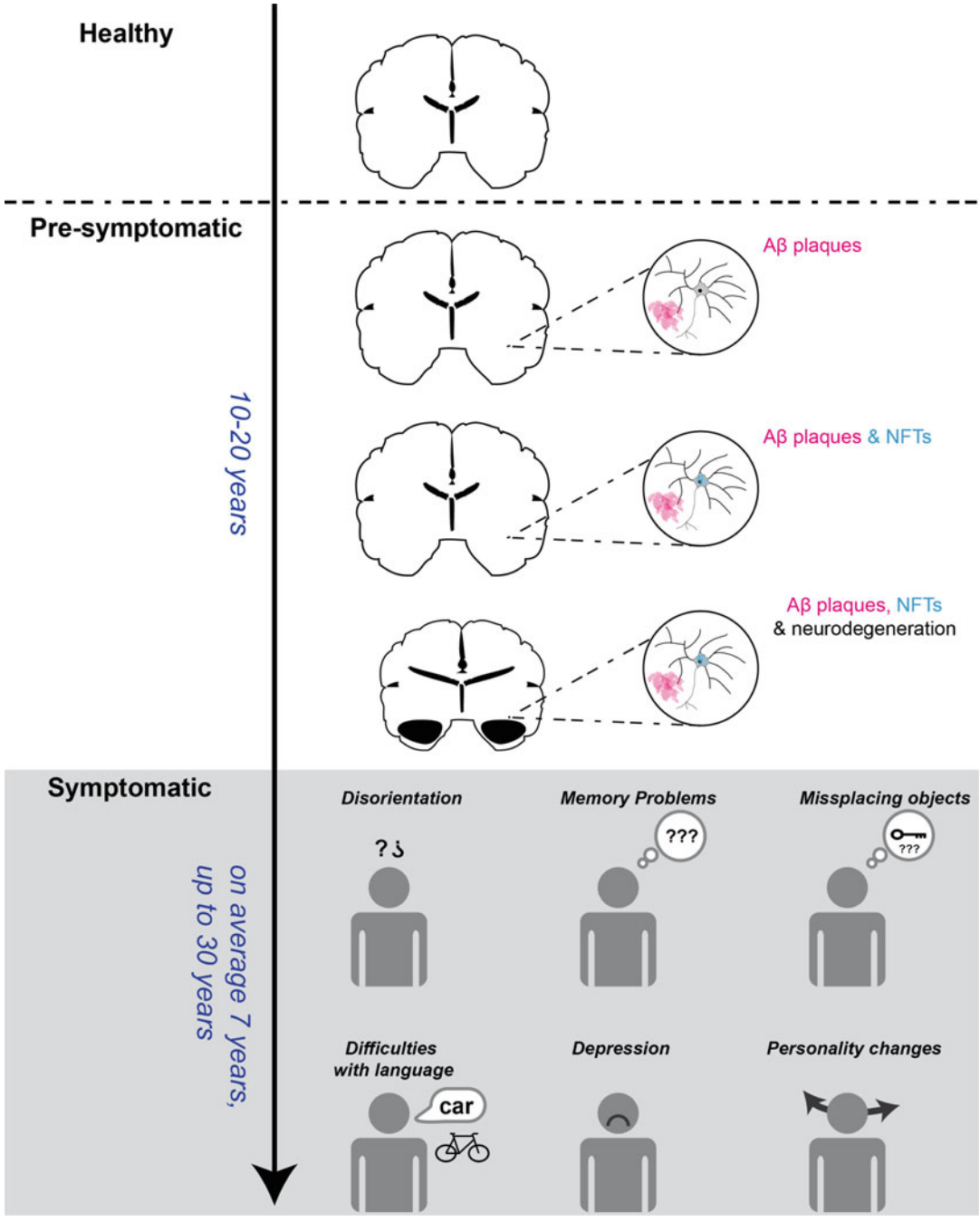
Familial Alzheimer's Disease

In rare cases, AD has a genetic cause and generally follows an autosomal dominant inheritance pattern. This hereditary form of AD is called familial Alzheimer's disease (FAD). FAD symptoms manifest earlier than in sporadic AD (before the age of 65), and it is therefore classified as early-onset AD; however, clinical symptoms and the overall course of the disease are very similar in both forms. To date, mutations in three genes have been identified to cause FAD: *amyloid precursor protein (APP), presenilin 1 (PSEN1), and presenilin 2 (PSEN2)* (Bohm et al. 2015).

Aβ Production, Clearance, and Aggregation

While there is no well-described physiological function of Aβ, its production and clearance are processes that occur in healthy organisms. Aβ is derived from the sequential proteolysis of the amyloid precursor protein (APP) by a process called regulated intramembrane proteolysis. APP is a type-I transmembrane protein that is part of an evolutionarily conserved protein family, which includes the amyloid precursor-like proteins 1 (APLP1) and 2 (APLP2). APP and APLPs are functionally redundant; however, the Aβ sequence in APP is unique and is not conserved in APLP1 or APLP2.

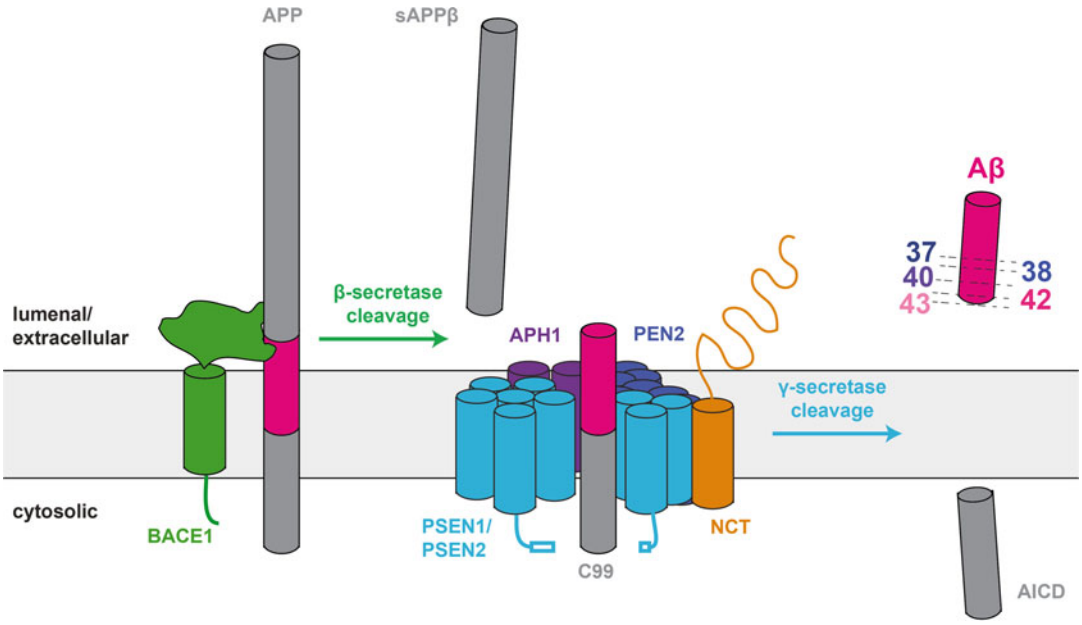
The initial step in Aβ production is catalyzed by the β-secretase (BACE1), which cleaves APP in its ectodomain to generate soluble APPβ (sAPPβ) and a C-terminal membrane-bound fragment with 99 amino acid residues (C99). BACE1 activity is pH-regulated (optimal at pH 4.5), and therefore, the bulk of BACE1-mediated APP cleavage takes place in the endolysosomal system. In a subsequent step, the γ-secretase [a multiprotein complex, consisting of presenilin 1 (PSEN1) or presenilin 2 (PSEN2), anterior



Alzheimer's Disease, Fig. 1 The preclinical phase of Alzheimer's disease is characterized by the sequential appearance of amyloid-β (Aβ) plaques, neurofibrillary tangles (NFTs), and signs of neurodegeneration. Once neurodegeneration is advanced, patients transition into the clinical phase of the disease, and cognitive symptoms appear. Progressive worsening of neurodegeneration and cognitive impairment continue until an independent life becomes impossible

pharynx defective 1 (APH1), presenilin enhancer 2 (PEN2), and nicastrin (NCT)] recognizes C99 as a substrate and generates the APP intracellular domain (AICD) and Aβ peptides of varying lengths. The γ-secretase can cleave at various sites in a consecutive manner, generating Aβ peptides mainly with 37–43 amino acids that can be detected in body fluids and serve as potential biomarkers. The most abundant Aβ forms are comprised of 38 (Aβ38), 40 (Aβ40), or 42 (Aβ42) amino acids (Fig. 2). These various Aβ peptides are produced by a sequential proteolytic cleavage mechanism. The cuts at the cytoplasmic ends of the transmembrane sequence (i.e., the ε-site, which can occur at two different positions) produce AICD (residues 50–99 or 49–99) and membrane-bound Aβ49 or Aβ48. The membrane-bound fragments are further processed into Aβ46 or Aβ45 (at the ζ-site). Aβ46 is further processed into Aβ43 and Aβ40, whereas Aβ45 is the direct precursor of Aβ42, implying that Aβ42 and Aβ40 belong to two different product lines (Munter et al. 2007).

While both Aβ40 (minor species) and Aβ42 (major species) exist in amyloid plaques, Aβ42 is more prone to form oligomers, which are thought to contribute to cognitive decline in AD (Glabe 2006). Within the brain, the concentration of Aβ is severalfold higher than in the periphery, and several mechanisms, such as interstitial fluid drainage, cellular uptake, passive elimination, and enzymatic degradation, are responsible for Aβ clearance from the brain and ultimately from the body (Bates et al. 2009). Thus, it has been proposed that in many cases of FAD, Aβ (especially Aβ42) is chronically overproduced, the Aβ42/Aβ40 ratio is increased, or the Aβ aggregation kinetics are altered. In sporadic AD, impaired Aβ clearance may be responsible for its increased cerebral retention.



Alzheimer's Disease, Fig. 2 Amyloid-β (Aβ) is derived from the amyloid precursor protein (APP). First, the β-secretase (BACE1) cleaves APP within its ectodomain, generating sAPPβ and C99. Second, C99 is cleaved by the γ-secretase complex, generating Aβ peptides of varying lengths, with Aβ40, Aβ38, and Aβ42 being the most abundant species detected in the brain, among others like Aβ37 and Aβ43

Amyloid Hypothesis

Based on pathological findings from sporadic AD and genetic evidence from FAD, the Aβ peptide (especially Aβ42 or even longer, more hydrophobic forms) has a central role in the cause of the disease (Hardy and Selkoe 2002). Accordingly, the entire cascade is initiated by cerebral Aβ oligomer formation (which is followed or accompanied by the appearance of Aβ plaques), which subsequently leads to synaptic dysfunction, inflammation, NFT pathology, and neurodegeneration.

Pharmacological Intervention

General

There is currently no cure for AD. Several symptomatic treatments are approved and used to improve the clinical state of patients; however, these interventions do not delay the progression of the disease. There is an urgent need for disease-modifying interventions, and while several strategies were successful in transgenic animal models of AD, none of these treatments proved efficacious for AD patients.

Symptomatic Treatment

Acetylcholinesterase inhibitors, such as galantamine, rivastigmine, donepezil, tacrine, and others, are used to treat the cognitive symptoms of AD. These drugs counteract the decreased levels of acetylcholine in the brain of AD patients, which is caused by a loss of cholinergic neurons. Acetylcholinesterase inhibitors delay the breakdown of acetylcholine into acetate and choline, thereby increasing the level and duration of action of the neurotransmitter (Mehta et al. 2012).

Memantine is a noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist, which prevents excessive receptor activation and Ca^{2+} influx. In AD and other neurodegenerative diseases, excitatory networks tend to get over-activated (a process called excitotoxicity), and drugs such as memantine can counteract this process by decreasing the effect of the excitatory neurotransmitter glutamate (van Marum 2009).

In addition to acetylcholinesterase inhibitors and NMDA receptor antagonists, AD patients can receive antidepressant, anxiolytic, and antipsychotic medications.

Approaches Toward Disease-Modifying Treatment

Numerous approaches for potentially disease-modifying AD treatments are pursued, and while several attempts were successful in preclinical models (transgenic animal models of AD), none of these could be translated into the clinic yet.

Major strategies aim at a reduction of Aβ production by directly targeting the secretases that are involved in its formation (De Strooper et al. 2010). BACE1 inhibitors can effectively lower Aβ production; however, no beneficial outcome was observed in patients (Barao et al. 2016). Besides its essential role in Aβ production, BACE1 has additional important functions, such as ectodomain shedding of APP-unrelated substrates and even in vivo Aβ clearance (Barao et al. 2016; Liebsch et al. 2019). Such crucial enzymatic roles challenge the BACE1 inhibition approach. Furthermore, γ-secretase inhibitors and modulators have been developed that can either block its entire activity or specifically reduce the formation of Aβ42 by interfering with the sequential proteolytic cleavage mechanism (Olsson et al. 2014). While γ-secretase inhibitors showed strong side effects (likely due to the importance of γ-secretase cleaving other substrates, which would ultimately interfere with essential functions of membrane proteins/receptors), γ-secretase modulators did not alter the cognitive decline in AD patients (Bursavich et al. 2016).

Alternative approaches aim at removing or neutralizing cerebral Aβ by passive immunization (Mo et al. 2017). Antibodies targeting specific regions of the peptide can facilitate Aβ clearance from the brain; however, until today, these are not approved for therapeutic use.

Taking up the challenges, trials with anti-Aβ antibodies and secretase inhibitors and modulators are continued. Ideally, Aβ-targeting treatments should begin in the pre-symptomatic phase of the disease, before overt and possibly

nonreversible neurodegeneration occurs. Therefore, such approaches may be beneficial for disease prevention and must be started decades before symptom onset.

Cross-References

- ▶ Cholinesterases
- ▶ Diabetes Mellitus

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AMP-Activated Protein Kinase

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Synonyms

AMPK

Definition

AMP-activated protein kinase (AMPK) is a member of the serine/threonine protein kinase family, which modify other proteins (*target proteins*) by attaching phosphate groups to the side chains of serine or threonine, thus modifying their function. The primary role of AMPK is to sense energy status by monitoring the cellular ratios of AMP:ATP and ADP:AMP. Once activated by energy stress, AMPK acts to restore homeostasis by switching off downstream processes consuming ATP (such as cell growth and proliferation) while switching on catabolic processes generating ATP (such as glucose uptake and mitochondrial biogenesis). AMPK is also involved in regulating whole-body energy balance and has been identified as a key target in treating disorders such as obesity and type 2 diabetes.

Basic Characteristics

Structure

AMPK occurs universally in the form of heterotrimeric complexes containing a catalytic α subunit and regulatory β and γ subunits (Ross et al. 2016). Humans express multiple isoforms of each subunit (α1, α2; β1, β2; γ1, γ2, γ3), encoded by distinct genes. These can give rise to up to 12 heterotrimeric combinations that display differences in regulatory properties, subcellular locations, tissue-specific expression, and/or interactions with drugs.

Regulation

AMPK complexes are normally only significantly active when phosphorylated at a threonine residue within the kinase domain of the α subunit, usually referred to as Thr172 (Hawley et al. 1996). Thr172 phosphorylation is a widely used biomarker for AMPK activation and is primarily catalyzed by a complex containing the upstream kinase, LKB1 (Hawley et al. 2003; Woods et al. 2003). The gene encoding LKB1 (*STK11*) was originally identified by human genetics to be a tumor suppressor, introducing a link between AMPK and cancer that is still being investigated (Vara-Ciruelos et al. 2019).

Canonical Regulation by Adenine Nucleotides

The AMPK-γ subunits contain four tandem repeats of sequence motifs known as CBS repeats, which are involved in binding of the regulatory ligands AMP, ADP, and ATP. They form a pseudosymmetrical disk-shaped structure with one repeat in each quadrant. In the center of this disk are four potential binding sites for regulatory nucleotides, although only three are utilized (Xiao et al. 2007). The critical site is formed by repeat 3 (CBS3); binding of AMP at this site causes conformational changes in the AMPK complex that trigger activation by three complementary mechanisms: (i) allosteric activation; (ii) promotion of Thr172 phosphorylation by LKB1; and (iii) inhibition of Thr172 dephosphorylation by protein phosphatases (Ross et al. 2016). Binding of ATP at CBS3 opposes all of these effects, so that the AMPK complex is activated by increases in the cellular AMP:ATP ratio. Binding of ADP to CBS3, at higher concentrations than AMP, also mimics the effects of AMP, except that it does not cause allosteric activation. Increases in cellular ADP:ATP ratios indicate that the energy status of the cell has been compromised but are always accompanied by even larger changes in AMP due to displacement of the reaction catalyzed by adenylate kinases ($2ADP \leftrightarrow ATP + AMP$). For

this reason, increases in AMP:ATP are a particularly sensitive indicator of cellular energy stress.

Noncanonical Regulation by Hormones Involved in Regulation of Whole-Body Energy Balance

A second upstream kinase that phosphorylates Thr172 is the Ca^{2+}/calmodulin-dependent kinase, CaMKK2, which is activated by hormones that trigger intracellular release of Ca^{2+} ions. These include ghrelin, which is released from the stomach during fasting, promoting appetite and hence whole-body energy intake by activation of AMPK in specific neurons of the hypothalamus (Yang et al. 2011). Some hormones have the opposite effect, including leptin that is released from adipocytes containing high stores of fat. Leptin inhibits AMPK in specific hypothalamic neurons, possibly by triggering phosphorylation of a regulatory sequence near the C-terminus of the α subunit (Dagon et al. 2012), thus repressing appetite and whole-body energy intake.

Noncanonical Activation by Glucose Starvation

AMPK is also activated by depriving cells of glucose – in some cell types, this is associated with increases in AMP:ATP/ADP:ATP ratios and can thus occur in part via the canonical mechanism described above. However, in other cells, AMPK is activated by glucose starvation without accompanying changes in adenine nucleotides, as long as an alternative carbon source such as glutamine is available; AMPK is also activated in livers of fasted mice without any apparent changes in nucleotides (Zhang et al. 2017). Under these circumstances, activation involves the translocation of a complex between LKB1 and the adapter protein Axin and of AMPK, to the surface of the lysosome where they associate with each other as well as the resident lysosomal proteins, p18/LAMTOR1 and the vacuolar ATPase (Zhang et al. 2017). The absence of glucose is sensed as a drop in the binding of the glycolytic intermediate fructose-1,6-bisphosphate (FBP) to the glycolytic enzyme FBP aldolase; this appears to cause inhibition of Ca^{2+} release by TRPV channels that occur at ER/lysosome contact sites, thus affecting interaction of the channels with the vacuolar ATPase (Li et al. 2019).

Downstream Targets of AMPK

One activated, AMPK can phosphorylate numerous downstream targets at specific serine or threonine residues within well-defined sequence motifs, with over 60 being currently identified (Hardie et al. 2016). A full description of these is beyond the scope of this article, but in general AMPK switches off ATP-consuming anabolic pathways required for cell growth while switching on catabolic processes that generate ATP. These effects can occur both via direct phosphorylation of metabolic enzymes and also via phosphorylation of proteins affecting gene expression. A good example is the ACC1 isoform of acetyl-CoA carboxylase, the first enzyme in the pathway of de novo fatty acid synthesis from acetyl-CoA. While ACC1 is inactivated by direct phosphorylation by AMPK (Munday et al. 1988), expression of its mRNA is also switched off via phosphorylation of multiple transcription factors (Li et al. 2011; Kawaguchi et al. 2002). On the other hand, phosphorylation by AMPK of the ACC2 isoform, which is associated with mitochondria, is involved in acute activation of fatty acid oxidation (Merrill et al. 1997). Thus, AMPK activation switches on fatty acid oxidation, a catabolic pathway that plays an important role in whole-body energy expenditure, while switching off fatty acid synthesis, an energy-consuming anabolic pathway required for cell growth. AMPK also switches on glucose uptake involving the glucose transporters GLUT1 and GLUT4, in part via phosphorylation and degradation of the α-arrestin family member TXNIP, which otherwise promotes ubiquitination and consequent reuptake of the glucose transporters from the plasma membrane via endocytosis (O'Donnell and Schmidt 2019). Finally, AMPK enhances mitochondrial function in cells not only by promoting biogenesis of new mitochondrial components (Zong et al. 2002) but also by triggering removal of damaged

components, by promoting mitochondrial fission (Toyama et al. 2016) and recycling of the resulting fragments by *mitophagy*, a specialized form of autophagy (Egan et al. 2011).

Drugs

Interest in the AMPK system greatly increased when it was reported to be activated by metformin, still the primary drug used to treat type 2 diabetes today (Zhou et al. 2001). Metformin mainly acts on the liver and gut due to its cellular uptake by organic cation transporters expressed in those cells. It is now clear that its ability to acutely inhibit hepatic glucose production does not require AMPK (Foretz et al. 2010), but another important component of metformin action is its long-term ability to enhance hepatic insulin sensitivity. In mice this requires the phosphorylation of both the ACC1 and ACC2 isoforms of acetyl-CoA carboxylase by AMPK, thus reducing the elevated lipid storage in the liver that is strongly associated with insulin resistance (Fullerton et al. 2013).

Numerous drugs and natural products have now been shown to activate AMPK in preclinical studies, and they can now be divided into three groups according to the mechanisms by which they cause its activation (Fig. 1).

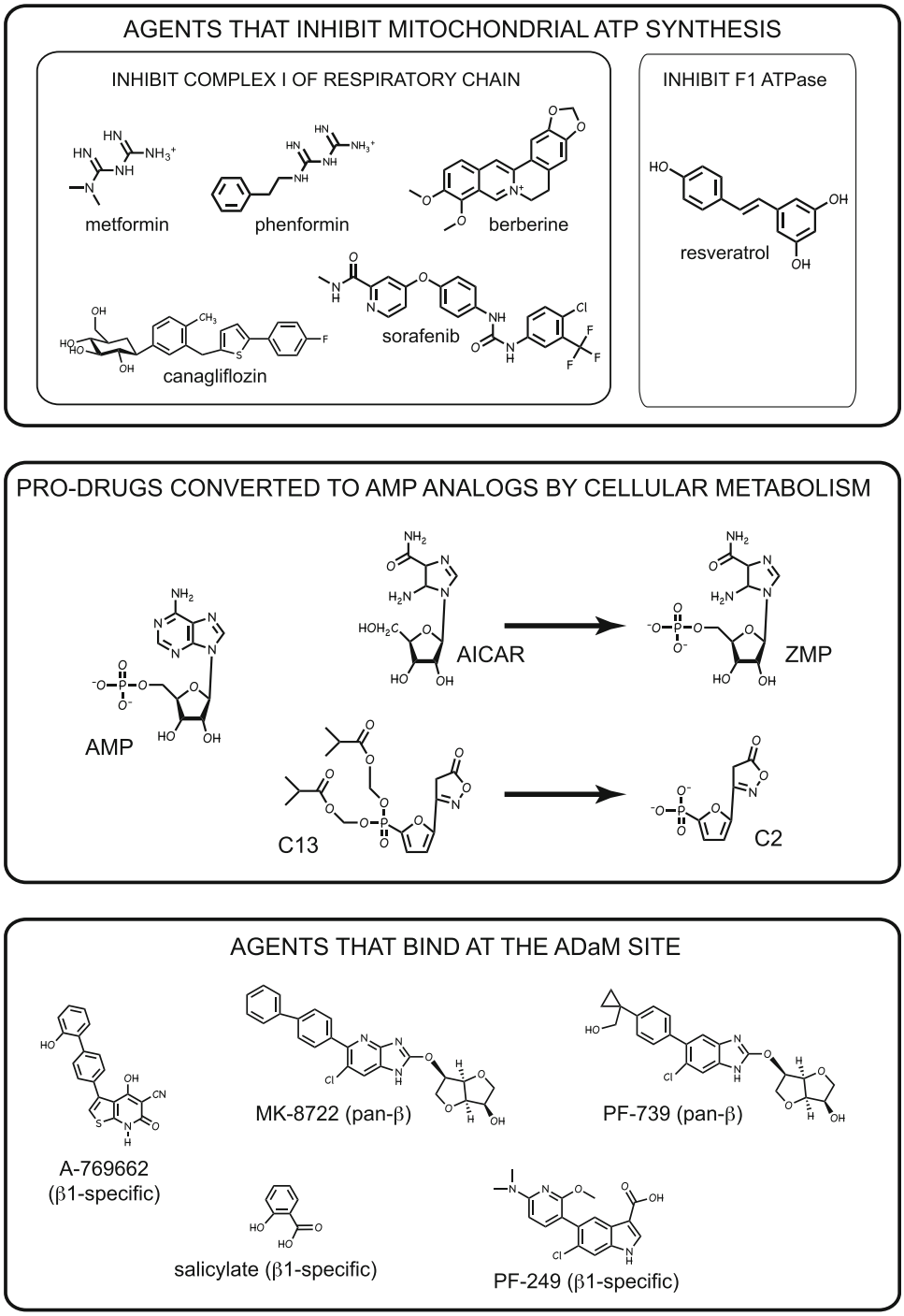
Agents That Act Indirectly by Inhibiting Mitochondrial Function

In most cells, mitochondria produce much of the cellular ATP, so agents that inhibit their function will activate AMPK indirectly by the canonical AMP-dependent mechanism. Agents that act in this this manner can be identified by their failure to activate AMPK complexes containing arginine-glycine (RG) mutations in the critical CBS3 site of the γ subunit (Hawley et al. 2010). They include the antidiabetic drug metformin, which inhibits complex I of the mitochondrial respiratory chain. Metformin and a related biguanide, phenformin, are derivatives of *galegine*, originally extracted from the medicinal herb *Galega officinalis*. AMPK is also activated by many other natural plant products, including *resveratrol* and *berberine* (Hawley et al. 2010). Berberine is used in traditional Chinese medicine, and, remarkably, more than 400 papers now describe compounds from traditional medicines that activate AMPK. Although the mechanisms by which most of these activate AMPK are not known, we suspect that many may be inhibitors of mitochondrial function. Most are so-called secondary plant metabolites (meaning that they are not essential for growth under ideal conditions) and may be defensive compounds produced to deter infection by pathogens or grazing by insects or other herbivores. Interestingly, they are often stored either in the vacuole or the cell wall of plant cells, where they would not come into contact with the plant's own mitochondria (Shitan 2016).

Finally, AMPK is also activated via "off-target" mitochondrial inhibition by at least two drugs that are in use clinically, although previously thought to be solely directed at other targets. These are the SGLT2 inhibitor *canagliflozin*, used to treat diabetes (Hawley et al. 2016), and the protein-tyrosine kinase inhibitor *sorafenib*, used to treat some cancers (Ross et al. 2017).

Prodrugs That Are Converted by Cellular Metabolism into AMP Analogs

The first compound reported to activate AMPK in intact cells was 5′-aminoimidazole-4-carboxamide riboside (AICAR), which is taken up into cells via adenosine transporters and converted by adenosine kinase to the monophosphorylated ribotide, ZMP (Fig. 1). ZMP mimics all three effects of AMP to activate AMPK and has been widely used as an experimental tool to study downstream consequences of AMPK activation. However, ZMP is around 50-fold less potent than AMP in activating AMPK (Corton et al. 1995) and only works because ZMP accumulates inside cells to millimolar concentrations. At those concentrations it is perhaps no surprise that ZMP should have "off-target," AMPK-independent effects. For example, its ability to cause acute hypoglycemia in mice is due to the ability of ZMP to mimic the inhibition



AMP-Activated Protein Kinase, Fig. 1 AMPK activators grouped according to their mechanism of action. See text for details

of the gluconeogenic enzyme, fructose-1,6-bisphosphatase, by AMP (Hunter et al. 2018).

An agent that activates AMPK by a related mechanism is compound 13 (C13), a diisopropyl phosphonate ester that is taken up into cells and converted by cellular esterases to the AMP analog C2 which, surprisingly, is a more potent activator of AMPK than AMP itself (Gomez-Galeno et al. 2010). C2 does not affect other AMP-sensitive enzymes such as fructose-1,6-bisphosphatase and is also highly specific for AMPK complexes containing α1, with no activation of α2 complexes (Hunter et al. 2014).

Agents That Activate AMPK by Binding the Allosteric Drug and Metabolite (ADaM) Site

The ADaM site is located between one surface of the carbohydrate-binding module (CBM) on the AMPK-β subunits and the surface of the small lobe of the kinase domain on the α subunit, opposite to the catalytic site (Xiao et al. 2013). This site is of great interest because, being formed by the juxtaposition of two different subunits, it is unique to AMPK. The first compound shown to bind there was A-769662, derived from a high-throughput screen that searched for allosteric activators of AMPK (Cool et al. 2006). A-769662 and most other ADaM site activators cause a large allosteric activation of AMPK, with just a modest increase in Thr172 phosphorylation caused by inhibiting its dephosphorylation.

A curious feature of the ADaM site is that almost all compounds currently known to bind there are synthetic molecules, rather than natural products. The one exception is salicylate (Hawley et al. 2012), but salicylate is a plant product and is not known to occur in animals unless it is ingested. The ADaM site is therefore a type of *orphan receptor* – most researchers in the field assume there is a natural ligand that binds there (hence the "*a*nd Metabolite" element of the ADaM acronym), but such a ligand has not yet been identified. Salicylate (in the form of willow bark extract) has been used by humans as a medicine since ancient times and is usually now administered in the form of acetylsalicylic acid (ASA or aspirin). While ASA itself acts by inhibiting cyclooxygenases and hence the synthesis of prostanoid messengers, salicylate is rapidly generated in vivo from ASA by cellular esterases, and some therapeutic effects of aspirin might be mediated by AMPK (Hawley et al. 2012).

A-769662 has poor oral availability, but subsequent screens produced more potent and orally available ADaM site activators, such as MK-8722 (Myers et al. 2017), PF-739 (Cokorinos et al. 2017), and PF-249 (Salatto et al. 2017). All of these compounds are more potent activators of β1- rather than β2-containing complexes, and some (like A-769662 and PF-249) are essentially β1-specific. However, MK-8722 and PF-739 are *pan-β activators* that do activate β2-containing complexes in vivo. This is significant because it means that they activate AMPK in skeletal muscle, which only expresses β2 and not β1. This enabled the direct demonstration that drugs that activate AMPK and thus promote glucose uptake in skeletal muscle would significantly lower plasma glucose in animal models of insulin resistance, both in mice and nonhuman primates (Myers et al. 2017; Cokorinos et al. 2017). None of these compounds have yet proceeded into human clinical trials, possibly because they cause the potentially adverse effect of cardiac hypertrophy in animals (Myers et al. 2017). Promisingly, however, more short-acting ADaM site activators have been shown to be just as effective as MK-8722 in lowering plasma glucose and insulin in rodent models of insulin resistance and diabetes, although they did not cause cardiac hypertrophy (Muise et al. 2019).

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AMPK

► AMP-Activated Protein Kinase

Amylin

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Synonyms

Diabetes-associated peptide; Islet amyloid polypeptide

Definition

Amylin was discovered in 1987 and was identified as the major component of islet amyloid, which is commonly found in patients with type 2 diabetes (T2D). Amylin belongs to the calcitonin peptide family, which includes calcitonin (CT), α- and β-calcitonin gene-related peptide (CGRP), adrenomedullin, and intermedin/adrenomedullin 2. Members of this family play important roles in the regulation of several important homeostatic processes and thus have been drug targets of interest (Hay et al. 2015).

Amylin is a naturally occurring ~4 kDa peptide hormone composed of 37 amino acids with an intramolecular disulfide bridge between cysteine residues at positions 2 and 7 and an amidated C-terminus. Both of these are essential for amylin to carry out its biological activity (Bower and Hay 2016).

The gene encoding human amylin is located on chromosome 12, which transcribes in humans as an 89-amino acid precursor. The precursor is then converted to the 67-amino acid proamylin with the removal of the signal peptide and is stored in β-cell granules with insulin and C-peptide. The biologically active 37-amino acid form of amylin is derived through proteolysis and posttranslational modifications of proamylin. The process involves proteolytic cleavage of proamylin at pairs of basic residues at the C-terminus and N-terminus with carboxypeptidase E and prohormone convertases 1/3 and 2, followed by the formation of a disulfide bond between positions 2 and 7 and amide formation at the C-terminal tyrosine (Westermark et al. 2011).

Amylin is primarily synthesized in the pancreatic islet β-cells. Expression of amylin has also been detected in the gastrointestinal system, lung tissue, and nervous system. It is co-synthesized and co-secreted with insulin by pancreatic β-cells at a ratio of approximately 10–100:1 (insulin:amylin) in response to nutrient stimuli (Zhang et al. 2016).

Amylin circulates in the plasma at concentrations of 3–5 pM in the fasting state of normoglycemic subjects and increases to 15–25 pM after eating (postprandially). Plasma amylin is deficient in type 1 diabetes (T1D) patients, due to a lack of functional β-cells. In contrast, amylin increases in the initial phase of T2D, followed by a diminished capacity to secrete amylin in late-stage T2D, corresponding to β-cell dysfunction (Hay et al. 2015).

Basic Characteristics

Physiological Role

Amylin is a neuroendocrine hormone that acts centrally. It is highly conserved phylogenetically, which often indicates a significant role physiologically. Amylin reportedly has several biological roles. These include regulation of energy homeostasis, acting as an adiposity signal, bone metabolism, blood pressure regulation, and locomotor activity (Hay et al. 2015). However, most evidence indicates that the main role of amylin is in complementing insulin in the regulation of nutrient influx into the circulation and maintenance of energy homeostasis.

Glucose Regulation

Amylin is co-secreted with insulin in response to nutrient stimuli, and the role of insulin as a regulator of blood glucose is well-known. It is suggested that amylin works synergistically with insulin rather than directly to suppress

postprandial blood glucose levels. Amylin is the third most important and active pancreatic islet hormone behind insulin and glucagon for the regulation of glucose homeostasis. Studies have found that amylin regulates postprandial glycemia by several mechanisms including slowing gastric emptying, decreasing postprandial glucagon secretion, and promoting satiety thus preventing postprandial spikes in blood glucose levels (Zhang et al. 2016). Insulin promotes absorption of glucose from the bloodstream into peripheral tissues, while amylin controls the entry of glucose from ingested food into the bloodstream by slowing gastric emptying. The rate of gastric emptying is important in blood glucose homeostasis, as a slower rate delays carbohydrate from entering the small intestine, thus limiting the inflow of meal-derived glucose into the bloodstream. Amylin is well established to inhibit gastric emptying. This effect is observed in humans; administration of an amylin analogue, pramlintide, delays gastric emptying in T1D and T2D patients (Ryan et al. 2009). Another key step of postprandial glucose regulation is to halt further endogenous production of glucose from energy stores. Amylin has been shown to hinder glucagon release which in turn stops hepatic gluconeogenesis and glycogenolysis (Lutz 2010).

Satiety

Amylin is well documented to play a role in reduced eating by inducing meal-ending satiation. The anorectic actions of amylin represent a physiological effect as it is rapidly released following a meal, and exogenous amylin administration reduces eating within minutes. Studies have shown that the effect of reduced meal size is dose-dependent and chronic administration of amylin resulted in a sustained reduction in food intake. Furthermore, this action is blocked by an amylin receptor antagonist, AC187. The effects are likely to be mediated via the area postrema (AP) of the brainstem as the anorectic effect of amylin was abolished in rats with lesions in the AP region (Lutz 2010).

Adiposity Signal

It has been proposed by Morton et al. (2006) that there are three criteria to fulfill to be considered as an adiposity signal. The hormone should (1) circulate in the blood at a level proportionate to body adiposity and able to access to the brain and (2) promote weight loss through neural systems which regulate energy homeostasis, and (3) inhibition of these neuronal actions results in body weight and adiposity gain. Numerous studies have demonstrated amylin may play a role as an adiposity signal as it shares similar characteristics to well-known adiposity signal hormones, i.e., insulin and leptin. Higher basal plasma amylin is found in obese rats and overweight/obese humans compared to lean controls. Moreover, chronic peripheral or central amylin administration in rats resulted in weight reduction by lowering of adiposity, whereas amylin antagonist treatment increased food intake and body adiposity (Lutz 2010).

Energy Expenditure

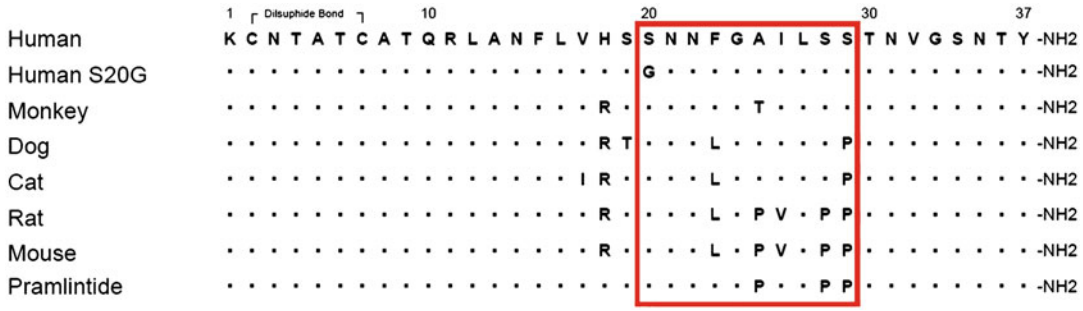
There are a number of studies on the effect of amylin on energy expenditure. The experimental observations are based on the assessment of energy expenditure by indirect calorimetry. Acute administration of the long-acting amylin receptor agonist salmon CT has been reported to increase energy expenditure in the rat. However chronic peripheral administration of amylin exhibited the same energy output as control rats but had lower food intake and weight (Lutz 2010).

Additionally, transgenic mice that overexpress either human amylin or receptor activity-modifying protein (RAMP) 1 of an amylin receptor complex have shown increased energy expenditure and higher body temperature. Mice overexpressing RAMP1 also had increased sympathetic nerve activity in thermogenic brown adipose tissue (regulator of energy expenditure and body fat in humans and rodents), and addition of a β-adrenergic receptor antagonist inhibits the activity (Lutz 2010).

Pathological Roles

It has been proposed that aberrant protein aggregation to form amyloid deposits is the underlying cause of several diseases such as T2D, Alzheimer's, and Parkinson's disease.

Amylin's amino acid sequence is highly conserved through evolution. Amylin shares at least



Amylin, Fig. 1 Single-letter code amino acid sequences of amylin in the human, human variant (S20G) monkey, dog, cat mouse, rat, and pramlintide. Box highlights the amyloidogenic region. Dots correspond to conserved residues with human amylin sequence

80% sequence identity across mammalian species. However, human, feline, and nonhuman primate forms of amylin have the propensity to form amyloid, while rat/mouse amylin lacks fibrillogenicity (Fig. 1). Studies have demonstrated that the variations detected in the region between amino acids 20 and 29 are critical for amyloid formation because this region contains five of six differences found between human and rat amylin. Most notably, rodent amylin has three proline substitutions at positions 25, 28, and 29, which are known as β-*sheet breakers* that greatly attenuate fibril formation. It is now recognized that amylin fragments 8–20, 10–19, and 30–37 also form amyloid fibrils (Westermark et al. 2011).

Islet amyloid formation starts with soluble monomeric amylin associating into soluble β-sheet oligomeric states. These oligomers then subsequently assemble into protofibrils arranged in parallel or antiparallel β-pleated sheets. Once protofibrils are formed, it rapidly aggregates into insoluble fibrils, and further aggregation of fibrils results in islet amyloid (Westermark et al. 2011).

Numerous transgenic rodent models overexpressing human amylin in islet β-cells develop diabetes, and increasing transgene expression resulted in increased β-cell apoptosis and earlier onset of diabetes (Westermark et al. 2011). The current consensus is that the aggregation intermediate of the soluble oligomer of human amylin is the main culprit that constitutes the toxic species and not the fibrils. A study using time-lapse atomic force microscopy captured human amylin oligomers forming, thus confirming their existence in vitro. Human amylin preparations with few preformed fibrils are highly toxic to β-cells, and conversely, amylin preparations with ample preformed fibrils are low in toxicity. Furthermore, dissociating the pre-existing aggregates into monomers restored the high cytotoxic potential. Rifampicin is a potent inhibitor of fibril development, but not an inhibitor of the formation of amylin oligomers. It did not prevent β-cells from apoptosing, supporting the notion that the amylin oligomers are the toxic species (Raleigh et al. 2017).

Several mechanisms of human amylin cytotoxicity have been proposed, but the exact causes of cell death are still not completely defined. Currently, human amylin aggregates which mediate apoptosis by triggering several apoptotic signals and membrane disruption through the formation of ion channels and/or direct interaction to affect membrane integrity are the two main widely accepted hypotheses (Westermark et al. 2011). However, one must take into consideration that in vitro and transgenic animal model studies may not fully reflect the slow process of islet amyloid deposition in human T2D patients. It is also important to note that islet cell cytotoxicity and islet amyloidosis are not universal features that occur in all T2D patients. Thus, human amylin cytotoxicity could be one of the many contributors to T2D pathogenesis in humans (Raleigh et al. 2017).

Islet Amyloid and T2D Progression

Human amylin is a major component of islet amyloid deposition. It has been documented that between 40% and 100% of T2D patients have amyloid deposits in their pancreatic islets. It has

been proposed that the formation of islet amyloid is related to β-cell dysfunction and reduced β-cell mass, causing diminishing insulin secretion, contributing to the pathogenesis of T2D. Additionally, extended exposure of pancreatic islets to high glucose environments has been shown to selectively favor amylin secretion, thus increasing the risk of islet amyloid formation and β-cell deterioration (Westermark et al. 2011).

Recent studies have also reported that amylin deposition was found in the kidney and heart of T2D patients. Renal amylin deposition was found in postmortem tissue from patients with diabetic nephropathy, and the amount of deposition was correlated with disease severity. Significant accumulation of large amylin oligomers, fibrillar tangles, and plaques was also found in failing hearts from patients with obesity and/or T2D, but not in normal hearts and failing hearts from lean humans without diabetes (Despa and DeCarli 2013).

Human Amylin and Alzheimer's Disease

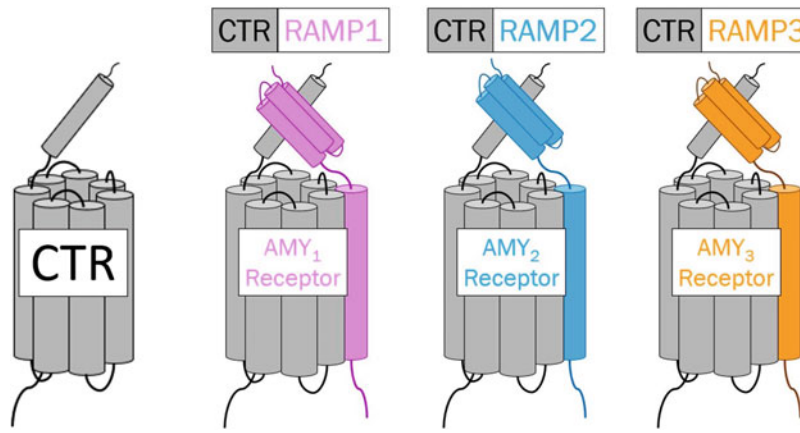
There has been interest in the connection between amylin and Alzheimer's disease (AD). T2D and AD are more prevalent with aging and share many clinical and biochemical features indicating common pathogenic mechanisms. Both diseases feature amyloid deposits: human amylin in the pancreas and amyloid β (Aβ) in the brain, respectively. Human amylin aggregation has been found to be linked to β-cell death, while Aβ formation causes neuronal cell loss. A meta-analysis also revealed that T2D patients have a 60% greater risk of developing AD (Vagelatos and Eslick 2013). There have been several studies into the potential involvement of amylin in AD. However, these are conflicting as to whether it is a beneficial or contributory factor to AD. Therefore, more work is required to understand this.

Amylin Receptors

Amylin receptors designated as AMY_1, AMY_2, and AMY_3 are comprised of heterodimerized complexes formed from the CT receptor (CTR) interacting with one of three accessory proteins called RAMPs (Fig. 2). CTR is a seven-transmembrane-domain Class B G protein-coupled receptor (GPCR) encoded on chromosome 12 in humans. There are two main splice variants of human CTR being $hCT_{(a)}$ as the major subtype and $hCT_{(b)}$. The RAMPs are single-domain transmembrane proteins with subtypes RAMP1, RAMP2, and RAMP3. Six amylin receptors can form from the two CTR isoforms plus one of three RAMPs (Hay et al. 2015).

Amylin receptors are widely distributed in the brain as identified through radioligand binding studies. The most abundant sites of the amylin receptors are found in the nucleus accumbens, the AP of the brainstem, and the hypothalamus. It is thought that the AP, which does not have a blood-brain barrier (thereby allowing access to circulatory peptides), could be an important site for amylin action (Hay et al. 2015).

To date, extensive studies have reported on the pharmacological properties of these receptors.



Amylin, Fig. 2 A schematic diagram of the amylin receptors AMY_1, AMY_2, or AMY_3. Receptors are generated from the interaction of the calcitonin receptor with RAMP1, RAMP2, or RAMP3

Amylin, Table 1 Development of amylin receptor agonist-based drugs

Drug/company	Therapy area	Status of drug
Pramlintide acetate (Amylin Pharmaceuticals, AstraZeneca)	Type 1 and 2 diabetes	Launched
Pramlintide + A21G human insulin (Adocia)	Type 1 diabetes	Phase 2 clinical
AM-833 (Novo Nordisk)	Obesity	Phase 2 clinical
BI-473494 (Boehringer)	Cardiovascular disease; diabetes mellitus; metabolic disorder; obesity	Phase 1 clinical
DACRA-089 (Eli Lilly & Co)	Diabetes mellitus; nonalcoholic steatohepatitis; obesity	Phase 1 clinical
Pramlintide + human insulin (BioChaperone), (Adocia)	Type 1 diabetes	Phase 1 clinical
Pramlintide + metreleptin (Amylin/ Takeda)	Obesity	Discontinued
Davalintide	Obesity	Discontinued
Pramlintide development for Alzheimer's disease (WE Biosciences Llc)	Alzheimer's disease	National Institute on Aging funded We Biosciences Llc for development

However, the physiological relevance of each of the amylin receptor subtypes is still not fully elucidated. Study of amylin receptor function is complicated by lack of antagonists that can block specific subtypes of amylin receptors and the fact that some amylin receptors can also be activated by CGRP. Hence, it is still unknown which combination of CTR and RAMP is the functional amylin receptor in AP or the other brain regions.

Drugs

Pharmaceutical companies have been focusing on drug development based on amylin function in glycemic and energy regulation (Table 1). Preclinical studies have considered inhibition of amylin aggregation as a potential therapeutic strategy against T2D.

Amylin Receptor Agonist-Based Drugs

Approved Drugs

Pramlintide was the first FDA-approved amylin-based drug, which is approved for use as an adjunct to insulin for T1D and insulin-treated T2D. Human amylin's self-aggregation properties to form an insoluble compound in a number of solvents make it unsuitable for pharmaceutical use directly. However, this issue is circumvented by substituting proline for the positions 25 (alanine), 28 (serine), and 29 (serine) of amylin to result in a soluble, stable synthetic analogue, pramlintide (Fig. 1). Most importantly, this analogue possesses similar beneficial action to naturally occurring amylin (Bower and Hay 2016). Various clinical data in T1D and T2D patients showed that pramlintide therapy decreases A1C by 0.2–0.6% and the 2-h postprandial hyperglycemia by 3.4–5 mmol/L. Pramlintide was also found to reduce the weight of diabetic patients after 1-year of study (Ryan et al. 2009). One of the issues with pramlintide is incompatibility with insulin due to the formulation pH. Pramlintide is formulated at pH 4 to maintain solubility, whereas insulin is formulated at a neutral pH. Thus patients are required to take both drugs separately, and multiple injections per day can become a burden (Bower and Hay 2016). This currently limits its clinical use and has driven the search for novel amylin mimetics.

Exploratory Drugs

There is strong interest in improving the potency and pharmacokinetics of amylin agonists to

Amylin, Table 2 Development of amylin aggregation inhibitor-based drugs

Drug	Active indication	Status of drug
Antihuman amylin oligomer antibodies (Tel Aviv University)	Type 2 diabetes	Discovery
Human amylin aggregation inhibitor (Zyentia)	Type 2 diabetes	Discontinued

achieve a more patient-friendly treatment of metabolic diseases.

Lipidation, PEGylation, and glycosylation are common strategies to modify peptides by the covalent attachment of polyethylene glycol (PEG), oligosaccharides, or fatty acids. Each aims to improve drug properties such as half-life, metabolic stability, and receptor binding. PEGylation and glycosylation have been tested so far, but further investigation is warranted (Bower and Hay 2016). Other amylin mimetics include davalintide and DACRA-089 (Bower and Hay 2016).

Combination therapy of amylin agonists with other hormones such as leptin, PYY, and cholecystokinin could also prove to be useful for weight loss. Pramlintide plus leptin has been investigated extensively. Leptin plays a role in the long-term signal of energy balance. Yet, obese rodents and humans are largely nonresponsive to exogenous leptin treatment. Studies have shown that amylin may improve leptin sensitivity and restore leptin responsiveness in obesity. Pramlintide with metreleptin, an analogue of leptin, has been selected to advance toward phase 3 clinical trial. However, it was halted due to antibody development against metreleptin in patients (Hay et al. 2015).

The strategies aforementioned lay the foundation for continuing trials examining drug design, development, and therapy for amylin-based pharmacotherapy that may prove to be a promising approach for weight loss, diabetes, and potentially other disorders.

Amylin Aggregation Inhibitor-Based Drug

There has been some success with small peptides that interact with human amylin to attenuate the aggregation process in vitro and in vivo to inhibit amyloidosis (Table 2). Alternative inhibitors like tetracycline, Congo red, and rutin have been reported to be effective in vitro and in vivo. Moreover, aromatic and polyphenol compounds such as resveratrol (a substance in red wine) and epigallocatechin-3-gallate (a substance in green tea), respectively, have been suggested as potential inhibitors (Raleigh et al. 2017).

Cross-References

► Diabetes Mellitus

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Amylin Receptor

► Calcitonin Family Receptors

Anakinra

► Interleukin-1 (IL-1)

Analgesics

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Synonyms

Pain medication

Definition

Analgesics interfere with the generation and/or transmission of impulses following noxious stimulation (nociception) in the nervous system. This can occur at peripheral and/or central levels of the neuraxis. The therapeutic aim is to diminish the perception of pain.

Nonsteroidal Anti-inflammatory Drugs
Opioid Systems

Mechanisms of Action

Analgesics aim at modulating the generation of noxious chemicals (e.g., prostaglandins) or the activation of neuronal receptors/ion channels transducing/transmitting noxious stimuli (e.g., peptide receptors, Ca^{++} channels). Clinically used drugs include opioids, nonsteroidal anti-inflammatory drugs (NSAIDs), antiepileptics, and antidepressants. Local anesthetics are used for local and regional anesthetic techniques. Mixed drugs (e.g., tramadol, tapentadol, serotonin/noradrenaline reuptake inhibitors) combine various mechanisms (Table 1). Acute and chronic pain are substantially different conditions. The treatment of chronic pain must include non-pharmacological approaches and address psychosocial influences (Dowell et al. 2016; Kaiser et al. 2017).

Opioids

Opioids act on heptahelical G-protein-coupled receptors. Three main types of opioid receptors (μ, δ, κ) have been identified. Additional subtypes (e.g., μ_1, μ_2), possibly resulting from gene polymorphisms, splice variants, or alternative processing, have been proposed. Opioid receptors are localized and can be activated along all levels of the neuraxis including peripheral and central processes of primary sensory neurons (nociceptors), spinal cord (interneurons, projection neurons), brainstem, midbrain, and cortex. All opioid receptors couple to G-proteins (mainly G_i/G_o) and arrestins. G-protein activation leads to inhibition of adenylyl cyclase, decreased conductance of voltage-gated Ca^{++} channels, and opening of rectifying potassium channels, and arrestin coupling induces receptor internalization. Subsequently, neuronal activity and the release of excitatory (pronociceptive) neurotransmitters are reduced. A prominent example is the suppression of tachykinin (substance P) release from primary sensory neurons within the spinal cord and from their peripheral terminals in injured tissue. At the postsynaptic membrane, opioids produce hyperpolarization by opening K^+ channels, thereby preventing excitation or propagation of action potentials in second-order projection neurons. In addition, opioids inhibit sensory neuron-specific tetrodotoxin-resistant Na^+ channels, TRPV1 channels, and excitatory postsynaptic currents evoked by glutamate receptors in the spinal cord. The result is decreased transmission of nociceptive stimuli at all levels of the neuraxis and profoundly reduced perception of pain (Stein 2016). Endogenous opioid receptor ligands are derived from the precursors pro-opiomelanocortin (encoding β-endorphin), pro-enkephalin (encoding Met-enkephalin and Leu-enkephalin), and prodynorphin (encoding

Analgesics, Table 1 Analgesics

Drugs	Targets	Mechanisms	Functional consequences	Side effects
Opioids	G-protein-coupled μ-, δ-, κ-receptors	↓ cAMP ↓ Ca^{++} currents ↑ K^+ currents	↓ excitability of peripheral and central neurons ↓ release of excitatory neurotransmitters	μ, δ: sedation, nausea, euphoria/reward, respiratory depression, constipation κ: dysphoria/aversion, diuresis, sedation
NSAIDs	Cyclooxygenases (COX-1, COX-2)	↓ prostaglandins ↓ thromboxanes	↓ sensitization of sensory neurons ↑ inhibition of spinal neurons	Gastrointestinal ulcers, perforation, bleeding, renal impairment, myocardial infarction, stroke
Antiepileptics	Na^+, Ca^{++} channels GABA receptors	↓ Na^+ currents ↓ Ca^{++} currents ↑ GABA receptor activity	↓ excitability of peripheral and central neurons ↓ release of excitatory neurotransmitters	Sedation, dizziness, cognitive impairment, ataxia, hepatotoxicity, thrombocytopenia
Antidepressants Serotonergic drugs	Noradrenaline/5-HT transporters Na^+, K^+ channels, 5-HT receptors	↓ noradrenaline/5-HT reuptake ↓ Na^+ currents ↑ K^+ currents, cAMP modulation	↓ excitability of peripheral and central neurons ↓ release of excitatory neuropeptides ↓ neurogenic inflammation ↑ vasoconstriction	Cardiac arrhythmia, myocardial infarction, peripheral vascular occlusion, sedation, constipation, dizziness, blurred vision

dynorphins). These peptides contain the common Tyr-Gly-Gly-Phe-[Met/Leu] sequence at their amino terminals, known as the opioid motif. β-Endorphin and the enkephalins are potent antinociceptive agents acting at μ and δ receptors. Dynorphins can elicit both pro- and antinociceptive effects via κ-opioid and/or glutamate receptors. A fourth group of tetrapeptides (endomorphins) with yet unknown precursors do not contain the pan-opioid motif but bind to μ-receptors with high selectivity (Stein 2016).

Nonsteroidal Anti-inflammatory Drugs (NSAIDs)

NSAIDs inhibit cyclooxygenases (COX), the enzymes that catalyze the transformation of arachidonic acid (a ubiquitous cell component generated from phospholipids) to prostaglandins and thromboxanes. Two isoforms, COX-1 and COX-2, are expressed in peripheral tissues and in the central nervous system. In response to injury and inflammatory mediators (e.g., cytokines, growth factors), both isoforms can be upregulated, resulting in increased concentrations of prostaglandins. In the periphery, prostaglandins (mainly PGE_2) sensitize nociceptors by phosphorylation of ion channels (e.g., Na^+, TRPV1) via EP receptor activation. As a result, nociceptors become more responsive to noxious mechanical (e.g., pressure, hollow organ distension), chemical (e.g., acidosis, bradykinin, neurotrophic factors), or thermal stimuli. In the spinal cord, PGE_2 blocks glycinergic neuronal inhibition, enhances excitatory amino acid release, and depolarizes ascending neurons. These mechanisms facilitate the generation of impulses in nociceptors and their transmission through the spinal cord to higher brain areas. By blocking COX, prostaglandin formation diminishes. Subsequently nociceptors become less responsive to noxious stimuli, and spinal neurotransmission is attenuated (Brune and Patrignani 2015).

Antiepileptics

Antiepileptics are primarily used in neuropathic pain resulting from lesions to the peripheral (e.g., diabetes, herpes) or central nervous system (e.g., stroke) (Wiffen et al. 2013). Such syndromes have been attributed to ectopic activity in sensitized nociceptors from regenerating nerve sprouts, recruitment of previously "silent" nociceptors, and/or spontaneous neuronal activity. This can result in sensitization of primary afferents and subsequent sensitization of ascending neurons. In addition, increased expression and trafficking of ion channels (e.g., Na^+, Ca^{++}, TRP) and increased activity at glutamate receptor sites have been demonstrated. Proposed mechanisms of action of antiepileptics include neuronal membrane stabilization by blockage of pathologically active voltage-sensitive Na^+ channels (e.g., carbamazepine, lacosamide, lamotrigine), blockage of voltage-dependent Ca^{++} channels (gabapentin, pregabalin, topiramate), inhibition of presynaptic release of excitatory neurotransmitters (gabapentin, lamotrigine), and enhancing the activity of γ-aminobutyric acid (GABA) receptors (valproate, clonazepam, pregabalin, topiramate) (Finnerup et al. 2015; Wiffen et al. 2013).

Antidepressants/Serotoninergic Drugs

Antidepressants are used for the treatment of neuropathic pain and headache. They include the classic tricyclic compounds and are divided into nonselective noradrenaline/serotonin reuptake inhibitors (e.g., amitriptyline, imipramine, clomipramine, venlafaxine), preferential noradrenaline reuptake inhibitors (e.g., desipramine, nortriptyline), and selective serotonin reuptake inhibitors (e.g., citalopram, paroxetine, fluoxetine). Serotonin (5-hydroxytryptamine; 5-HT) is a monoamine neurotransmitter in the sympathetic nervous system, in the gastrointestinal tract, and in platelets. It acts on 5-HT receptors expressed at all levels of the neuraxis and in blood vessels. Within the spinal cord, serotoninergic neurons contribute to endogenous pain inhibition. With the exception of 5-HT_3 (a ligand-gated ion channel), 5-HT receptors are G-protein-coupled receptors. The reuptake block leads to a stimulation of endogenous monoaminergic pain inhibition in the spinal cord and brain. In addition, tricyclics have glutamate receptor antagonist, opioid enhancing, Na^+ channel blocking, and K^+ channel opening effects, which can suppress peripheral and central sensitization (Finnerup et al. 2015).

$5\text{-HT}_{1B/1D}$ agonists (triptans) are effective against neurovascular (migraine, cluster) headaches. Migraine is related to the release of neuropeptides (e.g., calcitonin gene-related peptide; CGRP) from trigeminal sensory neurons innervating meningeal and intracranial blood vessels. This leads to vasodilation, an inflammatory reaction, and subsequent pain. Triptans inhibit neurogenic inflammation via 5-HT_{1D} receptors on trigeminal afferents, with possible additional sites of action on thalamic neurons and in the periaqueductal gray. The activation of vascular 5-HT_{1B} receptors constricts meningeal (and coronary) vessels. The latter effects have stimulated the search for non-vasoconstrictor approaches such as selective 5HT_{1F} agonists and CGRP antagonists (Gonzalez-Hernandez et al. 2018).

Clinical Use and Side Effects

Whereas acute pain (e.g., associated with surgery or trauma) is usually amenable to drug therapy, chronic pain is a complex disease in its own right and needs to be differentiated into malignant (cancer-related) and noncancer (e.g., musculoskeletal, neuropathic) pain. Acute and cancer-related pain are commonly treatable with opioids, NSAIDs, and/or local anesthetic blocks. Chronic noncancer pain (CNCP) requires a multidisciplinary approach including non-pharmacological (e.g., psychological, physiotherapeutic) treatment strategies (Reinecke et al. 2015; Kaiser et al. 2017; Dowell et al. 2016). Various routes of drug administration (e.g., oral, intravenous, subcutaneous, intrathecal, epidural, topical, intraarticular, transnasal) are used, depending on the clinical circumstances. Local anesthetics are used topically and in regional (e.g., epidural, perineural) anesthetic techniques for the treatment of acute (e.g., associated with surgery or child birth) and some cancer-related chronic pain syndromes.

Opioids

Opioids are the most effective drugs for severe acute and cancer-related chronic pain. They are not appropriate as sole analgesics in CNCP (Reinecke et al. 2015). Lack of fundamental knowledge and misconceptions about the management of CNCP have contributed significantly to the current opioid crisis (Dowell et al. 2016; Babu et al. 2019). All commonly available agents (e.g., morphine, codeine, methadone, fentanyl) are μ-agonists. Naloxone is a nonselective antagonist at all three receptors (μ, δ, κ). Partial agonists must occupy a greater fraction of the available functional receptors than full agonists to induce a response (e.g., analgesia) of equivalent magnitude. Mixed agonist/antagonists (e.g., buprenorphine, butorphanol, nalbuphine, pentazocine) may act as agonists at low doses and as antagonists (at the same or a different receptor) at higher doses. Such compounds typically exhibit ceiling effects for analgesia, and they may elicit an acute withdrawal syndrome when administered together with a pure agonist. All three receptors mediate analgesia but differing side effects. μ-Receptors mediate respiratory depression, sedation, reward/reinforcement, euphoria, nausea, and constipation. Animal studies have shown that δ-agonists produce similar side effects, whereas κ-agonists elicit dysphoric, aversive, sedative, and diuretic effects. Tolerance and physical dependence usually occur with prolonged administration of pure agonists, and abrupt discontinuation or antagonist administration can evoke a withdrawal syndrome. Because G-protein activation occurs not only in sensory neurons (leading to pain relief) but also in neurons driving respiration, arousal, and intestinal peristalsis and in neurons suppressing reward/reinforcement, systemically administered μ-opioids can elicit respiratory depression (apnea), sedation, constipation, dependence, and addiction (Stein 2018; Fields and Margolis 2015; Negus and Freeman 2018).

Opioids are effective in the periphery (e.g., topical or intraarticular administration), at the spinal cord (intrathecal or epidural administration), and systemically (e.g., intravenous or oral administration). The clinical choice of particular compounds is based on pharmacokinetic considerations (route of administration, desired onset or duration, lipophilicity) and on side effects associated with the respective route of drug delivery. Dosages are dependent on patient characteristics, type of pain, and route of administration. Recent guidelines recommend to limit opioid treatment to the lowest dose and shortest duration possible (Dowell et al. 2016; Babu et al. 2019). Systemically and spinally administered μ-opioids can produce similar side effects, depending on dosage and rostral/systemic redistribution. Side effects (e.g., respiratory depression) can be minimized by careful dose titration and close patient monitoring or can be treated by co-medication (e.g., with laxatives) or naloxone (Babu et al. 2019). No adverse effects have been reported upon local/peripheral application of small, systemically inactive doses.

NSAIDs

Less severe pain states (e.g., early arthritis, menstruation, headache, minor surgery) are commonly treated with NSAIDs (e.g., aspirin, ibuprofen, indomethacin, diclofenac). NSAIDs are mostly used orally. Some agents are available for parenteral, rectal, or topical application. Over-the-counter availability and self-medication have led to frequent abuse and toxicity. All available NSAIDs, including acetaminophen and aspirin, are associated with gastrointestinal and/or cardiovascular side effects, related to their relative selectivity for COX-1 and COX-2. Inhibition of COX-1 leads to blockade of thromboxane production and impairment of platelet function (bleeding), decrease of tissue-protective prostaglandins (gastrointestinal ulcers, perforation), and decrease of renal vasodilatory prostaglandins (nephrotoxicity). In contrast to the other NSAIDs, aspirin (acetylsalicylic acid) causes irreversible inactivation of COX-1 and COX-2 in most patients, which underlie its prolonged suppression of platelet activation and unique cardioprotective effects. Acetaminophen has no measurable anti-inflammatory effects at therapeutic doses, but inhibits pain primarily through suppression of PGE_2 synthesis. The development of selective COX-2 inhibitors was driven by the assumption that COX-2 expression is selectively induced in inflamed tissue. It is now clear that COX-2 is constitutively expressed in several tissues (e.g., gastrointestinal epithelium, vascular endothelium, spinal cord) and that

COX-2 inhibition decreases formation of vasodilatory prostacyclin. Thus, COX-2 inhibitors confer an increased risk of thrombosis, myocardial infarction, hypertension, and stroke. COX inhibitors can cause anaphylactic reactions, and diverse interactions with co-administered drugs (e.g., steroids, coumarin, serotonin reuptake inhibitors) have to be observed in clinical practice (Brune and Patrignani 2015).

Antiepileptics

Antiepileptics are used for neuropathic pain and for migraine prophylaxis (Gonzalez-Hernandez et al. 2018; Wiffen et al. 2013). They are frequently co-administered with antidepressants. The most common adverse effects are impaired mental (somnolence, dizziness, cognitive impairment, fatigue) and motor function (ataxia) which limit clinical use, particularly in elderly patients. Other side effects include hepatotoxicity, thrombocytopenia, and sometimes life-threatening dermatologic and hematologic reactions. Plasma drug concentrations should be monitored. Evidence from clinical trials particularly supports the use of gabapentin and pregabalin (Finnerup et al. 2015; Wiffen et al. 2013).

Antidepressants/Serotoninergic Drugs

Antidepressants are used for neuropathic pain and migraine prophylaxis (Finnerup et al. 2015; Gonzalez-Hernandez et al. 2018). Tricyclics may require monitoring of plasma drug concentrations to achieve optimal effect and avoid toxicity. Block of cardiac ion channels by tricyclics can lead to life-threatening arrhythmias. In patients with ischemic heart disease, there may be increased mortality, and in patients with recent myocardial infarction, arrythmia, or cardiac decompensation, tricyclics are contraindicated. Tricyclics also block histamine and cholinergic and adrenergic receptor sites. Further adverse events include sedation, nausea, constipation, dizziness, and blurred vision (Gonzalez-Hernandez et al. 2018). Triptans narrow coronary arteries via 5-HT_{1B} receptors at clinical doses and should not be administered to patients with risk factors or manifest hypertensive, coronary, cerebrovascular, or peripheral vascular disease. Many triptans have the potential for drug-drug interactions (e.g., with monoamine oxidase inhibitors, propranolol, cimetidine, hepatic P450-metabolized medications). Triptans should be restricted to patients with migraine-associated disability (Gonzalez-Hernandez et al. 2018).

Other Drugs

Ergot alkaloids are still in use as antimigraine drugs. They produce cranial and systemic vasoconstriction and can interact with serotonin, dopamine, and α-adrenoreceptors. For the prophylaxis of migraine, β-blockers, antidepressants, antiepileptics, Ca^{++} channel blockers, and reuptake inhibitors of noradrenaline/serotonin are also used (Gonzalez-Hernandez et al. 2018). Cannabinoids have attracted huge public attention, but systematic reviews and prospective studies have not confirmed their efficacy, tolerability, or safety in pain treatment (Häuser et al. 2018). CGRP antagonists/antibodies and opioid ligands leading to preferential (biased) activation of G-proteins are under intense investigation, but superiority over currently available drugs or significant reduction of side effects has not been demonstrated to date (Negus and Freeman 2018; Gonzalez-Hernandez et al. 2018; Stein 2018). The combined administration of different classes of drugs may increase the risk of overdose (Babu et al. 2019).

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Anchoring Protein

► Adaptor Proteins

Angiotensin-1 Converting Enzyme Inhibitors

► ACE Inhibitors

Anion Channels

► Cl^- Channels and Cl^-/H^+ Exchangers

Anomalous Rectifiers

► Inward Rectifier Potassium Channels

Anterograde Amnesia

► GABAergic System

Antiarrhythmic Drugs

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Synonyms

AADs, Antiarrhythmic agents

Definition

Antiarrhythmic drugs (AADs) are a group of pharmaceuticals used to treat or prevent heart rhythm disorders (cardiac arrhythmias) by altering the electrophysiological properties of the heart, typically through direct interaction with ion channels, receptors, or pumps/exchangers (Dan et al. 2018).

Basic Characteristics

Clinical Use

During a normal heartbeat, electrical impulses generated in the sinoatrial node conduct across the atria to the atrioventricular node. After a brief delay, the electrical impulse rapidly conducts along the His-Purkinje conduction system, resulting in synchronous activation of both ventricles that is necessary for optimal pump function of the heart. During cardiac arrhythmias, electrical impulse formation and/or conduction is disturbed, leading to excessively fast, slow, or irregular electrical activity of one or more parts of the heart. Although cardiac arrhythmias can be completely asymptomatic, they often lead to palpitations, dizziness, or fatigue, decreasing quality of life. Moreover, arrhythmias are associated with increased morbidity and mortality, with more than 50% of

sudden cardiac deaths attributed to ventricular arrhythmias (Camm 2017).

Despite a growing trend toward non-pharmacological therapy (e.g., electrical cardioversion and catheter ablation), it is not possible to apply invasive interventions to all patients due to the very large size of the patient population. Thus, AADs remain the cornerstone of antiarrhythmic therapy. AADs are used for the acute termination of arrhythmias (restoration of normal sinus rhythm, i.e., cardioversion), prevention of new arrhythmia occurrence (long-term sinus rhythm maintenance), or management of persistent arrhythmias (e.g., reducing ventricular rate in atrial fibrillation [AF]) (Dan et al. 2018). Acute pharmacological cardioversions are typically performed through intravenous application of AADs, with the exception of cardioversion of AF in a subset of patients without structural heart disease, in whom high-dose oral formulations are used (a so-called "pill-in-the-pocket" approach). Long-term sinus rhythm maintenance or arrhythmia management (rhythm and rate control) is performed with oral AADs in patients who do not qualify for more invasive catheter ablation procedures or in conjunction with these procedures (Camm 2017; Dan et al. 2018).

Mechanisms of Action

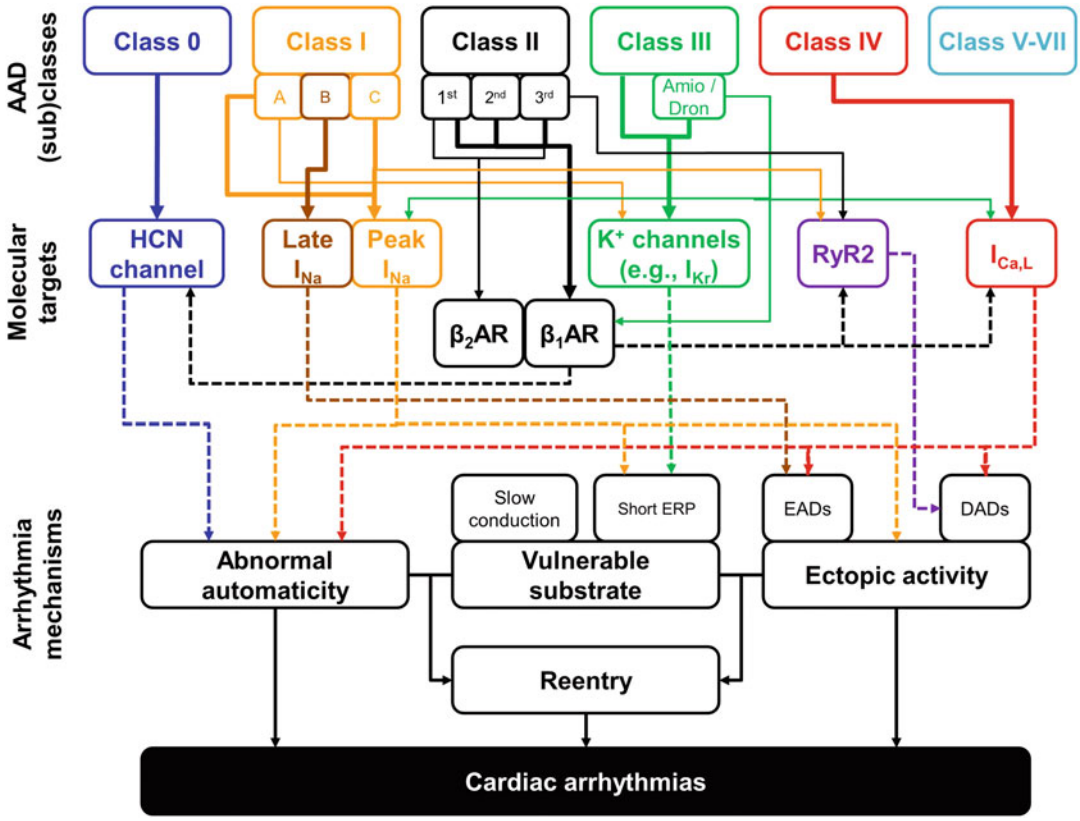
Antiarrhythmic Mechanisms

Conceptually, cardiac arrhythmias require a vulnerable substrate resulting from genetic predisposition, advancing age, or cardiovascular and systemic diseases, as well as a dynamic factor (e.g., sympathetic activation, inflammation, or electrolyte disturbances) explaining their sudden initiation (Heijman et al. 2017). Abnormal automaticity, triggered activity, and reentry are considered the major arrhythmogenic mechanisms (Mankad and Kalahasty 2019). At the cellular level, triggered activity is promoted by secondary depolarizations occurring during (early afterdepolarizations, EADs) or after (delayed afterdepolarizations, DADs) the normal electrical activation. EADs typically occur during conditions of excessive prolongation of cardiac electrical activity (prolonged repolarization duration), whereas DADs are favored by conditions of Ca^{2+} overload. Triggered activity can itself maintain an arrhythmia when occurring repetitively at sufficiently high frequency, or can initiate reentry, the foremost arrhythmia-maintaining mechanism, in a vulnerable substrate. This substrate can be structural (e.g., scar tissue leading to slow, heterogeneous electrical conduction) and/or functional (e.g., functional disturbance in electrical cell-to-cell conduction or short effective refractory periods [ERP]) (Heijman et al. 2017).

AADs can exert antiarrhythmic effects by inhibiting acute initiating factors (e.g., in the case of β-adrenoceptor blockers), reducing the likelihood of ectopic activity by reducing cellular excitability (e.g., Na^{+}-channel blockers), or decreasing substrate vulnerability (e.g., prolonging ERP; Fig. 1) (Heijman et al. 2017). As discussed below, most of the currently available drugs affect multiple electrophysiological components and arrhythmia mechanisms. Of note, although several cardiovascular drugs (e.g., inhibitors of the renin-angiotensin-aldosterone system) may also slow the development of a vulnerable substrate and thus may have indirect antiarrhythmic effects, they typically do not have direct electrophysiological effects and are therefore not considered classical AADs, although they have been incorporated as upstream therapy in more recent classification systems (Lei et al. 2018).

(Proarrhythmic) Side Effects

Ventricular proarrhythmia is a well-known adverse side effect of AADs. In the early 1990s, the Cardiac Arrhythmia Suppression Trial (CAST) and Survival with Oral D-Sotalol (SWORD) studies showed increased mortality in patients treated with AADs, highlighting the potentially severe consequences of these proarrhythmic side effects (Camm 2017). AAD-induced proarrhythmia is attributed to excessive prolongation of cardiac electrical activity (refractoriness), promoting EAD generation, or due to slowing of conduction velocity. However, there is substantial heterogeneity in both the antiarrhythmic efficacy and occurrence of proarrhythmic side effects, making the optimal clinical use of AADs



Antiarrhythmic Drugs, Fig. 1 Schematic overview of antiarrhythmic drug (AAD) classes, major molecular targets, and their impact on fundamental arrhythmia mechanisms (AAD classes are adapted from the 2018 classification by Lei et al. 2018). Primary targets of Class 0–IV AADs are indicated with thick solid lines and share the same color as the AAD class. Additional molecular targets of Class I, II, and III AADs are indicated with thin lines. Arrhythmia mechanisms affected by inhibition of a specific molecular target are connected with dashed lines. For example, inhibition of the L-type Ca^{2+} current ($I_{Ca,L}$) reduces abnormal automaticity in nodal tissue and reduces the likelihood of early and delayed afterdepolarizations (EADs and DADs, respectively). 1st/2nd/3rd refers to different generations of β-adrenoceptor (βAR) blockers. Amio/Dron, amiodarone/dronedarone; HCN, hyperpolarization-activated cyclic nucleotide-gated; I_{Na}, Na^+ current; I_{Kr}, rapidly activating delayed rectifier K^+ current; RyR2, type 2 ryanodine receptor channel

challenging (Dan et al. 2018; Heijman et al. 2017). For example, despite comparable AAD efficacy in men and women, the risk of pro-arrhythmic side effects is greater in women (Dan et al. 2018). The causes underlying these diverse effects remain incompletely understood and likely involve a combination of genetic susceptibility, heterogeneous cardiac remodeling as a result of multiple interacting comorbidities, and altered pharmacokinetic properties due to drug-drug interactions or disease-induced alterations in drug metabolism (including differential electrophysiological effects of metabolites) or drug elimination. In addition, acute and long-term drug effects may differ due to drug-induced remodeling of the electrophysiological properties of the heart.

Molecular Targets

Most clinically available AADs directly block one or more ion channel subtypes in the sarcolemmal membrane of cardiomyocytes, thereby reducing the corresponding ion currents and altering the electrophysiological properties of the heart. Common electrophysiological targets are hyperpolarization-activated, cyclic

nucleotide-gated (HCN) channels, the cardiac Na^+ current (I_{Na}), the L-type Ca^{2+} current ($I_{Ca,L}$), and the rapidly activating delayed rectifier K^+ current (I_{Kr}) (Fig. 1) (Heijman et al. 2017; Mankad and Kalahasty 2019). HCN channels contribute to cardiomyocyte automaticity, and their inhibition reduces spontaneous depolarization in the sinoatrial and atrioventricular nodes, His-Purkinje system, or diseased myocardium. Inhibition of I_{Na} reduces excitability, making triggered activity less likely, and prolongs ERP, reducing the likelihood of reentry. However, inhibition of I_{Na} may also slow conduction velocity, promoting reentrant arrhythmias. $I_{Ca,L}$ inhibition can reduce Ca^{2+} overload, decreasing the occurrence of DADs, and inhibits atrioventricular conduction, lowering the ventricular response rates during supraventricular arrhythmias. On the other hand, $I_{Ca,L}$ inhibition may decrease ERP, increasing the likelihood of reentry. Several K^+ currents are involved in the electrical recovery (repolarization) of the heart. I_{Kr} is a major target for AADs. Inhibition of I_{Kr} prolongs ERP, but excessive prolongation may promote EADs and so-called "torsade des pointes" (TdP) arrhythmias. Of note, many other drugs (non-AADs and non-cardiovascular drugs) also target I_{Kr} and may produce drug-induced proarrhythmia. Assessing I_{Kr} inhibition is therefore a standard component of safety screening during drug development.

Cardiac β-adrenoceptors are another important AAD target (Grandi and Ripplinger 2019). Their acute inhibition indirectly affects cardiac electrophysiology through modulation of cyclic nucleotide-gated channels and ion channel phosphorylation, reducing automaticity and lowering atrioventricular conduction. In addition, β-adrenoceptor blockers can reduce DAD-promoting intracellular Ca^{2+}-releases, and long-term use may have antiarrhythmic effects by improving cardiac metabolism and limiting adverse remodeling.

Besides these classical molecular targets, several others have been modulated by new experimental AADs (Heijman et al. 2017). These include Ca^{2+}-handling proteins contributing to proarrhythmic DADs, including the ryanodine receptor channel type 2 (RyR2) and Na^+/Ca^{2+}-exchanger, as well as gap junctions responsible for electrical cell-to-cell communication, key intracellular signaling molecules such as Ca^{2+}/calmodulin-dependent protein kinase-II (CaMKII), and additional cardiac ion channels. In particular, inhibition of I_{Na}, I_{Kr}, $I_{Ca,L}$, and β-adrenoceptors affects electrophysiological properties throughout the heart. Recently, significant efforts have been made to identify region-specific targets. For example, a number of relatively selective blockers of atrial-predominant ion channels, including the ultrarapid delayed rectifier K^+ current (I_{Kur}), the acetylcholine-activated inward rectifier K^+ current ($I_{K,ACh}$), and the two-pore domain K^+ channel 3.1, have been developed. Such drugs hold promise for selective therapy of AF without the risk of ventricular proarrhythmia, but so far initial clinical trials have been unsuccessful, and no compounds have been approved for clinical use.

Drugs

Based on the seminal work of Singh and Vaughan Williams, AADs have traditionally been divided into Na^+-channel blockers (Class I), β-adrenoceptor blockers (Class II), K^+-channel blockers (Class III), and non-dihydropyridine Ca^{2+} channel blockers (Class IV) (Dan et al. 2018; Lei et al. 2018). Several extensions of this AAD classification have been developed to better reflect the multiple mechanisms of action of each AAD (e.g., in the Sicilian Gambit classification) or to include newer compounds (e.g., in the 2018 extension of the Vaughan Williams classification by Lei et al. (2018)). The molecular mechanisms, key pharmacological properties, and clinical applications of commonly used AADs, grouped according to this last classification, are briefly discussed below and are summarized in Table 1. In general, few of these AADs were specifically developed for their antiarrhythmic properties. Indeed, most compounds were derived from naturally occurring substances (e.g., cardiac glycosides, quinidine) or originally developed for the treatment of other cardiovascular diseases (e.g., amiodarone and ranolazine for angina pectoris)

Antiarrhythmic Drugs, Table 1 Overview of commonly used antiarrhythmic drugs (AADs) belonging to Classes 0–IV, their targets, mechanisms, and indications

	Drug	Molecular targets	Mechanisms targeted	Common indications/uses
Class 0: HCN channel blockers				
0	Ivabradine	HCN channels	↓ Abnormal automaticity	Management of angina Inappropriate sinus tachycardia
Class I: Na^+ channel blockers				
IA	Ajmaline	I_{Na}, I_{Kr}	N/A	Diagnosing Brugada syndrome
IA	Disopyramide	I_{Na}, I_{Kr}	↓ Ectopic activity (↓ excitability)	Rhythm control of vagal AF Hypertrophic obstructive cardiomyopathy (combined with Class II or IV AADs)
IA	Procainamide	I_{Na}, I_{Kr}	↓ Ectopic activity (↓ excitability) ↓ Reentry (↑ repolarization)	Acute cardioversion of hemodynamically stable VT Preexcited AF
IA	Quinidine	I_{Na}, I_{Kr}, I_{to}	↓ Ectopic activity (↓ excitability) ↓ Reentry (↑ repolarization)	Brugada syndrome, short-QT syndrome and idiopathic VF
IB	Lidocaine	I_{Na}	↓ Ectopic activity (↓ excitability)	Local anesthesia VT (i.v. only)
IB	Mexiletine	I_{Na}, $I_{Na,late}$	↓ Ectopic activity (↓ excitability, ↓EADs)	VT (notably in LQTS3) Suppression of PVCs Chronic pain
IB	Ranolazine	$I_{Na,late}$, I_{Kr}, RyR2, I_{Na}	↓ Ectopic activity (↓ EADs and ↓ DADs)	Angina pectoris Rhythm control of AF VT (e.g., in LQTS3)
IC	Flecainide	I_{Na}, I_{Kr}, RyR2	↓ Ectopic activity (↓ excitability) ↓ Reentry (↑ post-repolarization refractoriness)	Acute cardioversion (w.o. structural heart disease) Rhythm control of AF (w.o. structural heart disease)
IC	Propafenone	I_{Na}, βAR, I_{Kr}, RyR2	↓ Ectopic activity (↓ excitability) ↓ Reentry (↑ post-repolarization refractoriness)	Acute cardioversion (w.o. structural heart disease) Rhythm control of AF (w.o. structural heart disease)
Class II: autonomic inhibitors				
II-1	Propranolol	β_1AR, β_2AR	Multiple mechanisms including ↓ remodeling, ↓ ectopic activity (repolarization stability, DADs), ↓ reentry (repolarization heterogeneities)	Heart failure Rate control of supraventricular tachycardia and AF Prevention of postoperative AF (Prevention of) VT (e.g., in LQTS) Angina pectoris and myocardial infarction
II-2	Atenolol	β_1AR		
II-2	Bisoprolol	β_1AR		
II-2	Metoprolol	β_1AR		
II-3	Carvedilol	β_1AR, β_2AR, α_1AR, RyR2		
	Digitalis	I_{NaK} + parasympatho-mimetic effects	↓ AV conduction	Rate control of AF
Class III: K^+ channel inhibitors				
	Amiodarone	I_{Kr}, $I_{Ca,L}$, I_{Na}, I_{Kur}, I_{to}, $I_{K,ACh}$, I_{K2P}, βAR, αAR, M_2R	↓ Ectopic activity (↓ excitability) ↓ Reentry (↑ repolarization)	Rhythm control of AF Hemodynamically stable VT Suppression of PVCs Reducing ICD intervention rates

(continued)

Antiarrhythmic Drugs, Table 1 (continued)

	Drug	Molecular targets	Mechanisms targeted	Common indications/uses
	Dofetilide	I_{Kr}	↓ Reentry (↑ repolarization)	Acute cardioversion of AF Rhythm control of AF
	Dronedarone	I_{Kr}, $I_{Ca,L}$, $I_{K,ACh}$, I_{Na}, I_{Kur}, I_{to}, I_{K2P}, βAR, αAR, M_2R	↓ Ectopic activity (↓ excitability) ↓ Reentry (↑ repolarization)	Rhythm control of paroxysmal or persistent AF (w.o. structural heart disease)
	Ibutilide	I_{Kr}	↓ Reentry (↑ repolarization)	Acute cardioversion of AF or atrial flutter
	Sotalol	I_{Kr}, βAR	↓ Reentry (↑ repolarization)	Acute cardioversion of AF Rhythm control of AF VT
Class IV: Ca^{2+} channel inhibitors				
	Diltiazem	$I_{Ca,L}$	↓ AV conduction ↓ Chronotropy ↓ Ectopic activity (↓ DADs)	Angina pectoris High blood pressure Rate control of supraventricular tachycardia and AF
	Verapamil	$I_{Ca,L}$, I_{Kr}	↓ AV conduction ↓ Chronotropy ↓ Ectopic activity (↓ DADs)	Angina pectoris High blood pressure Rate control of supraventricular tachycardia and AF

AF, atrial fibrillation; i.v., intravenous; LQTS, long-QT syndrome; VF, ventricular fibrillation; VT, ventricular tachycardia; w.o, without

(Heijman et al. 2017). Please note that the AADs presented below are not available in every country and some regional preferences in their use exist.

Class 0: Hyperpolarization-Activated Cyclic Nucleotide-Gated (HCN) Channel Blockers

Ivabradine is the only clinically approved HCN channel blocker. It blocks the pacemaker (HCN) current, reducing diastolic depolarization and pacemaker activity in the sinoatrial node, and is primarily used to lower heart rate (and thereby energy demand) in patients with stable angina pectoris not fully managed by β-adrenoceptor blockers (Koruth et al. 2017). In line with its primary negative chronotropic properties, a potential role for off-label use in the treatment of inappropriate sinus tachycardia has also been suggested (Koruth et al. 2017). Furthermore, in recent studies using heterologous expression systems, ivabradine blocks I_{Kr} and I_{Na} at therapeutically relevant concentrations, potentially explaining its effects on ventricular rate in patients with AF. Of note, in other AAD classifications, ivabradine is sometimes included in a class with miscellaneous drugs (Class V) (Dan et al. 2018; Heijman et al. 2017).

Class I: Na^+ Channel Blockers

Class I AADs bind Na^+ channels in their open and/or inactivated states, inhibiting the inflow of Na^+. As such, the extent of I_{Na} inhibition by Class I AADs depends on channel activity, a concept termed "use dependence." As a result, Class I AADs are more potent at fast rates (frequent opening of Na^+ channels) or during ischemia, when depolarization of the resting membrane potential promotes Na^+ channel inactivation. Different Na^+ channel blocker subtypes have been defined based on their relative affinities for individual states and their on/off kinetics (Lei et al. 2018).

Class IA drugs such as ajmaline, disopyramide, procainamide, and quinidine typically exhibit a high affinity for the open state of the Na^+ channel, intermediate dissociation kinetics with time constants of 1–10 s, and concomitant I_{Kr} inhibition (Aguilar and Nattel 2015). Quinidine was the first drug used to treat symptoms of

cardiac arrhythmias (palpitations). Besides I_{Na}, it inhibits I_{Kr} and the transient outward K^+ current, significantly prolonging repolarization duration. Quinidine was initially used for sinus rhythm maintenance in AF patients and prevention of recurrences of ventricular arrhythmias by reducing ectopic activity and prolonging repolarization duration. However, adverse gastrointestinal effects and syncopal events ("quinidine syncope") due to drug-induced TdP arrhythmias were relatively common (Aguilar and Nattel 2015; Camm 2017). Quinidine has also been associated with increased mortality, resulting in a decrease in its use. Although it is no longer available in several countries, a renewed interest in quinidine has developed recently for the treatment of several inherited arrhythmogenic disorders (Brugada syndrome, short-QT syndrome, and idiopathic ventricular fibrillation) (Mankad and Kalahasty 2019). Procainamide also inhibits I_{Na} and I_{Kr} and is used for the acute cardioversion of hemodynamically stable ventricular tachycardias and in patients with accessory pathways and preexcited AF, slowing conduction across the accessory pathway and lowering ventricular rate (Dan et al. 2018). Although classified as Class I AADs, ajmaline and disopyramide are not commonly used for antiarrhythmic therapy (Dan et al. 2018). Ajmaline is used to unmask concealed arrhythmogenic phenotypes regulated by I_{Na} dysfunction (e.g., Brugada syndrome). Disopyramide is a Class IA AAD with negative inotropic effects and can be used to suppress ventricular ectopy (Dan et al. 2018) or in combination with β-adrenoceptor or Ca^{2+}-channel blockers in patients with hypertrophic obstructive cardiomyopathy. In addition, disopyramide has significant anticholinergic effects, responsible for most of its adverse effects.

Class IB AADs block the Na^+ channel in the inactivated state with rapid dissociation kinetics (time constants of 0.1–1 s (Aguilar and Nattel 2015)), thereby having limited effect under normal physiological conditions but pronounced I_{Na}-blocking effects in depolarized tissue (Mankad and Kalahasty 2019). Lidocaine is a local anesthetic used intravenously for the prevention or treatment of ventricular arrhythmias by decreasing cardiomyocyte excitability. For example, a lidocaine bolus is used prior to aortic cross-clamp removal during bypass surgery to prevent cardiac arrhythmias. Its extensive first-pass effects preclude oral use. Mexiletine is an orally available Class IB AAD with similar electrophysiological effects as lidocaine (Mankad and Kalahasty 2019). It can suppress ventricular ectopy and tachycardia and has particular relevance in the treatment of long-QT syndrome type 3 (Dan et al. 2018), which is linked to gain-of-function mutations in the cardiac Na^+ channel, where it directly inhibits the dysfunctional I_{Na} responsible for QT prolongation and EAD-mediated arrhythmogenesis.

Class IC AADs flecainide and propafenone block Na^+ channels in both the open and inactivated states with slow on/off kinetics and are commonly used for the management of supraventricular tachycardias and AF (Aguilar and Nattel 2015). Both AADs reduce ectopic activity, prolong ERP, and destabilize reentrant activity. ERP prolongation is due to a combination of I_{Kr} inhibition and increased post-repolarization refractoriness (i.e., the period after repolarization during which the tissue is unexcitable). In addition, the *S*-enantiomer of propafenone has β-adrenoceptor-blocking properties (Aguilar and Nattel 2015). Flecainide and propafenone are used for acute cardioversion of AF (either through intravenous application or as "pill-in-the-pocket" approach with high oral doses) as well as long-term sinus rhythm maintenance in patients without structural heart disease (Aguilar and Nattel 2015; Dan et al. 2018). In addition, recent experimental data have suggested that both AADs also stabilize RyR2 gating, potentially exerting antiarrhythmic effects by preventing Ca^{2+}-handling abnormalities and providing rationale for the clinical use of flecainide in patients with catecholaminergic polymorphic ventricular tachycardia due to RyR2 mutations (Heijman et al. 2017).

Ranolazine is an antianginal drug with high affinity for the late (persistent) component of I_{Na} and additional I_{Kr} and RyR2-blocking properties, which may prevent proarrhythmic Ca^{2+}-handling abnormalities and stabilize repolarization (Aguilar and Nattel 2015; Heijman et al. 2017).

In the 2018 AAD classification, it is designated as a Class ID drug, although other classifications have listed it as Class IB (due to the structural similarity to lidocaine) or Class V ("other drugs") (Lei et al. 2018). A number of experimental and clinical studies have demonstrated the antiarrhythmic potential of ranolazine for atrial and ventricular arrhythmias. These include patients with long-QT syndrome type 3 in whom ranolazine can directly inhibit the disease-causing increase in late I_{Na}, similar to mexiletine, as well as patients with AF (Dan et al. 2018; Heijman et al. 2017). Of note, studies with a more selective late I_{Na} inhibitor (eleclazine) were recently halted (Heijman et al. 2017).

Class II: Autonomic Inhibitors

Chronic autonomic dysfunction promotes hypertrophy, apoptosis, and fibrosis and contributes to the progression of multiple cardiovascular diseases, whereas acute sympathetic/parasympathetic imbalance is a well-accepted trigger of cardiac arrhythmias (Grandi and Ripplinger 2019). Excessive sympathetic stimulation may produce Ca^{2+} overload due to elevated heart rates and hyperphosphorylation of Ca^{2+}-handling proteins, thereby promoting DADs and triggered activity. Under physiological conditions, repolarization-prolonging effects of $I_{Ca,L}$ hyperphosphorylation are offset by augmentation of the slowly activating delayed rectifier K^+ current (I_{Ks}). However, in the diseased heart, sympathetic stimulation can cause proarrhythmic repolarization instability when I_{Ks} is downregulated or dysfunctional due to genetic mutations (in the case of long-QT syndrome types 1 and 5).

In accordance with the diverse proarrhythmic potential of autonomic dysfunction, the protective effects of β-adrenoceptor blockers have been established in a wide range of cardiovascular diseases and include several antiarrhythmic actions (Grandi and Ripplinger 2019). Inhibition of β-adrenoceptors has negative chronotropic effects and inhibits abnormal automaticity. Due to their negative dromotropic effects (inhibition of atrioventricular conduction), they are commonly used for rate control in AF. In addition, β-adrenoceptor blockers reduce the likelihood of ventricular tachyarrhythmias and sudden cardiac death in patients with myocardial infarction and/or heart failure by prolonging ERP, reducing triggered activity due to Ca^{2+} overload and attenuating electrophysiological heterogeneity due to inhomogeneous hyper-/hypo-innervation. Finally, long-term β-adrenoceptor blocker use may prevent proarrhythmic remodeling by reducing myocardial energy consumption and oxidative stress.

β-adrenoceptor blockers are typically divided into three subgroups (Grandi and Ripplinger 2019). First-generation β-adrenoceptor blockers (e.g., propranolol) have similar affinity for β_1- and β_2-adrenoceptor subtypes, whereas the second generation (e.g., metoprolol, atenolol, or bisoprolol) have a higher affinity for β_1-adrenoceptors. Most of the proarrhythmic effects of sympathetic stimulation have been attributed to β_1-adrenoceptors. Finally, third-generation β-adrenoceptor blockers have vasodilatory effects. For example, carvedilol inhibits both β_1- and β_2-adrenoceptor isoforms and α_1-adrenocepters. It also has anti-inflammatory and antioxidant effects. Finally, experimental work has suggested that direct RyR2-stabilizing effects of carvedilol may contribute to its antiarrhythmic effects (Heijman et al. 2017).

Digitalis and other cardiac glycosides inhibit the Na^+-K^+-ATPase, increasing intracellular Na^+ levels, leading to inhibition of the Na^+/Ca^{2+} exchanger and elevation of intracellular Ca^{2+}, resulting in increased contractility (positive inotropic effect). In addition, digitalis has parasympathomimetic effects, resulting in inhibition of atrioventricular condition, and is used for rate control in AF patients (Scalese and Salvatore 2017). This is the reason that cardiac glycosides, which traditionally belonged to the category of "other drugs," are included in Class II in the 2018 AAD classification. On the other hand, digitalis-induced Ca^{2+}-handling abnormalities and related DADs may have proarrhythmic effects, and its safety remains a topic of intense debate (Scalese and Salvatore 2017).

Class III: K^+ Channel Blockers

Dofetilide is a selective I_{Kr} blocker approved in the United States for the acute cardioversion of

atrial fibrillation and for long-term rhythm control. Its primary antiarrhythmic mechanism of action is prolongation of repolarization, and it is available in oral and intravenous forms (Mankad and Kalahasty 2019). Ibutilide is a Class III agent approved for acute cardioversion that is only available in intravenous form because of extensive first-pass metabolism (Mankad and Kalahasty 2019), similar to lidocaine. Besides inhibition of I_{Kr}, ibutilide activates a slow inward Na^+ current, further promoting repolarization prolongation. Sotalol is a 1:1 racemic mixture of a selective I_{Kr} blocker (D-sotalol) and a combined I_{Kr}/β-adrenoceptor blocker (L-sotalol) approved for the acute and long-term treatment of both atrial and ventricular arrhythmias (Mankad and Kalahasty 2019). Most Class III drugs, including dofetilide, ibutilide, and sotalol, have the largest repolarization-prolonging effects at slow rates, when baseline action potential duration is already long, a concept known as reverse use dependence (Mankad and Kalahasty 2019). Class III AADs have been associated with drug-induced proarrhythmia due to excessive repolarization prolongation, particularly at slow rates or under conditions favoring abnormal repolarization (e.g., hypokalemia). As such, treatment initiation should be performed under ECG monitoring and is contraindicated in patients with congenitally prolonged repolarization (long-QT syndrome) (Mankad and Kalahasty 2019). Experimental work has suggested that chronic application of dofetilide and other Class III drugs associated with increased risk of TdP arrhythmias increases the late component of I_{Na}, contributing to excessive prolongation of repolarization that may cause TdP.

Although originally developed as an antianginal agent, amiodarone is generally considered the most effective AAD available. It is associated with relatively low proarrhythmic risks compared to other Class III AADs and is the only AAD approved for treatment of patients with heart failure (Heijman et al. 2013). Amiodarone is used for cardioversion and rhythm control of AF, and to treat and prevent ventricular tachycardia (e.g., to reduce the intervention rate of implantable cardioverter defibrillators). Its efficacy and safety likely result from a complex interaction between numerous molecular targets. Indeed, amiodarone inhibits a wide range of ion channels and receptors, including I_{Kr}, I_{Na}, $I_{Ca,L}$, and β-adrenoceptors, thus exhibiting effects of all four original Vaughan Williams AAD classes (Lei et al. 2018; Mankad and Kalahasty 2019). Amiodarone also has less pronounced reverse use-dependent effects. Amiodarone is highly lipophilic, resulting in a very long half-life (30–100 days) and necessitating long and high oral loading doses to accelerate the onset of drug activity, although intravenous application has a rapid onset of action. Of note, there are significant electrophysiological differences between intravenous amiodarone and chronically administered oral amiodarone, including a smaller repolarization prolongation, little effect on sinus rate, and more potent antiadrenergic activity with intravenous application. These differences are likely in part due to additional effects of metabolites and due to electrical remodeling that develops during chronic amiodarone treatment. Despite its potent antiarrhythmic effects, the use of amiodarone is limited by potentially severe extra-cardiac toxicity, including thyroid dysfunction and pulmonary toxicity, resulting in drug discontinuation in up to 20% of patients during long-term therapy.

The striking antiarrhythmic efficacy combined with extra-cardiac toxicity of amiodarone prompted the development of safer derivatives. Dronedarone lacks the iodine moieties partially responsible for amiodarone's toxicity and is significantly less lipophilic (Heijman et al. 2013). Like amiodarone, dronedarone inhibits a wide range of ion channels, exhibiting effects of all four Vaughan Williams classes. However, there are important electrophysiological differences between both AADs, including a significantly higher affinity for $I_{K,ACh}$ with dronedarone (Heijman et al. 2013). A direct comparison of both drugs in patients with AF showed fewer side effects but also higher arrhythmia recurrence rates with dronedarone. Nonetheless, the randomized, double-blind Athena trial comparing dronedarone to placebo for the treatment of atrial fibrillation has been the only trial with AADs showing prognostic benefit (reduction of the

composite primary endpoint hospitalization due to cardiovascular events or death) (Heijman et al. 2013). On the other hand, in later trials in AF patients with concomitant heart failure (Andromeda) or in patients with permanent AF (Pallas), dronedarone was associated with increased mortality (Heijman et al. 2013). In the Pallas trial, this was attributed to pharmacokinetic interactions between dronedarone and digoxin.

Vernakalant is the most recent AAD approved for clinical use in Europe and Canada. It is available for intravenous pharmacological cardioversion of recent-onset AF and acts by prolonging ERP (Dan et al. 2018). Vernakalant is considered an AAD with atrial-predominant effects due to inhibition of I_{Kur} and $I_{K,ACh}$ and preferential inhibition of atrial I_{Na} due to a state-dependent block favoring the inactivated state of the channel (Aguilar and Nattel 2015; Dan et al. 2018). In experimental studies vernakalant's antiarrhythmic effects are primarily due to increased post-repolarization refractoriness (due to I_{Na} inhibition) and conduction slowing, suggesting that it should be considered a Class I AAD rather than a Class III AAD (Aguilar and Nattel 2015).

Class IV: Ca^{2+} Channel Blockers and Ca^{2+}-Handling Modulators

Verapamil and diltiazem inhibit $I_{Ca,L}$ and are primarily used to reduce atrioventricular conduction, lowering the ventricular response rate during supraventricular tachycardia and AF (Mankad and Kalahasty 2019). In addition, they can be used for the treatment of high blood pressure and angina. Verapamil additionally inhibits I_{Kr} and is sometimes used for the treatment of migraine and cluster headaches (off-label use). Both AADs can have significant negative inotropic effects due to inhibition of cardiomyocyte $I_{Ca,L}$ and are therefore contraindicated in patients with reduced cardiac function (Mankad and Kalahasty 2019). Combinations with other drugs inhibiting sinoatrial or atrioventricular nodal activity (e.g., β-adrenoceptor blockers) are also contraindicated due to the increased risk of symptomatic bradycardia or atrioventricular block. Due to their short half-lives, verapamil and diltiazem are administered as extended-release preparations. Both AADs are also moderate inhibitors of P-glycoprotein and CYP3A4, potentially resulting in pharmacokinetic interactions with other drugs. For example, dose reduction of the non-vitamin-K oral anticoagulant dabigatran is recommended when taken in conjunction with verapamil (Dan et al. 2018).

In the recent 2018 AAD classification, modulators of Ca^{2+}-handling proteins other than L-type Ca^{2+} channels have been included in Class IV. Although RyR2 stabilizers and inhibitors of the Na^{+}/Ca^{2+}-exchanger have indeed shown antiarrhythmic properties in experimental models, no clinically approved compounds are currently available (Heijman et al. 2017).

Classes V–VII

Classes V–VII in the 2018 AAD classification contain mechanosensitive channels (Class V), gap junction modulators (Class VI), and upstream therapy (Class VII) (Lei et al. 2018). Although some compounds in Class V (including GsMtx-4, a nonselective inhibitor of stretch-activated channels from tarantula spider venom) and Class VI (including rotigaptide and danegaptide) have shown antiarrhythmic potential in experimental studies (Woods and Olgin 2014), there are currently no ongoing clinical trials or clinically approved drugs available. Upstream therapy with angiotensin-converting enzyme inhibitors, angiotensin-receptor blockers, aldosterone antagonists, or statins may prevent development or worsening of the vulnerable substrate, thus indirectly exerting antiarrhythmic effects (Woods and Olgin 2014). However, direct antiarrhythmic effects of these drugs appear limited and studies in patients have produced mixed results regarding prevention of arrhythmic death, likely in part because of differences in preexisting irreversible remodeling.

Conclusion

AADs remain the cornerstone of antiarrhythmic therapy. Although numerous AADs are available, they were not developed based on specific arrhythmia mechanisms and have complex

electrophysiological effects, including a significant risk of proarrhythmic side effects. New insights into arrhythmia mechanisms have recently been obtained, and promising experimental compounds have emerged, but no new AADs for the clinical management of cardiac arrhythmias are currently foreseen.

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Antibody Drug Conjugates

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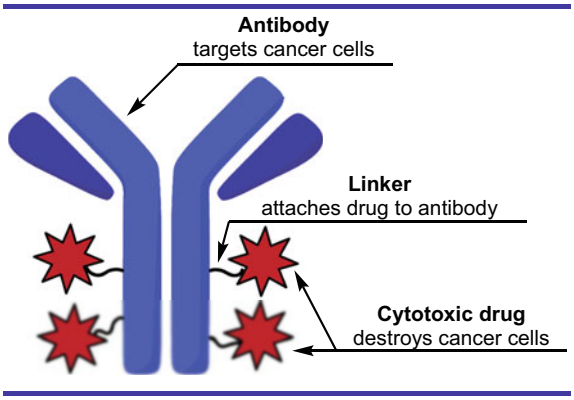
Synonyms

ADC; ADCs

Definition

Antibody drug conjugates (ADCs) are hybrid therapeutics that combine the specificity of monoclonal antibodies with the potent cytotoxicity of small-molecule drugs. ADCs are designed for targeted delivery of the cytotoxic payload directly to the desired site of action to increase efficacy and minimize off-target effects. ADCs consist of three essential components – the antibody, the drug, and the chemical linker connecting the two (Fig. 1). Accordingly, careful consideration is given to the design of each part. An optimal ADC has high target cell specificity, a long circulation half-life, minimal immunogenicity, and low off-target toxicity (McCombs and Owen 2015).

(a) ***Antibody:*** Monoclonal antibodies are the targeting agent in ADC therapies. The development of a successful antibody for an ADC therapy requires an antigen that is prevalent on the target cells and absent in normal tissue in order to allow for specific targeting to the tumor (Chau et al. 2019). It is important to



Antibody Drug Conjugates, Fig. 1 Critical components of an ADC

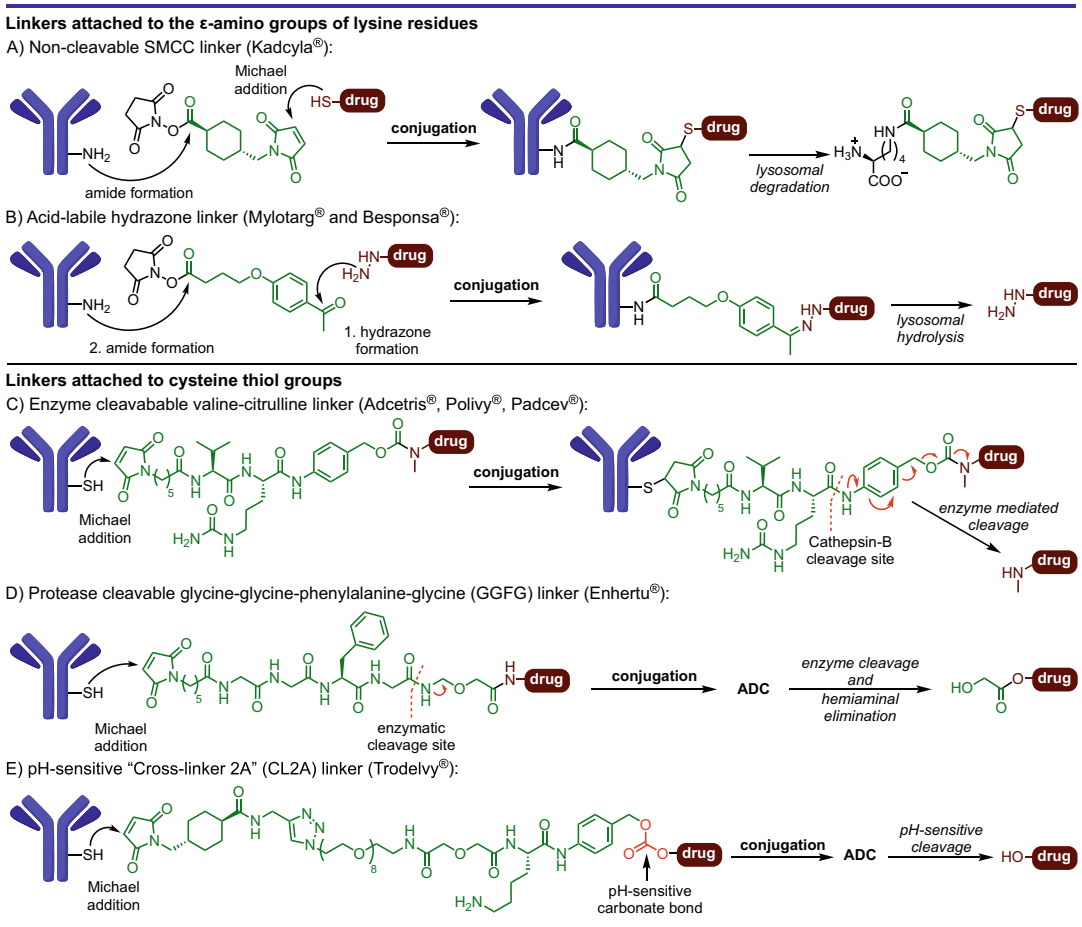
note that serious ADC side effects can arise from antigen expression on unrelated cell types. For example, HER2 expression in heart tissue can lead to cardiotoxicity in trastuzumab-based ADCs (Kondapalli 2016). After antigen selection, antibodies are developed against the desired target. It is critical for the antibody to be able to induce receptor-mediated internalization for the ADC to be effective. The majority of antibodies in approved ADCs are of the IgG1 isotype, with IgG4 as the second isotype used (Mylotarg® and Besponsa®) (Khongorzul et al. 2020). Importantly, either of these isoforms impart long half-life, providing pharmacokinetic advantages to the ADC product compared to the free small-molecule drug. In order to minimize immunogenicity, these antibodies are humanized, with the exception of one chimeric ADC (Adcetris®) that has been approved. Minimal antibody engineering is required since drug conjugation can occur with native amino acid residues (Khongorzul et al. 2020).

(b) ***Payload:*** Several criteria must be met in choosing an appropriate payload for ADCs including: solubility, amenability to conjugation, stability, and potency. The drug must be hydrophilic enough to allow for both conjugation to the antibody in aqueous conditions and stability of the resulting conjugate. Many potential drugs suffer from low solubility, which can be somewhat ameliorated by employing hydrophilic linkers. The payloads must also bear chemically functional handles, such as amines, to allow for conjugation. Consideration is given to how modification to contain a functional handle may affect the efficacy of the drug. Further, the payload must remain stable during circulation and cellular processing in order to maintain efficacy after reaching the cytosolic target (McCombs and Owen 2015). Finally, the site-specific delivery of ADCs allows for the use of extremely potent drugs; in fact, these drugs are generally too toxic to be given as a single agent with potencies 100- to 10,000-fold greater than standard chemotherapeutics (Nejadmoghaddam et al. 2019). The drug classes that have been employed by clinically approved ADCs include calicheamicins (Mylotarg® and Besponsa®), mertansines (Kadcyla®), auristatins (Adcetris®, Polivy®, and Padcev®), and camptothecins (Enhertu® and Trodelvy®) (Chau et al. 2019; Khongorzul et al. 2020; Nicolaou and Rigol 2019).

(c) ***Linker*:** The linker has two critical roles: (1) stable attachment of the drug to the antibody without premature release in circulation and (2) release of the drug after localization to disease cells. With respect to release mechanisms, linkers are divided into two classes: noncleavable and cleavable. Noncleavable linkers depend on the degradation of the antibody once internalized into the lysosome and release drug with the linker still covalently

attached, as with Kadcyla® (McCombs and Owen 2015). More commonly, cleavable linkers are broken down by components of the lysosomal environment to release the payload. Several cleavage mechanisms have been investigated, including acidic pH hydrolysis, thiol reduction, and protease cleavage (McCombs and Owen 2015; Nejadmoghaddam et al. 2019). The most prevalent cleavage mechanism occurs from the specific recognition of a dipeptide (such as valine-citrulline) by lysosomal enzymes, which results in the release of free drug (Dubowchik et al. 2002).

(d) ***Conjugation Chemistry:*** Final construction of the ADC requires conjugation of the linker and drug to the native antibody. Two conjugation methods are seen in FDA-approved ADC products to date: (1) lysine conjugation and (2) cysteine conjugation. For lysine conjugation, an activated N-hydroxysuccinimide ester on the linker reacts with accessible lysine residues on the surface of the ADC to produce a stable amide bond. Similarly, native cysteines are commonly used for conjugation. First, interchain disulfide bonds are reduced to provide a free cysteine for conjugation. Next, the side-chain thiol undergoes Michael addition into a linker bearing a pendant maleimide, creating a stable linkage. The proposed advantages of cysteine conjugation include a more controllable drug to antibody



Antibody Drug Conjugates, Fig. 2 Conjugation and lysosomal cleavage mechanisms of lysine and cysteine conjugated ADCs

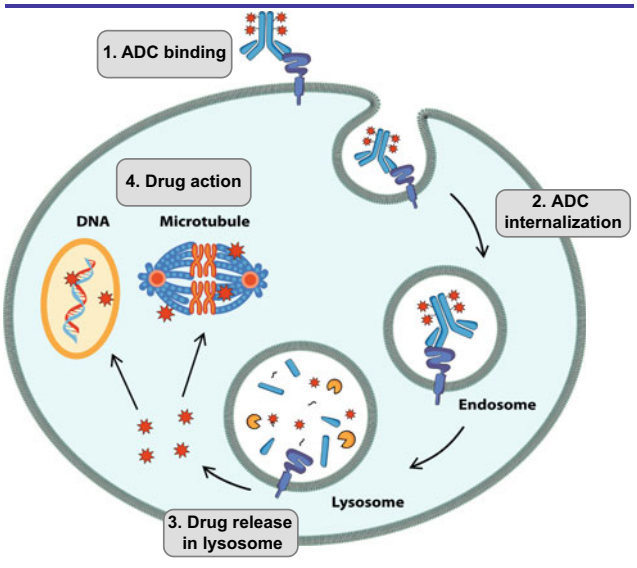
ratio (DAR) and more homogenous product. Figure 2 outlines the linkers, conjugation strategies, and release mechanisms of the current FDA-approved ADCs (McCombs and Owen 2015; Nicolaou and Rigol 2019).

Mechanism of Action

(a) ***ADC mechanism:*** Antibody drug conjugates work through the antibody-mediated targeted delivery of cytotoxic payloads directly to tumor cells (Chau et al. 2019). As illustrated in Fig. 3, once an ADC is in circulation, the antibody will bind to cells bearing the targeted antigens. After antigen binding, internalization of the antigen-ADC complex occurs, passing through early endosomes and endosomes, ultimately reaching the lysosome. Once inside the lysosome, cleavage of the drug from the ADC occurs. This can happen via several different mechanisms. For non-cleavable linkers, proteolytic degradation of the antibody itself releases the linker-drug complex. In the case of cleavable linkers, several lysosomal pathways can occur, depending on the type of linker employed (see Fig. 2 for details).

By this mechanism, the ADC acts as a prodrug, sparing normal tissue from the toxic effects of the drug while the ADC is in circulation. Instead, lysosomal release of the free drug within the target cells results in targeted tumor cell killing.

(b) ***Drug mechanism of action:*** The cytotoxic warheads on the clinically approved ADCs can be broken down into two categories: (1) DNA damaging agents and (2) antimitotic agents. Mylotarg® and Besponsa® both bear a calicheamicin γ_1^I-derived payload that causes double-stranded DNA damage through single and double strand cuts. Enhertu® and Trodelvy® are equipped with camptothecin derivatives that work by inhibiting topoisomerase 1 by stabilizing its complex with DNA, prohibiting religation and ultimately triggering apoptosis. The other FDA-approved ADCs employ antimitotic agents. Adcetris®, Polivy®, and Padcev® bear monomethyl auristatin E (MMAE), which binds to tubulin and inhibits polymerization, ultimately inhibiting cell division.



Antibody Drug Conjugates, Fig. 3 Mechanism of action of an ADC at a tumor cell

Antibody Drug Conjugates, Table 1 Summary of FDA-approved antibody drug conjugates

Drug name (trade name)	Disease indication	Molecular target		Conjugation site	Linker	Side effects
		mAb (target)	Drug (target)			
Gemtuzumab ozogamicin (Mylotarg®)	Newly diagnosed CD33-positive acute myeloid leukemia (AML) or relapsed/refractory AML	Humanized IgG4k (anti-CD33)	Calicheamicin derivative (causes DNA strand scission)	Native lysine	Acid-labile acetyl butyrate linker (hydrazone)	Hemorrhage, infection, fever, nausea, vomiting, constipation, headache, increased alanine and aspartate aminotransferase, rash, mucositis Risk: hepatotoxicity
Brentuximab vedotin (Adcetris®)	Relapsed Hodgkin lymphoma and relapsed systemic anaplastic large cell lymphoma	Chimeric IgG1k (anti-CD30)	MMAE (blocks tubulin polymerization)	Interchain cysteine	Protease cleavable linker (Val-Cit)	Neutropenia, anemia, peripheral sensory neuropathy, nausea, fatigue, constipation, diarrhea, vomiting, pyrexia
Trastuzumab emtansine (Kadcyla®)	HER2-positive metastatic breast cancer previously treated with trastuzumab and a taxane	Humanized IgG1k (anti-HER2)	DM1 – mytansine derivative (inhibits microtubules)	Native lysine	Noncleavable thioether linker (SMCC)	Fatigue, nausea, increased transaminases, musculoskeletal pain, hemorrhage, thrombocytopenia, headache, peripheral neuropathy, arthralgia
Inotuzumab ozogamicin (Besponsa®)	Relapsed or refractory CD22-positive B-cell precursor acute lymphoblastic leukemia	Recombinant humanized IgG4 (anti-CD22)	Calicheamicin derivative (causes DNA strand scission)	Native lysine	Acid-labile acetyl butyrate linker (hydrazone)	Thrombocytopenia, neutropenia, nausea, leukopenia, infection, abdominal pain, febrile neutropenia, anemia, fatigue, hemorrhage, pyrexia, headache, liver damage, hyperbilirubinemia Risk: hepatotoxicity

Polatuzumab vedotin-piiq (Polivy®)	Relapsed or refractory diffuse large B-cell lymphoma in combination with bendamustine and rituximab	Humanized IgG1 (anti-CD79b)	MMAE (blocks tubulin polymerization)	Interchain cysteine	Protease cleavable linker (Val-Cit)	Neutropenia, thrombocytopenia, anemia, peripheral neuropathy, fatigue, diarrhea, pyrexia, decreased appetite, pneumonia
Enfortumab vedotin-ejfv (Padcev®)	Locally advanced or metastatic urothelial cancer who have received a PD-1 or PD-L1 inhibitor and a platinum-containing chemotherapy	Human IgG1 (against Nectin-4)	MMAE (blocks tubulin polymerization)	Interchain cysteine	Protease cleavable linker (Val-Cit)	Fatigue, peripheral neuropathy, decreased appetite, rash, dry skin, alopecia, nausea, dysgeusia, diarrhea, dry eye, pruritus Risk: diabetic ketoacidosis
Trastuzumab deruxtecan-nxki (Enhertu®)	Unresectable or metastatic HER2-positive breast cancer who have received two or more prior anti-HER2 based regimens in the metastatic setting	Humanized IgG1k (anti-HER2)	Deruxtecan – exatecan derivative (topoisomerase I inhibitor)	Interchain cysteine	Lysosomal protease cleavable tetrapeptide (Gly-Phe-Leu-Gly; GFLG)	Nausea, fatigue, vomiting, alopecia, constipation, cough, decreased appetite, anemia, neutropenia, diarrhea, leukopenia, thrombocytopenia Risk: interstitial lung disease, embryo-fetal toxicity
Sacituzumab govitecan-hziy (Trodelvy®)	(adult patients) Metastatic triple negative breast cancer (mTNBC) who have received at least two prior therapies	Humanized IgG1k (anti-TROP2)	SN-38 – active metabolite of irinotecan (topoisomerase I inhibitor)	Interchain cysteine	pH-sensitive CL2A	Nausea, vomiting, neutropenia, anemia, alopecia, diarrhea, fatigue, constipation, decreased appetite, rash, abdominal pain

Similarly, Kadcyla® utilizes a mytansine-derived payload, DM1, which also inhibits microtubule assembly by binding to tubulin (Nicolaou and Rigol 2019).

Clinical Use

To date, FDA-approved ADCs have only been developed to treat cancer with specific indications for lymphomas and leukemias (Table 1). The majority of ADCs are designated to treat solid tumors with three ADCs indicated to treat breast cancer (Kadcyla® (U.S. Food and Drug Administration 2019a), Enhertu® (U.S. Food and Drug Administration 2019b), and Trodelvy® (U.S. Food and Drug Administration 2020)), and one ADC each for Hodgkin lymphoma (Adcetris® (U.S. Food and Drug Administration 2018a)), large B-cell lymphoma (Polivy® (U.S. Food and Drug Administration 2019c)), and urothelial cancer (Padcev® (U.S. Food and Drug Administration 2019d)). Two ADCs are designated to treat liquid tumors, one for acute myeloid leukemia (Mylotarg® (U.S. Food and Drug Administration 2017)) and one for acute lymphoblastic leukemia (Besponsa® (U.S. Food and Drug Administration 2018b)). Regardless of indication, ADCs are supplied as lyophilized powders, formulated in acidic buffer with various excipients. Following reconstitution in saline or specified diluent, ADCs are administered by IV injection or infusion. Several side effects have been reported for ADCs, with nausea, neutropenia, and fatigue among the most common (refer to Table 1 for details).

ADCs are actively being researched in order to further address the major challenges including low payload potency, immunogenicity, and off-target toxicity. Further, ADCs are being explored for applications outside of cancer therapy.

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Anticoagulants

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Synonyms

Blood thinners

Definition

Anticoagulants are drugs that alter the biochemical processes of blood clotting resulting in the prevention or reduction of blood coagulation. They are the mainstay of pharmacotherapy for thrombosis prevention and treatment. The coagulation system is a homeostatic regulation process by which the blood is balanced between states of clotting (coagulation) and bleeding (free flow). This complex process is an integral part of maintaining a healthy cardiovascular system, ensuring that any trauma to the vasculature is sufficiently controlled in order to avoid excessive blood loss and hemodynamic compromise. Conversely, excessive activation of this system may result in thrombosis, an intravascular clot formation that disrupts blood circulation.

Hypercoagulability, stasis, and injury/trauma (otherwise referred to as Virchow's triad) contribute to the formation of a thrombus (Esmon 2009). Hypercoagulability, or thrombophilia (an increased tendency of blood to form thrombus), is common in cancer, pregnancy, patients who use oral contraceptives, and in patients with certain genetic disorders including antiphospholipid syndrome, homocysteinemia, or those with genetic deficiencies in antithrombin III, protein C or S, and/or factor V. Blood stasis (the tendency for blood pooling or stagnation) is associated with increased risk of clotting cascade activation. Prolonged periods of immobility (traveling or bedridden individuals) and cardiovascular disorders including arrhythmias and heart failure predispose patients to stasis and thrombosis. Trauma and injuries, such as accidents or surgeries, promote the activation of the coagulation cascade due to physiologic changes in the blood vessel wall (Esmon 2009). When triggered, the clotting cascade may result in thrombosis, often referred to as venous thromboembolism (VTE). VTE includes both deep vein thrombosis (DVT) and pulmonary embolism (PE).

A DVT occurs most commonly in the veins of the legs and if left untreated, a portion of the thrombus may break off and travel in the blood stream, now called an embolus, to another part of the body. Among the worst locations for an embolus to lodge is in the pulmonary veins. This is known as a PE, which has a high morbidity and mortality rate, as it blocks the flow of blood through the pulmonary system, rendering a patient unable to adequately perform oxygen exchange. About half of all DVT cases are asymptomatic, contributing to large differences in prevalence estimates (Haines 2003). Symptomatic VTE, including either DVT and/or PE, is thought to affect between 300,000 and 600,000 Americans yearly, and of those cases, an estimated 10–30% of them result in death (Beckman et al. 2010). VTE also has numerous complications and is associated with a significant economic burden (Beckman et al. 2010).

The presence of VTE and conditions that may predispose a patient to a VTE may indicate the need for pharmacological intervention. If

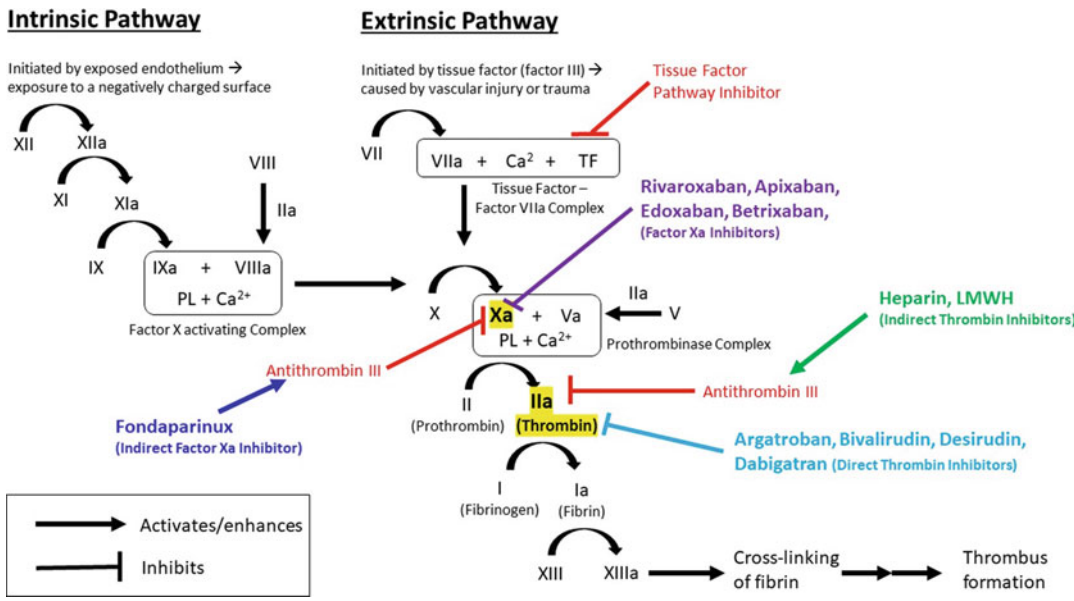
appropriate, some patients may be candidates for mechanical intervention to prevent VTE, such as the use of interpneumatic compression devices or compression stockings. However, mechanical intervention is not completely effective and is reserved for those with low VTE risk and high bleeding risk. Drugs that slow or halt the coagulation cascade are known as anticoagulants, and work through various mechanisms involving the numerous proteins, or clotting factors, that make up the coagulation cascade (Fig. 1). Regardless of their mechanism, anticoagulant drugs are indicated for the prevention and/or treatment of VTE.

Mechanism of Action

Anticoagulants mostly target the coagulation cascade. In order to fully understand the mechanism of action of the various anticoagulants, it is necessary to have a general understanding of the coagulation cascade (Fig. 1). There are two pathways – intrinsic and extrinsic, which are initiated by different factors. These pathways converge to form the common pathway (Davie et al. 1991). The intrinsic pathway is initiated by the exposure of factor XII to a negatively charged surface, mainly, an exposed endothelium. The extrinsic pathway is initiated by tissue factor (factor III). While the extrinsic pathway is largely involved in homeostasis and the intrinsic pathway is not, both play important roles in the pathogenesis of VTE. The two pathways converge at factor X, which is activated to Xa, and the common pathway proceeds to activate thrombin from prothrombin, followed by the activation of fibrin from fibrinogen (Smith et al. 2015). The cross-linking of fibrin strands forms the framework of the thrombus (Fig. 1). The majority of anticoagulant drugs binds and inhibits different factors or proteins that play a key role in the coagulation cascade. Table 1 provides a list of the available clinically relevant anticoagulants. Based on their mechanisms of action, anticoagulant drugs are classified into four types – direct and indirect thrombin inhibitors, vitamin K antagonists, and factor Xa inhibitors.

Indirect Thrombin Inhibitors

Indirect thrombin inhibitors include heparins and their synthetic analogue, fondaparinux. Heparin is a naturally occurring polymer of



Anticoagulants, Fig. 1 Description of the clotting cascade indicating the targets for the various anticoagulants

Anticoagulants, Table 1 An overview of medically approved anticoagulant drugs, adapted from Kustos and Fasinu 2019

General Class/MOA	Drug Name and Year of First Approval	Labeled Indications	Adult Dosing (with normal renal & hepatic function)	Route of Administration	Approved Reversal Agent
Vitamin K Antagonist	Warfarin (1954)	• VTE prophylaxis and treatment (associated with Afib or cardiac valve replacement) • Adjunct to reduce the risk of systemic embolism after MI	INR-adjusted-based dosing Goal INR is 2–3 for most patients Goal INR for mitral valve replacement is 2.5–3.5	Oral	Vitamin K and/or Prothrombin Complex Concentrate
Indirect Thrombin Inhibitors	Heparin (1940s)[a]	• VTE prophylaxis and treatment (associated with thromboembolic disorders or Afib) • Prevention of clotting in arterial or cardiac surgery • Anticoagulant for extracorporeal circulation or dialysis procedures	*VTE Treatment*: 80 unit/kg IV bolus, then 18 unit/kg/h IV infusion *VTE Prophylaxis*: 5000 units q8h Target anti-Xa level 6 h post-dose: 0.3–0.7 units/mL	Injectable Intravenous or Subcutaneous	Protamine ➔ 100% reversal
	Low Molecular Weight Heparins (LMWH): • Dalteparin (1994) • Enoxaparin (1993) • Tinzaparin (2000)	• VTE prophylaxis (in hip, knee, abdominal, thoracic, cardiac, or neurosurgery; in patients with restricted mobility; trauma; pregnancy) • Thrombosis treatment and secondary prophylaxis (wide variety of indications) • Thromboprophylaxis in acute coronary syndrome (unstable angina, NSTEMI, STEMI) or cardioversion in Afib/atrial flutter	*DVT Treatment*: 1 mg/kg q12h OR 1.5 mg/kg q24h *VTE Prophylaxis*: 40 mg q24h Target anti-Xa level 4 h post-dose: 0.5–1.1 units/mL	Injectable Subcutaneous	Protamine ➔ 60% reversal
Direct thrombin Inhibitors	Argatroban (2000)	• Prophylaxis or treatment of thrombosis in patients with HIT • Anticoagulant for percutaneous coronary intervention (PCI)	*Prophylaxis/treatment of thrombosis in HIT*: 2 mcg/kg/min and adjust based on aPTT (goal 1.5–3 times baseline) *PCI*: 350 mcg/kg bolus, then 25 mcg/kg/min infusion and adjust based on ACT	Injectable Intravenous	N/A
	Bivalirudin (2000)	• Anticoagulant for percutaneous coronary intervention (PCI), including patients with HIT	*Before PCI*: 0.1 mg/kg bolus, then 0.25 mg/kg/h until PCI *During PCI*: 0.75 mg/kg bolus, then 1.75 mg/kg/h for the duration of procedure	Injectable Intravenous	N/A

(continued)

Anticoagulants, Table 1 (continued)

General Class/MOA	Drug Name and Year of First Approval	Labeled Indications	Adult Dosing (with normal renal & hepatic function)	Route of Administration	Approved Reversal Agent
	Dabigatran (2010)	• VTE treatment and prevention in patients who have been treated with a parenteral anticoagulant for 5 to 10 days • VTE prophylaxis in total hip arthroplasty (THA) • Stroke prevention in Afib	*VTE Treatment (after initial therapy with a parenteral anticoagulant for 5 days):* 150 mg BID *Afib*: 150 mg BID *THA*: 110 mg given 1–4 h after surgery, then 220 mg QD for 10–14 d	Oral	Idarucizumab (Praxbind)
	Desirudin (2003)	• DVT prophylaxis in hip-replacement surgery	15 mg q12h	Injectable Subcutaneous	N/A
	Lepirudin[b] (1998)	• HIT • Prevention of VTE in patients with HIT	0.4 mg/kg IV bolus, followed by 0.15 mg/kg/h IV infusion for 2–10 days (or as clinically indicated)	Injectable Subcutaneous	N/A
Indirect Factor Xa Inhibitor	Pentasaccharide – Fondaparinux (2001)	• DVT or PE treatment in conjunction with warfarin • VTE prophylaxis in surgical patients	*VTE Treatment:* *<50 kg ➔ 5 mg QD* *50–100 kg ➔ 7.5 mg QD* *>100 mg ➔ 10 mg QD* *VTE Prophylaxis*: 2.5 mg QD	Injectable Subcutaneous	N/A
Factor Xa Inhibitors	Apixaban (2012)	• Treatment of VTE and to reduce recurrence of VTE following initial therapy • Prevention of stroke and systemic embolism in NVAF • Prophylaxis of VTE post-op in hip or knee arthroplasty	*VTE Treatment:* 10 mg BID for 7 days, then 5 mg BID *Afib*: 5 mg BID; if patient has any 2 of the following then 2.5 mg BID ➔ age ≥ 80, weight ≤ 60 kg, or SCr ≥1.5 mg/dL *Knee/hip arthroplasty*: 2.5 mg BID starting 12–24 h after surgery *Secondary prevention*: 2.5 mg BID (following 6 months of initial therapy)	Oral Tablet	Andexanet Alfa
	Betrixaban (2017)	• Prophylaxis of VTE in medical patients	*VTE prophylaxis*: 160 mg as a single dose on day 1, followed by 80 mg once daily for 35 to 42 days	Oral Capsule	N/A
	Edoxaban (2015)	• Treatment and prevention of recurrent VTE following 5–10 days of parenteral anticoagulant • Prevention of stroke and systemic embolism in NVAF	*VTE*: If >60 kg ➔ 60 mg QD If ≤60 kg ➔ 30 mg QD *Afib*: 60 mg QD	Oral Tablet	N/A

	Rivaroxaban (2011)	• Treatment of VTE • Prophylaxis of VTE in total hip or knee arthroplasty • Prevention of stroke and systemic embolism in NVAF • Reduce the risk of cardiovascular events in CAD or PAD • Indefinite anticoagulation to reduce the risk of recurrent VTE	*VTE treatment*: 15 mg BID w/ food for 21 days, followed by 20 mg QD w/ food *Knee/hip arthroplasty*: 10 mg QD starting 6–10 h after surgery *Afib*: 20 mg QD w/ evening meal *CAD/PAD*: 2.5 mg BID (with or without aspirin) *Secondary prevention*: 10 mg QD (following 6 months of initial treatment) *Indefinite anticoagulation*: reduced intensity dosing	Oral Tablet	Andexanet Alfa

VTE = venous thromboembolism, *Afib* = atrial fibrillation, *MI* = myocardial infarction, *DVT* = deep vein thrombosis, *PE* = pulmonary embolism, *STEMI* = ST-elevated myocardial infarction, *NSTEMI* = non-ST-elevated myocardial infarction, *HIT* = heparin-induced thrombocytopenia, *CAD* = coronary artery disease, *PAD* = peripheral artery disease

Note: VTE includes DVT and PE

[a]Heparin was first described as being an effective anticoagulant as early as 1916, and was grandfathered in after the establishment of the US FDA

[b]Bayer, the pharmaceutical company that produced lepirudin, stopped further production of the drug since May 31, 2012

glycosaminoglycans first described in the 1940s and which has been well studied and characterized for its anticoagulant activity. It consists of chains of *d*-glucosamine alternating with a form of uronic acid, either glucuronic acid or iduronic acid. Unfractionated heparin (UFH) is a heterogeneous mixture of large polysaccharides with molecular weight ranging from 3000 to 30,000. The low-molecular-weight heparins (LMWHs) are made as fragments of the UFH through chemical and/or enzymatic depolymerization reactions (Table 2).

Heparins exert anticoagulant effect by binding and activating antithrombin III (Hirsh et al. 1995). Antithrombin III naturally inhibits thrombin as well as factor Xa and is involved in homeostasis. However, its inhibitory activity is potentiated (about 1000 times) by the binding of heparins, which causes a conformational change in antithrombin III. This is achieved through the action of the unique but randomly distributed pentasaccharide sequence in the heparin polymeric chains.

While both UFH and LMWHs are available for subcutaneous administration, UFHs are generally poorly bioavailable and often require large doses (>30,000 U/day). To achieve rapid anticoagulation, UFH can be given intravenously. By contrast, LMWHs, including enoxaparin, dalteparin, and tinzaparin (no longer available in the United States), have satisfactory bioavailability following subcutaneous administration. They also have a longer half-life with a more predictable dose-dependent therapeutic response. They are preferred over UFH in most clinical settings.

UFH is indicated for prophylaxis and treatment of thromboembolic disorders (including both DVT and PE) and thromboembolic complications associated with atrial fibrillation, prevention of clots in arterial and cardiac surgery, and as an anticoagulant for extracorporeal circulation and dialysis. Enoxaparin is indicated for the treatment of DVT, prophylaxis of VTE (including both DVT and PE), and for use during an acute coronary syndrome (including prophylaxis of complications due to unstable angina or non-ST-elevation myocardial infarction and treatment of acute ST-elevation myocardial infarction). Similar to enoxaparin, dalteparin is indicated for VTE prophylaxis, but is only indicated for non-ST elevation acute coronary syndromes. Its use for VTE treatment is limited to pediatric and cancer

Anticoagulants, Table 2 Examples of approved low molecular weight heparins

LMWHs	Trade name	Average molecular weight	Elimination half-life (h)	Bioavailability (%)	Anti-Xa/ anti-IIa ratio	Countries of approval
Ardeparin	Normiflo	6000	3.0	92	2.1	USA (withdrawn)
Certoparin	Sandoparin	5400	4.1	2.2	2.4	Germany
Bamiparin	Zibor	3600	5.5	96	8.0	Bulgaria, Poland
Dalteparin	Fragmin	5500	3–5	87	2.7	Germany, Japan, UK, USA
Enoxaparin	Levonox, Clexane	4500	4.5	92	3.8	Germany Spain, USA
Nadroparin	Fraxiparin	4500	3.7	89	3.3	France, Germany
Parnaparin	Fluxum	4500	4.3	90	2.3	Italy
Reviparin	Clivarin	4300	3.6	95	4.2	Canada, Germany
Tinzaparin	Innohep	6500	3.4	87	2.7	Denmark, Germany, USA

patients. Because they are effective, relatively inexpensive, have a rapid onset of action, and have a variety of indications, heparins are often used as first-line agents in an inpatient setting for VTE prophylaxis and treatment (Serhal and Barnes 2019). In general, UFH is administered subcutaneously for VTE prophylaxis and intravenously for other indications. Enoxaparin is only administered intravenously for acute coronary syndrome and subcutaneously for all other anticoagulation indications. Dalteparin is only administered subcutaneously.

Both UFH and LMWH are safe for use in hepatic impairment and do not require dose adjustments. However, dose adjustments are required for LMWHs (unlike UFH) in patients with renal impairment. UFH is monitored using the anticoagulation lab tests called the activated prothrombin time (aPTT) and anti-factor Xa level. Doses are adjusted based on results. While it is not required, anti-factor Xa levels may be monitored while using LMWHs in certain high-risk populations. Heparins should be avoided in patients with history of heparin-induced thrombocytopenia and uncontrolled active bleeding, and in circumstances where appropriate blood coagulation tests cannot be performed. The effect of heparins can be reversed, in cases of toxicity or severe bleeding, with protamine sulfate.

Fondaparinux is a synthetic sulfated pentasaccharide developed from the antithrombin pharmacophoric portions of heparin. The substitution of some of the sulfate groups on fondaparinux has yielded idraparinux (a drug in development), which has a longer half-life (5–6 times) allowing for a potential once-weekly administration. Fondaparinux potentiates the effects of antithrombin III, but in a manner that is more selective for the inhibition of factor Xa (Paolucci et al. 2002). Its dose-response is predictable like LMWHs. Fondaparinux is indicated for the treatment of VTE (both DVT and PE) and VTE prophylaxis specifically in surgical patients. Fondaparinux is contraindicated in patients with severe renal impairment, defined as a creatinine clearance (CrCl) of less than 30 mL/min. Renal dose adjustments are required for a CrCl of 30–50 mL/min. Administered subcutaneously, the dose of fondaparinux is also titrated based on anti-factor Xa levels.

Direct Thrombin Inhibitors

Direct thrombin inhibitors (DTIs) directly bind to and inhibit thrombin to prevent the progression of the clotting cascade (Fig. 1). DTIs inhibit both soluble thrombin and fibrin-bound thrombin and offer advantages over heparins and warfarin. The anticoagulant response to DTIs is more predictable because they do not bind to other plasma proteins nor induce immune-mediated thrombocytopenia (unlike heparins), and they possess antiplatelet activity. There are currently four DTIs approved in the United States for parenteral administration: bivalirudin, desirudin, lepirudin, and argatroban (Table 3). Dabigratran is the only orally available DTI with several others in development. Along with factor Xa inhibitors (see section below), dabigatran is referred to as a direct-acting oral anticoagulants.

Anticoagulants, Table 3 Comparative overview of the parenteral direct thrombin inhibitors

	Relative affinity for thrombin	Plasma half-life (h)	Clearance	Dose adjustment requirement
Argatroban	Lowest	0.75	Liver	30–50% reduction in liver failure; avoid in severe heart failure
Bivalirudin	Intermediate	0.4	20% renal excretion; liver	15–50% reduction if CL_{CR} is 15–60 mL/min; avoid if $CL_{CR} < 15$ mL/min
Desirudin	Highest	2.0	Kidney	If $CL_{CR} < 60$ mL/min
Lepirudin	Highest	1.5	Kidney	15–50% reduction if CL_{CR} is 15–60 mL/min; avoid if $CL_{CR} < 15$ mL/min

Desirudin and lepirudin are products of recombinant technology in *Saccharomyces cerevisiae* using hirudin, a naturally occurring peptide originally isolated and characterized from the salivary glands of blood-sucking leeches (*Hirudo medicinalis*). They have high affinity for thrombin, binding to both the active site and an exosite domain to form irreversible complexes. Bivalirudin is a synthetically engineered polypeptide analogue of hirudin with relatively weaker and reversible thrombin binding compared to desirudin and lepirudin. Argatroban is a small molecule that effectively but reversibly binds and inhibits thrombin. Dabigatran etexilate is a small molecule prodrug that is rapidly metabolized to dabigatran after administration. It is a potent and reversible orally bioavailable DTI. It has a rapid onset of action and has lower risk for drug interactions, as it is not metabolized by the cytochrome P450 enzymes. Furthermore, it does not require routine monitoring, and has a wider safety margin and a fixed-dose administration (Di Nisio et al. 2005).

Argatroban and bivalirudin are administered intravenously specifically in instances of heparin-induced thrombocytopenia (HIT). Argatroban may be used as thrombosis prophylaxis or treatment in patients with HIT as well as for percutaneous coronary intervention in patients at high risk for developing HIT. Bivalirudin is indicated specifically for percutaneous coronary intervention in patients at high risk for developing HIT. Dabigatran's indications include treatment and prevention of VTE, VTE prophylaxis in total hip arthroplasty, and prevention of stroke in nonvalvular atrial fibrillation. In order to use dabigatran to treat VTE, the patient must have been treated with a parenteral anticoagulant for 5–10 days before the initiation of dabigatran. Dabigatran is affected by renal impairment and is a substrate of p-glycoprotein, and as such, dosing should be adjusted based on renal function.

Vitamin K Antagonists

Most anticoagulants exert their activity on certain factors at various steps in the coagulation cascade with the exception of the vitamin K antagonists (VKAs). VKAs inhibit the vitamin K epoxy reductase complex (VKORC1), an enzyme required to convert vitamin K to its active form (Whitlon et al. 1978). The biochemical activation of the clotting factors II, VII, IX, and X involves gamma-glutaryl carboxylation, a process that requires the active reduced form of vitamin K, which forms a complex with the carboxylase enzyme. VKAs, by inhibiting VKORC1, indirectly prevent the activation of these clotting factors to reduce progression of the clotting cascade (Whitlon et al. 1978). Coumarins are the most relevant vitamin K antagonists, with warfarin being the only one approved for therapeutic anticoagulation in humans. Warfarin, as used in drug formulations, is a racemic mixture of the two (*R* and *S*) optically active isomers in roughly equal proportions. The *S*-warfarin isomer is about five times as potent as the *R*-isomer with regard to VKORC1 inhibition. Following oral administration, warfarin is well absorbed. It is metabolized in the liver through the actions of CYP2C9 (*S*-isomer) and CYP3A4 (*R*-isomer).

It is approved for oral administration for long-term anticoagulation following a thrombotic event and for prevention in at-risk patients. Major challenges to the use of warfarin include its narrow therapeutic index and interindividual variability in therapeutic response. It is a substrate of CYP2C9 and is therefore subjected to consequential drug interactions in the presence of concomitant medications. In maintaining adequate response and preventing deleterious side effect including intracranial bleeding, the international normalized ratio (INR) is used to monitor warfarin response, with recommended therapeutic ranges being 2.0–3.0 for most disease indications. The risk of hemorrhage is directly related to INR, with higher INR values corresponding to a higher risk of bleeding. The risk of serious bleeding is also higher in patients of advanced age, patients with chronic renal disease, liver failure, cardiovascular comorbidities, or those with concurrent antiplatelet medications. Another important consideration with warfarin use is the genetic variation in *CYP2C9* and *VKORC1* gene expression. It is now well known that individuals with the poor metabolizer *CYP2C9* phenotype are unable to metabolize warfarin, and the resulting accumulation

Anticoagulants, Table 4 Indications and year of approval of factor Xa inhibitors

	Rivaroxaban	Apixaban	Edoxaban	Betrixaban
Year of Approval	2011	2012	2015	2017
Administration with food	Depending on the dosage (high dose with food)	With or without food	With or without food	With food
Half-life (h)	5–13	8–15	10–14	19–27
T-max (h)	2–4	3–4	1–2	3–4
Clearance	50% renal, 50% metabolism	27% renal, 73% hepatic	50% renal, 50% hepatic/ enterobiliary	<10% renal, >80% hepatobiliary
Side effects	Bleeding, thrombocytopenia, agranulocytosis, hepatitis, hypersensitivity reaction	Bleeding, thrombocytopenia, hypersensitivity reaction	Bleeding, rash, anemia, abnormal liver enzymes levels	Bleeding, headache, dizziness

may precipitate deleterious bleeding. Phenotypic expression of poor-function *VCOR1* has been well documented resulting in therapeutic failure with warfarin.

In the event of toxicity or overdosage, the effect of warfarin can be neutralized by the administration of vitamin K or different combinations of clotting factors.

Factor Xa Inhibitors (Direct-Acting Oral Anticoagulants)

Along with dabigatran, factor Xa inhibitors are DOACs. They are small molecule compounds that inactivate factor Xa by binding directly to its active site. The first to be approved in this new class was rivaroxaban which binds reversibly and competitively to both unbound and clot-bound factor Xa. Other anticoagulants in this class include apixaban, edoxaban, and betrixaban. They are approved for a wide variety of indications (Table 1) and are gaining favorability in the outpatient setting due to their favorable pharmacokinetic and safety profile. DOACs and warfarin are now the mainstay of outpatient anticoagulation therapy. The DOACs are often preferred over warfarin because of their relatively reduced bleeding risk and lower tendency for drug interactions. They have wider margins of safety and a more predictable dose-response relationship. Furthermore, there is no required routine coagulation monitoring for patients taking DOACs, unlike for other anticoagulants. Some of the disadvantages to DOACS include cost and limited experience in clinical utility (Mekaj et al. 2015). The four currently available factor Xa inhibitors are compared in Table 4.

Safety Considerations and Reversal

Therapeutic interventions aimed at maintaining homeostatic balance and preventing blood clots have the expected tendency to tilt the balance towards bleeding. Thus, apart from the known side effects of the individual anticoagulants, severe and life-threatening bleeding is a serious consequence of anticoagulation. The degree to which an anticoagulant is prone to induce bleeding is one of the considerations in the clinical choice of anticoagulants. More importantly, the availability of reversal agents (antidotes) in cases of life-threatening bleeding, or the need for emergency surgery, is a determining factor for anticoagulant choice. Table 1 provides an overview of available anticoagulants and their reversal agents.

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Anticonvulsant

► GABAergic System

Anticonvulsant Drugs

► Antiepileptic Drugs

Anticonvulsants

► Antiepileptic Drugs

Anticonvulsive Drugs

► Antiepileptic Drugs

Antidepressant Drugs

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Synonyms

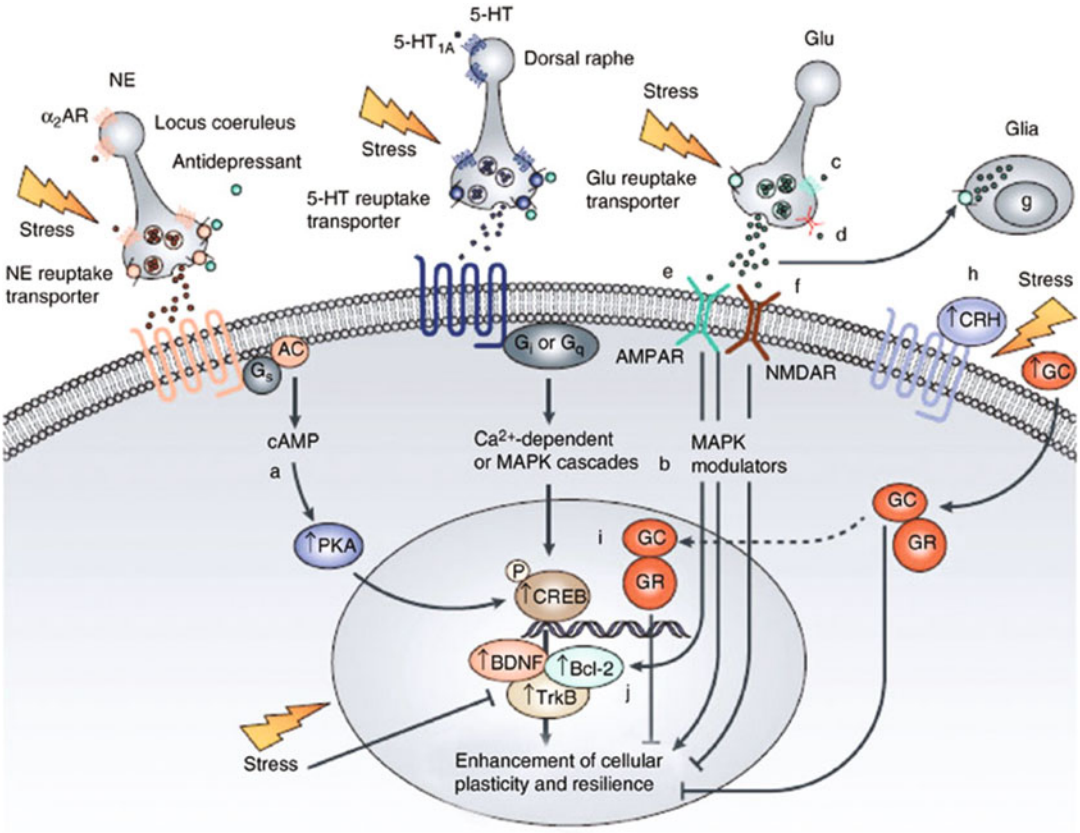
Antidepressants; Mood elevators; Psycho analeptics; Psycho energizers; Thymoleptics

Definition

Antidepressants are small heterocyclic molecules entering the circulation mostly after oral administration and passing the blood–brain barrier to bind at numerous specific sites in the brain. They are used for treatment of depression, panic disorders, generalized anxiety disorder, social phobia, obsessive compulsive disorder, and other psychiatric disorders and nonpsychiatric states.

Mechanism of Action

Most available antidepressants enhance neurotransmission of biogenic amines, mainly norepinephrine and serotonin, to a lesser extent dopamine. Once released from specialized vesicles at the presynaptic nerve terminal, neurotransmitters enter the synaptic cleft and bind to respective receptors at the postsynaptic cell membrane, thus modulating the associated signaling cascades (Fig. 1). Additionally, some of them bind to presynaptically localized receptors that regulate the amount of transmitter released. The



Antidepressant Drugs, Fig. 1 Effects of stress as a model for depression and the reversal by use of antidepressants. Multiple intracellular targets might be involved in the regulation of plasticity and resilience by antidepressants, which block extracellular transporters. (Adapted from Agid et al. 2007)

cell membrane of presynaptic nerve terminals also contains reuptake transporters that clear the synaptic cleft from biogenic amines. Once reshuffled into the presynaptic compartment, the neurotransmitter is degraded by monoamine oxidase (MAO). These two molecular processes, reuptake through specific transporters and enzymatic degradation by MAO, are targeted by most of the antidepressant drugs. For example, the selective serotonin reuptake inhibitors (SSRIs; citalopram, escitalopram, fluoxetine, fluvoxamine, paroxetine, sertraline) and the so-called dual acting drugs (the selective serotonin/noradrenaline reuptake inhibitors venlafaxine, duloxetine, and milnacipran and the noradrenergic/selective serotonergic drug mirtazapine), which became the mainstay for the treatment of the majority of depressed patients, either prevent clearance of serotonin and/or noradrenaline from the synaptic cleft by blocking the presynaptic transporter and thus amplify receptor-mediated events postsynaptically or antagonize specific pre- and postsynaptic receptors. Analogous effects are those by norepinephrine reuptake inhibitors, while MAO inhibitors act by reducing norepinephrine or serotonin degradation and thus increase the releasable amount of neurotransmitter from the respective vesicles. These drugs do not exert prompt antidepressant effects as it takes weeks or months until clinical amelioration occurs. The exact mode of action by which antidepressants work is still not resolved, but there is consensus that their primary

action, i.e., binding to cell membrane transporters, triggers a manifold of events, some of which are depicted here (Fig. 1; Agid et al. 2007).

One such hypothesis submits that most antidepressants enhance the expression of cyclo-AMP response element binding protein (CREB), which is a transcription factor that after phosphorylation binds to cyclo-AMP response elements localized in the promoter region of many genes including that coding for brain-derived neurotrophic factor (BDNF) (Berton and Nestler 2006). The latter neurotrophin was found to be decreased in the hippocampus of chronically stressed rats, serving as animal model of depression. When treated with antidepressants, BDNF expression increases, possibly through enhanced phospho-CREB-driven transactivation of the *BDNF* gene. Overexpression of mutant CREB prevented decreased neurogenesis in adult hippocampus. While many pieces of this hypothesis are in line with an antidepressant-induced enhancement of neurogenesis, evidence is lacking that this effect is the same through which antidepressants regulate emotional states. Morphological studies on brains of depressives failed to detect evidence for neuronal deterioration in the hippocampus. Moreover the increase of *BDNF* gene transcription as induced by antidepressants is possibly an unspecific response to a xenobiotic molecule. Whether increased transcription of BDNF conveys antidepressant effects is yet not proven, as mouse mutants where BDNF production is lowered by heterozygous gene deletion failed to show behavioral abnormalities. Also, data on drug-induced changes in BDNF peptide concentrations are not giving a clear picture.

Another hypothesis derives from the clinical observation that impaired stress hormone regulation is a cardinal symptom among patients with an acute major depressive episode. If stress hormones (primarily cortisol secreted by adrenocortical glands and corticotropin released from the pituitary) are monitored longitudinally in these patients, those who respond to drug treatment show a trend toward neuroendocrine normalization, while those where stress hormone regulation continues to be altered have a much worse outcome, i.e., they fail to respond or they relapse. Studies using transgenic mice with glucocorticoid receptor impairment show some behavioral and functional features reminiscent of depression. Some of these abnormalities disappear under antidepressants, which is in line with a drug-induced improvement of corticosteroid receptor function. When ligand-activated, these gluco- and mineralocorticosteroid receptors form homo- and heterodimers that interact with corticotropin-releasing hormone (CRH) in many ways. This is of relevance in this context because clinical and basic studies have shown that overexpression of CRH in many brain areas is causally related to development and course of depression. The effect of antidepressants has therefore consequences upon CRH secretion, and it is believed that these antidepressants may work through this corticosteroid receptor-driven signaling pathway, suppressing the depressogenic and anxiogenic effects of CRH acting through CRH type 1 receptors (CRHR1). CRH from the paraventricular nucleus (PVN) of the hypothalamus is released into the hypophyseal portal system and triggers the release of corticotrophin (ACTH) from the anterior pituitary via stimulation of CRH1 receptors. ACTH, in turn, stimulates the secretion of glucocorticoid hormones (cortisol in humans or corticosterone in rodents) from the adrenal cortex. Increased glucocorticoid levels suppress hypothalamic CRH expression via negative feedback through hippocampal and hypothalamic glucocorticoid receptors.

Thus, the antidepressant-induced behavioral and neuroendocrine changes in patients together with their observed molecular actions upon stress hormone signaling pathways have triggered the search for new pharmacological approaches to understand how antidepressants might work and ultimately to discover better drugs.

Novel antidepressants are rather directly acting on receptors or are so-called multimodal drugs. For instance, agomelatine is a melatonergic MT_1 and MT_2 receptor agonist and a selective serotonergic 5-HT_{2B} and 5-HT_{2C} receptor antagonist, vortioxetine is a serotonin receptor antagonist with serotonin reuptake inhibition, vilazodone is a serotonin 5-HT_{1A} autoreceptor partial agonist with serotonin reuptake inhibition, and trazodone

is a serotonin 5-HT_2 antagonist. Tianeptine seems to paradoxically increase serotonergic concentrations in the synaptic cleft. However, this does not represent its major mode of action. Opioidergic and glutamatergic mechanisms may play a major role for its efficacy as an antidepressant. Ingredients of St. John's wort have mild serotonin-/noradrenaline-/GABA- and glutamate reuptake inhibiting properties. The older tricyclic antidepressants like amitriptyline are also noradrenaline and/or serotonin reuptake inhibitors but also show antagonistic effects on different cerebral receptors like α1-adrenergic, cholinergic, histaminergic receptors. Figure 2 summarizes the major targets of most of the approved antidepressants.

N-methyl-D-aspartate (NMDA)-glutamatergic neurotransmission system blockers such as ketamine, CP-101,606 (traxoprodil), GLYX-13 (rapastinel), NRX-1074 (apimostinel), and riluzole gained much attention in the context of antidepressant treatment (Fasipe 2019). They increase glutamate release and finally enhance intracellular protein synthesis and synapse number and function. However, an opioidergic mechanism is also discussed with ketamine.

In the absence of a robust pathogenetic model for depression, hypotheses-driven research has limitations that hopefully can be overcome by unbiased approaches. However, some links between neurobiology and depression have already been found, e.g., low baseline hippocampal volume; disturbed connectivity and elevated anterior cingulated activity as measured by functional MRI, PET, and EEG; and activated microglia, hinting to an inflammatory background in major depression (Kraus et al. 2019). Immunological effects are part of the action of approved antidepressants (Eyre et al. 2016).

Moreover, minocycline, a broad-spectrum antibiotic, has generated considerable interest in neuroscience research because of its brain penetration and off-target anti-inflammatory effects, being currently under investigation as an antidepressant drug. The neurosteroid brexanolone, which is a positive allosteric modulator of γ-aminobutyric acid type A ($GABA_A$) receptors and needs to be injected, has been approved by the FDA for the treatment of postpartum depression.

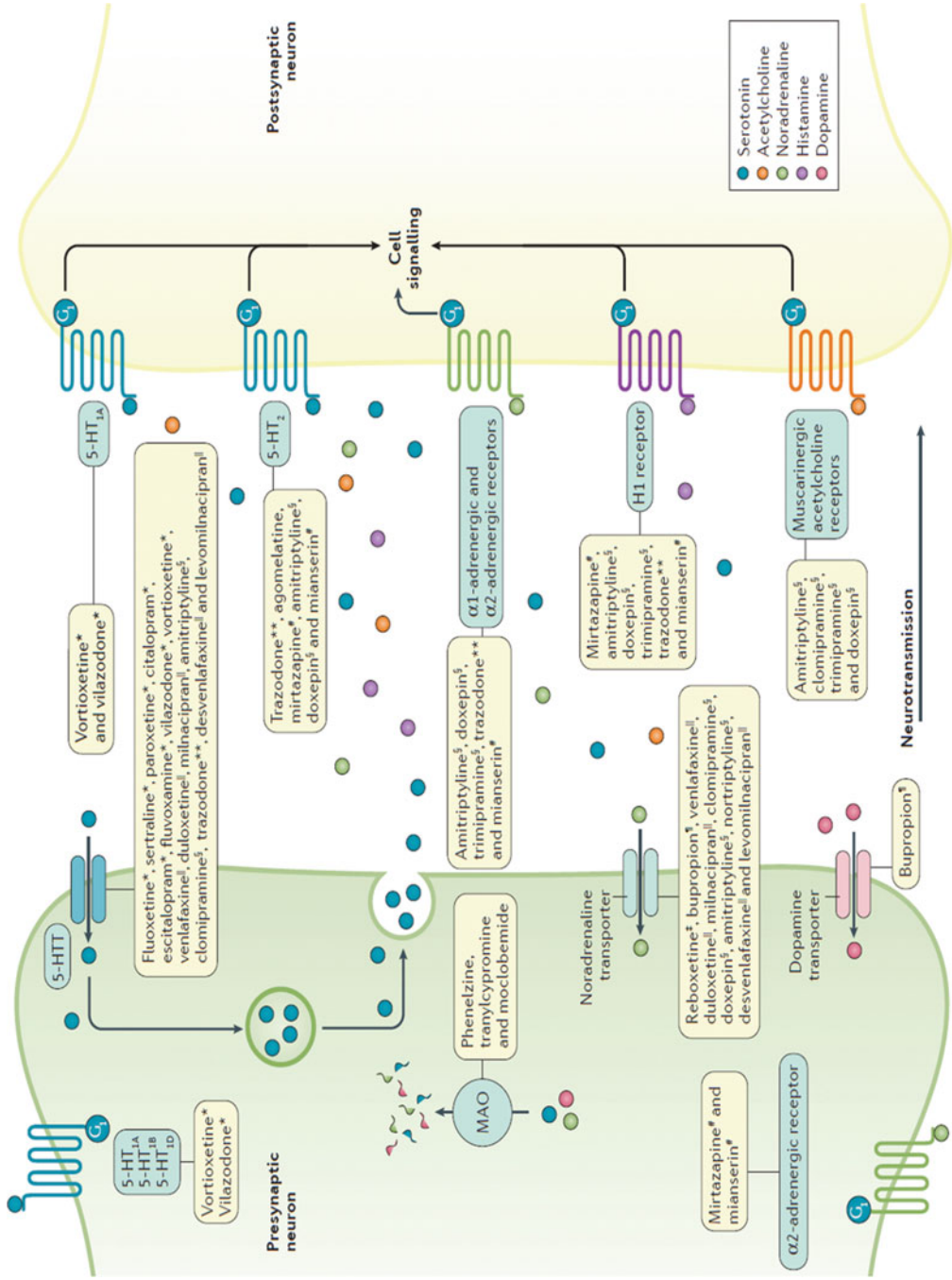
Genetic research in major depression has proven difficult. Like most complex diseases, depression is a polygenic disorder arising from the combined effect of many genetic variants with individually small effect sizes. By now, several sources provide evidence for the polygenic architecture of depression, despite a lack of genome-wide significant loci. Polygenic risk scoring uses association statistics from a discovery GWAS to weight the genotypes of individuals in an independent test sample and sums these effects across multiple SNPs into a polygenic risk score (PRS). Differences in PRS between cases and controls in the independent sample show that the PRS is capturing genetic susceptibility that is predictive of disease status. PRS for MDD shows modest, although significant, prediction for depression in independent samples (Mullins and Lewis 2017).

Clinical Use and Side Effects

Major depressive disorder is a serious mental health condition that affects up to 16% of people in the United States during their lifetimes and about 7% of the US population per year. In addition, patients with major depressive disorder have a substantial risk of suicidal behavior. Approximately two thirds of patients with major depressive disorder have a response to currently marketed antidepressant agents.

Antidepressants were serendipitously discovered in the 1950s, and the first generation of these drugs was constituted by tricyclic molecules. The refinement among the second and third generation of these drugs resulted in molecules that have less side effects, are better tolerated, and consequently enjoy much better acceptance. Given the personal and socioeconomic burden of depression, the undertreatment of this disabling and potentially life-threatening clinical condition seems neither ethical nor prudent.

While antidepressants have proven to be effective drugs, several drawbacks and caveats need to be resolved, such as fast, satisfactory response and higher proportion of remission. The NMDA receptor channel ionophore antagonist ketamine



Antidepressant Drugs, Fig. 2 Most of the receptor, transporter, or channel binding shown here represent functional antagonism. It is related either to effects or side effects. ∗SSRIs, §tricyclic antidepressants, #α2-adrenergic receptor antagonists, ∗∗serotonin antagonist and reuptake inhibitor, ‖SNRIs, ‡NRIs, ¶NDRIs. (Adapted from Otte et al. 2016)

and its enantiomer esketamine have been proposed as fast-acting substances able to overcome these impediments. Since the first randomized controlled trial in the early millennium, numerous studies have explored the antidepressant efficacy of ketamine. Clear evidence has emerged that a single infusion exerts a significant antidepressant and antisuicidal effect in both unipolar and bipolar depression. A few studies suggest that antidepressant response can be improved and maintained by repeated administration. Although intravenous application has been most common, subcutaneous, intramuscular, and intranasal application has also been successful, the latter obtaining an FDA approval for treatment-resistant depression. However, issues surrounding potential toxicity, such as hemodynamic effects, cognitive and dissociative side effects, and abuse potential, need to be addressed before precise positioning among the other antidepressants can occur.

Pharmacogenetics in combination with refined clinical research might improve prediction of effects and side effects. Matching patients to the antidepressant that is most likely to be effective and less likely to harm through adversive reactions is the main goal of all modern therapies. Patient characteristics including sex, age, anxiety level, premedication, and family history (genetic load) do not predict better or worse response to a particular antidepressant drug or drug class. However, the fact that all drugs are equally effective between comparison groups does not mean that they are equally effective for individual patients. It is now hoped that combination of clinical data, including functional assessments, e.g., neuroendocrine, neuroimaging, and neuropsychology together with information from genotyping, i.e., identification of a collection of single nucleotide polymorphisms (SNPs), will ultimately lead to choosing a first-line antidepressant based upon individual data. In practice, a genotype-guided medication selection is yet not in reach, but several minor innovations emerging from hypothesis-driven research are, such as glucocorticoid signaling (FKBP5) and serotonin neurotransmission (SLC6A4 and HTR2A) (Fabbri et al. 2017). Another new development of immediate clinical usefulness is the analysis of genetic variability in the cytochrome P450 enzyme system in patients, which may elucidate clinically relevant changes in drug metabolization and adverse reactions. For example, if a patient receives an SSRI such as fluoxetine, which blocks the P4502D6 enzyme, and an antiarrhythmic, which is metabolized by the same enzyme, a fatal increase of the cardiotropic drug may occur. Other possible candidates involved in pharmacokinetics are P-glycoproteins, which are important regulators of a drug's blood–brain barrier passage. It was recently shown that many antidepressants are substrates of P-glycoprotein, which, if overexpressed, can extrude the antidepressant out of the brain cells into the circulation, thus preventing central effects that may lead to therapy resistance. Polymorphisms in the ABCB1 gene leading to differential P-glycoprotein expression and activity are already used in certain clinical circumstances.

Side effects of antidepressants usually occur during the first days of treatment and tend to diminish over time. The side effect profile can be easily derived from the transporter binding profile. Serotonergic drugs might cause headaches, appetite loss, nervousness, sweating, and sexual dysfunction and noradrenergic drugs palpitations, sweating, anxiety, and drowsiness. Anticholinergic antidepressants show side effects like constipation, blurred vision, memory dysfunction, and dry mouth, while antihistaminergic drugs exhibit side effects like sedation, hypotension, and weight gain. Antiadrenergic properties are associated with postural hypotension and reflex tachycardia.

Cross-References

▶ Monoamine Oxidases
▶ Serotoninergic System

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Antidepressants

► Antidepressant Drugs

Antidiuretic Hormone (ADH)

► Vasopressin

Antiepileptic Drugs

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Synonyms

Anticonvulsant drugs; Anticonvulsants; Anticonvulsive drugs; Antiseizure drugs

Definition

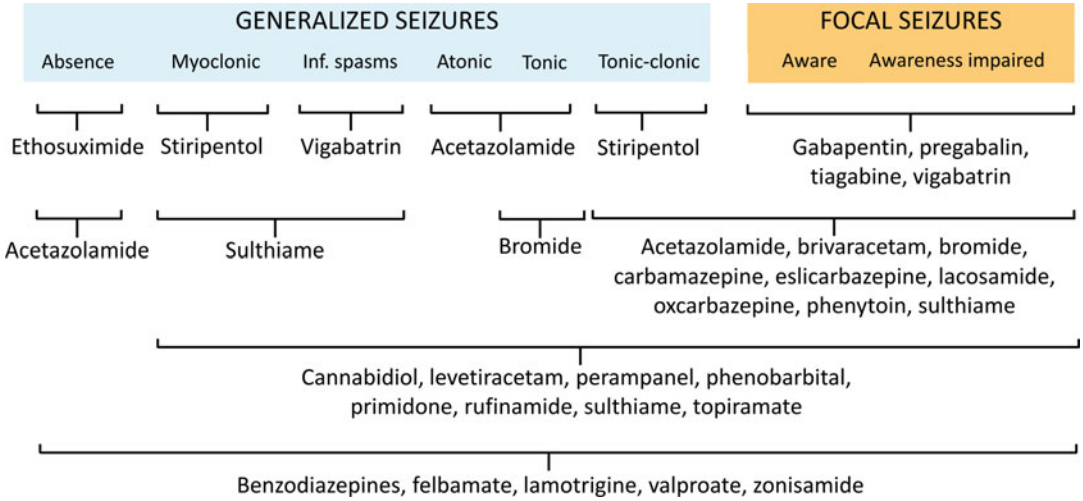
Antiepileptic drugs (AEDs) are used to treat epilepsy, i.e., the repeated occurrence of unprovoked epileptic seizures. They are also used in other conditions, e.g., bipolar disorder, neuropathic pain, or migraine. None of the available AEDs does cure epilepsy. Instead, they suppress epileptic discharges in the brain, thereby reducing seizure frequency. For this reason, they are sometimes called anticonvulsants or antiseizure drugs. Nevertheless, the official ATC code for these drugs as designated by the WHO is "N03A Antiepileptics."

The prevalence of epilepsy is about 0.7% worldwide, with equal distribution between sexes. Thus, epilepsy is among the four most common neurologic disorders. The incidence vs. age curve is U-shaped, meaning that the incidence of epilepsy is highest at both extremes of age. At young age, epilepsy is mostly due to connatal brain disease, hypoxia at birth, genetic, or unknown causes, while at old age, epilepsy is mostly caused by brain tumors or stroke. This has direct implications on treatment as different etiologies may lead to different types of epilepsy, and there are only few AEDs that are effective in all of them (Fig. 1).

Some AEDs may even aggravate certain seizure types and are thus contraindicated in these. Hence, it is of utmost importance that the patient's type of epilepsy is correctly identified as this will define the range of suitable AEDs.

Approximately 70% of people with epilepsy become seizure-free by medical treatment. Despite a large number of new AEDs launched during the past 30 years, many of them with new mechanisms of action (MOAs), this number has not changed. For these reasons, new AEDs are constantly being developed (Fig. 2). AEDs that came onto the market before 1990 are commonly referred to as first-generation AEDs, drugs launched thereafter as second generation. Drugs currently under clinical development include newly developed compounds as well as old, repurposed drugs, e.g., anakinra, cannabidivarin, fenfluramine, selurampanel, everolimus, ganaxolone, medium-chain fatty acids, padsevonil, and more. Many of these represent innovative MOAs, and some are discussed in this chapter.

AEDs are used both prophylactically (maintenance treatment) and acutely. Acute treatment means stopping ongoing prolonged seizure



Antiepileptic Drugs, Fig. 1 Spectrum of antiepileptic efficacy of commonly used antiepileptic drugs

activity such as status epilepticus or acute manic episodes.

All AEDs aim at reestablishing the balance between excitatory and inhibitory mechanisms in the brain, thereby reducing paroxysmal neuronal firing. Consequently, almost all AEDs modulate either glutamatergic or GABAergic neurotransmission or transmembrane ion currents (Fig. 3).

For most AEDs, more than one MOA has been documented (Table 1).

These diverse pharmacological actions may explain their clinical efficacy in different seizure types and a variety of non-epileptic medical conditions. Naturally, the number of identified MOAs is highest for the oldest AEDs because they have been more intensively studied than the newer ones. Some AEDs share certain structural similarities and MOAs, while other are structurally similar but have different MOAs and different clinical effects (Fig. 4a, b).

Acetazolamide

Mechanism of Action

The exact mechanism by which acetazolamide (ACZ) exerts its antiepileptic activity is not entirely clear. ACZ is a sulfonamide and a potent inhibitor of the enzyme carbonic anhydrase (CA) which exists in several subfamilies and isoforms. Other AEDs that also contain a sulfonamide structure like topiramate or zonisamide share this MOA. Inhibition of CA increases CO_2 and decreases HCO^{3-}concentrations, leading to reduced pH. Increased H^+-concentrations reduce cation currents through NMDA receptors and Ca^{++} currents through voltage-dependent calcium channels and, thus, neuronal excitability. Another proposed MOA of ACZ is activation of myosin light chain kinase (MLCK, an enzyme involved in synaptic vesicle recirculation), resulting in altered synaptic transmission (Bertone et al. 2017). Other data suggest that ACZ reduces neuronal firing by reducing $HCO3^-$ efflux through $GABA_A$ receptors, thereby stabilizing membrane potential and hampering depolarization (Hamidi and Avoli 2015).

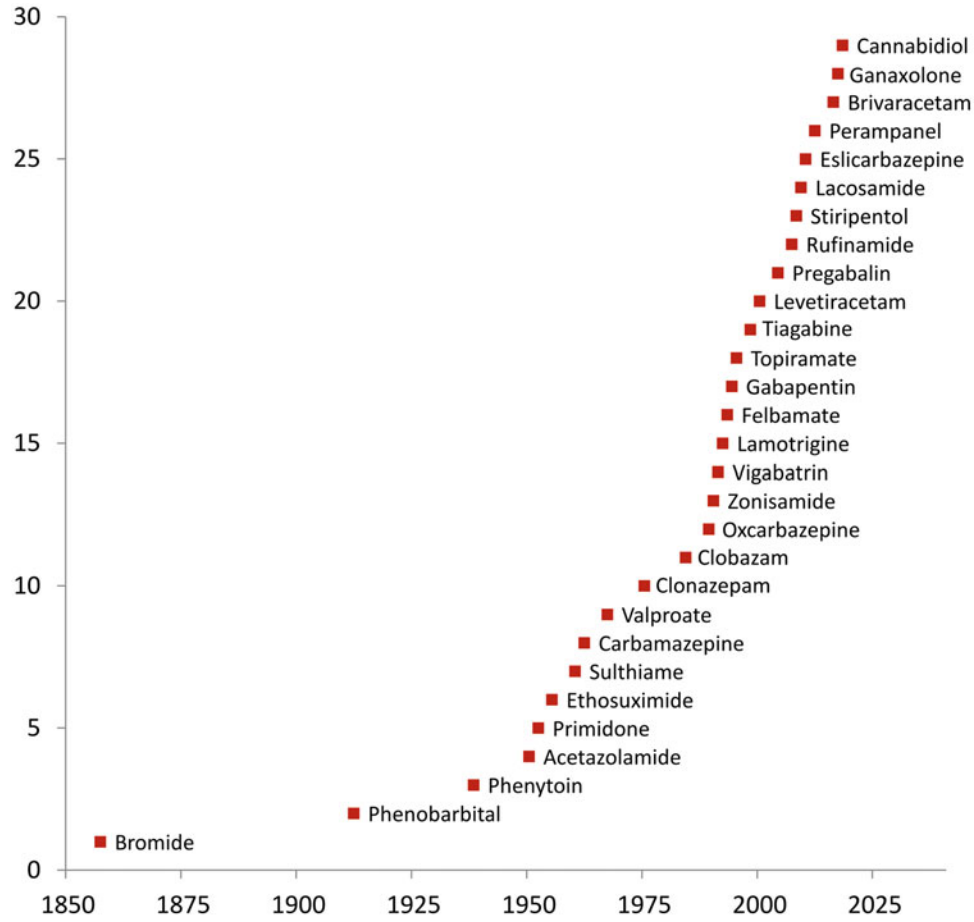
Clinical Use

AZA has been shown to be effective in treating partial, myoclonic, absence, and primary generalized tonic-clonic seizures (Reiss and Oles 1996). Besides, it is used for treating altitude sickness, glaucoma, and other medical conditions.

Brivaracetam

Mechanism of Action

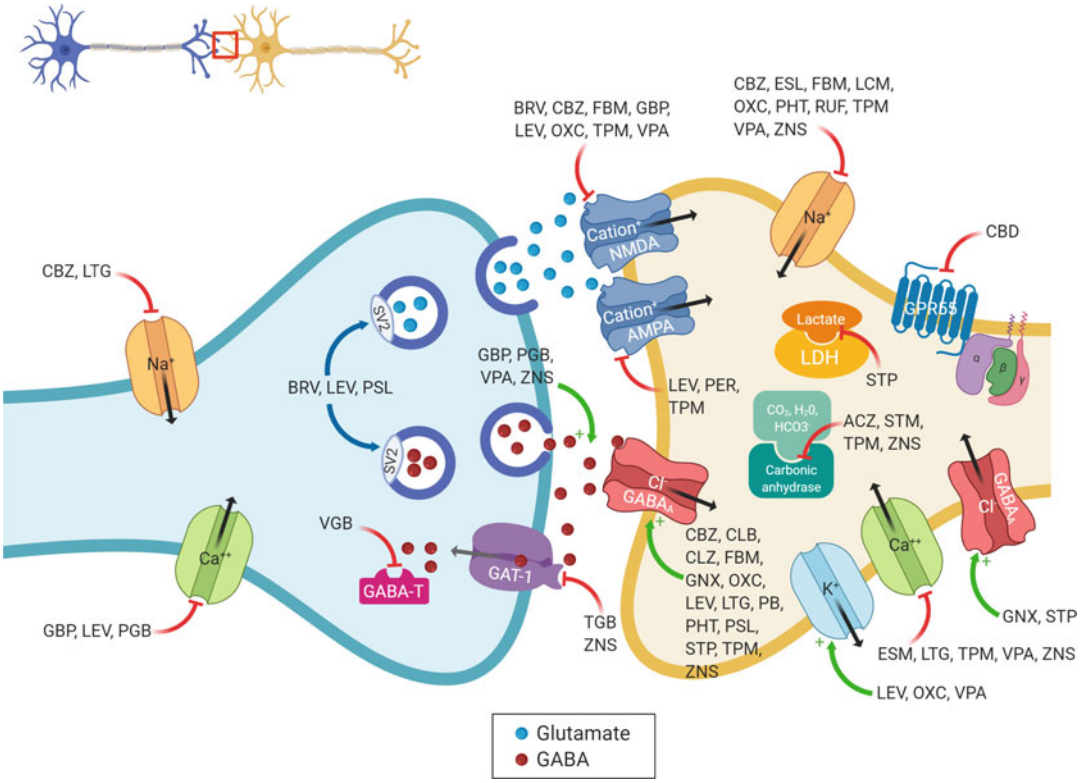
Brivaracetam (BRV) is an analogue of levetiracetam (LEV; Fig. 4). Both are derivatives



Antiepileptic Drugs, Fig. 2 Year of introduction of currently available antiepileptic drugs (AEDs). Several less used or abandoned AEDs (e.g., mephenytoin, trimethadione, retigabine) are not shown

of the older nootropic agent piracetam. BRV, like LEV, binds to the synaptic vesicle protein SV2A. This protein is located in the vesicle wall in terminal ends of neurons and in endocrine cells. It plays a central role in the exocytosis of neurotransmitter molecules into the synaptic cleft. BRV's binding to it results in decreased synaptic transmission. As this affects both excitatory and inhibitory neurotransmitters, it is not entirely clear how this MOA leads to reduced spreading of epileptic activity in the brain. BRV's affinity to the SV2A protein is reportedly 15–30-fold higher than LEV's. BRV is also a NMDA receptor antagonist and reduces excitatory glutamatergic transmission. It does not seem to act on AMPA and kainate receptors nor does it have GABAergic activity. Furthermore, it does not seem to have any effect on voltage-gated sodium, potassium, or calcium channels (Klein et al. 2018).

Owing to its differing pharmacokinetic profile, BRV may reach effective concentrations in brain tissue faster than LEV after IV administration. This is irrelevant for maintenance therapy but could be an advantage in acute situations like status epilepticus where the time to cessation of epileptic activity is negatively correlated with outcome.



Antiepileptic Drugs, Fig. 3 Schematic illustration of molecular targets of antiepileptic drugs. For a complete list of all drug actions, see Table 1. *ACZ* acetazolamide; *AMPA* α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; *BRV* brivaracetam; *CBD* cannabidiol; *CBZ* carbamazepine; *CLB* clobazam; *CLZ* clonazepam; *ESL* eslicarbazepine; *ESM* ethosuximide; *FBM* felbamate; *GABA$_A$* $GABA_A$ receptor; *GABA-T* GABA transaminase; *GAT-1* GABA transporter 1; *GBP* gabapentin; *GNX* ganaxolone; *GPR55* G protein-coupled receptor 55; *LDH* lactate dehydrogenase; *LEV* levetiracetam; *LTG* lamotrigine; *NMDA* N-methyl-D-aspartate receptor; *OXC* oxcarbazepine; *PB* phenobarbital; *PER* perampanel; *PGB* pregabalin; *PHT* phenytoin; *PSL* padsevonil; *RUF* rufinamide; *STM* sulthiame; *STP* stiripentol; *SV2* synaptic vesicle protein 2; *TGB* tiagabine; *TPM* topiramate; *VGB* vigabatrin; *VPA* valproate; *ZNS* zonisamide. Figure created with BioRender

Clinical Use

BRV is licensed for add-on treatment of partial seizures with or without secondary generalization in children and adults. It is available as an intravenous formulation for use in status epilepticus.

Bromide

Mechanism of Action

Bromide (BR) is a halogen and administered as its potassium or sodium salt. It exerts its antiepileptic effect by replacing up to 30% of the chloride atoms in cerebral intra- and extracellular fluids, which leads to neuronal hyperpolarization (Plumlee 2004).

Clinical Use

In several clinical studies, BR has shown moderate-excellent efficacy in focal, tonic, and tonic-clonic seizures. However, it frequently induces sedation as well as gastrointestinal and dermatological adverse effects and has unfavorable pharmacokinetics. Hence, it is mainly used as a drug of third choice in severe epilepsy syndromes of

Antiepileptic Drugs, Table 1 Molecular targets of antiepileptic drugs. *GABA* gamma-aminobutyric acid; *GLU* glutamate; *GPR55* G protein-coupled receptor 55; *HDAC* histone deacetylase; *TRPV1* transient receptor potential vanilloid 1; *VDAC1* mitochondrial voltage-dependent anion-selective channel 1

		Ion currents			Neurotransmission				
							GLU	GLU	
Antiepileptic drug	Abbr.	Na^+	Ca^{++}	K^+	$GABA_A$	SV2	NMDA	AMPA/ kainate	Other mechanisms of action
Acetazolamide	ACZ								Inhibition of carbonic anhydrase
Brivaracetam	BRV					+	+		
Bromide	BR								Replacement of chloride intra- and extracellularly
Cannabidiol	CBD								GPR55 antagonism, TRPV1 desensitization, VDAC1 modulation, adenosine reuptake inhibition, dopamine antagonism, serotonin agonism
Carbamazepine	CBZ	+	+	+	+		+		Adenosine receptor antagonism, increased dopaminergic, and serotonergic transmission
Clobazam	CLB				+				
Clonazepam	CLZ				+				
Eslicarbazepine	ESL	+	+						
Ethosuximide	ESM	+	+						
Felbamate	FBM	+	+		+		+		
Gabapentin	GBP		+		+		+		
Ganaxolone	GNX				+				
Lacosamide	LCM	+							Modulation of collapsin response mediator protein 2 (CRMP2)
Lamotrigine	LTG	+	+		+			+	$5\text{-}HT_3$ antagonism, σ-agonism, arachidonic acid cascade modulation
Levetiracetam	LEV		+	+	+	+	+	+	Increased glial GLU uptake, serotonergic, α_2-adrenergic, μ-opioid agonist
Oxcarbazepine	OXC	+	+	+	+		+		
Padsevonil	PSL				+	+			
Perampanel	PER							+	
Phenobarbital	PB				+				
Phenytoin	PHT	+	+		+				Na^+/K^+-ATPase enhancer, calcium-calmodulin inhibition, reduced cAMP/cGMP levels, reduced GLU levels, increased GABA levels

(continued)

Antiepileptic Drugs, Table 1 (continued)

Antiepileptic drug	Abbr.	Ion currents			Neurotransmission				Other mechanisms of action
		Na^+	Ca^{++}	K^+	$GABA_A$	SV2	GLU NMDA	GLU AMPA/ kainate	
Pregabalin	PGB		+		+				
Primidone	PRM				+				Biotransformed to phenobarbital
Rufinamide	RUF	+							
Stiripentol	STP				+				Neuronal LDH inhibitor
Sulthiame	STM	+							Inhibition of carbonic anhydrase, increased GABA levels, decreased GLU levels
Tiagabine	TGB								Synaptic GABA-reuptake inhibitor
Topiramate	TPM	+	+	+	+			+	Inhibition of carbonic anhydrase
Valproate	VPA	+	+	+	+				Aspartate inhibition, HDAC inhibition
Vigabatrin	VGB								Irreversible inhibition of GABA- transaminase
Zonisamide	ZNS	+	+	+	+		+		Inhibition of carbonic anhydrase

childhood. BR is also used by veterinarians to treat epilepsy in dogs and other animals.

Cannabidiol

Mechanism of Action

Cannabidiol (CBD) is one of the most abundant phytocannabinoids found in the plants *Cannabis sativa* and *C. indica*. In contrast to tetrahydrocannabinol (THC) which accounts for most of the psychotropic effects of cannabis, CBD has only weak affinity to, and rather antagonistic actions at, CB1 and CB2 receptors. Accordingly, it has been found that CBD does not induce a high, which is of importance for its safety profile including its abuse potential.

It has been shown that CBD reduces neuronal excitation by modulating intracellular calcium concentrations through antagonism at the G protein-coupled receptor 55 (GPR55). It also acts on other orphan GPRs like GPR3, GPR6, and GPR12. In addition, it desensitizes the transient receptor potential vanilloid (TRPV) 1 and probably other TRPV channels which may be of significance for its analgesic effects. Further, CBD inhibits adenosine reuptake at A1 and A2 receptors, blocks sodium channels, and acts on the equilibrative nucleoside transporter 1 (ENT-1) as well as the mitochondrial voltage-dependent anion-selective channel 1 (VDAC-1). Data also suggest that it has serotonergic properties and reduces dopaminergic transmission (Gaston and Friedman 2017). The plethora of pharmacological mechanisms may explain the broad spectrum of CBD's clinical effects.

Clinical Use

Purified CBD is currently licensed for the treatment of two severe forms of epilepsy, Lennox-Gastaut syndrome and Dravet syndrome. Current data show that it reduces the frequency of focal and generalized seizures including drop attacks, but not absences. Besides, it is widely used (with and without a prescription) for non-licensed indications including pain, anxiety, depression,

schizophrenia, and neurodegenerative diseases like amyotrophic lateral sclerosis, Parkinson's disease, Huntington's chorea, and Alzheimer's disease. Its neuroprotective efficacy in the latter indications is attributed to its anti-inflammatory and antioxidant properties. CBD is also part of nabiximols which is a 50/50 mixture of CBD and THC. Nabiximols is licensed for the treatment of MS-related spasticity. Like pure CBD, nabiximols is used for a variety of off-label indications.

Carbamazepine

Mechanism of Action

Carbamazepine (CBZ) has a tricyclic ring structure and was synthesized in 1953 in an attempt to repeat the success of the antipsychotic, chlorpromazine. It was, however, first marketed to treat trigeminal neuralgia and subsequently became a first-line treatment of epilepsy. CBZ is traditionally classified as a sodium channel blocker, but it has multiple MOAs. It binds to postsynaptic, voltage-dependent sodium channels when they are in their inactive state (i.e., when the membrane is depolarized), thereby prolonging this inactive state and reducing high-frequency repetitive firing. Normal, physiologic firing is affected only minimally. In addition, CBZ reduces synaptic signal transmission by blocking presynaptic sodium channels as well as glutamatergic NMDA receptors. CBZ also interacts with $GABA_A$ receptors, presumably via the benzodiazepine binding site (Ambrosio et al. 2002). Apart from these signaling systems typically involved in the generation of epileptic activity, CBZ has been shown to increase serotonin release and inhibit serotonin reuptake. It also modulates adenosine and dopaminergic neurotransmission. These effects may be relevant for its efficacy in psychiatric conditions such as bipolar disorder.

Clinical Use

CBZ is a first-line drug for focal and generalized tonic-clonic epileptic seizures. It worsens absence seizures, probably by enhancing $GABA_A$ receptor activity in the ventrobasal thalamus, and is therefore contraindicated in patients who have this type of seizures. Besides, CBZ is used in bipolar disorder and trigeminal neuralgia and to prevent seizures in alcohol withdrawal.

Clobazam

Mechanism of Action

Clobazam (CLB) is a 1,5-benzodiazepine, meaning that its nitrogen atoms are situated in the 1- and 5-positions of the diazepine ring instead of the usual 1- and 4-positions. CLB and its active metabolite, N-desmethylclobazam, bind to the $GABA_A$ receptor at the benzodiazepine binding site (Rudzinski et al. 2016; Riss et al. 2008). This increases the opening frequency of the ion channel when it is activated by GABA, without affecting the duration of the open state. The result is an enhanced influx of chloride (Cl^-) ions which leads to hyperpolarization of the neuronal cell membrane and, thus, reduced excitability of the neuron.

Clinical Use

CLB was originally introduced as an anti-anxiety drug. It is also effective against focal and generalized epileptic seizures, and it is licensed for the adjunctive treatment of seizures associated with Lennox-Gastaut syndrome (LGS) in patients 2 years of age or older. It allegedly has less pronounced sedative properties than other benzodiazepines, a characteristic that is ascribed to its 1,5-structure.

Clonazepam

Mechanism of Action

All benzodiazepines are effective against focal and generalized seizures. While lorazepam and diazepam are preferentially used to stop acute prolonged seizures including status epilepticus, few benzodiazepines are used for maintenance treatment. Clonazepam (CLZ) is the only benzodiazepine that by its ATC code (N03A E01) is classified as an AED. Like CLB, CLZ binds to the benzodiazepine binding site at the $GABA_A$ receptor and reduces neuronal

excitability through allosteric modulation (Riss et al. 2008).

Clinical Use

CLZ is licensed for monotherapy or as an adjunctive treatment of the Lennox-Gastaut syndrome. Due to its unfavorable safety profile (mainly sedation) and development of tolerance, it is a drug of second or third choice. Besides, it is widely prescribed for other psychiatric conditions, many of them off-label. There is also a considerable amount of drug abuse with CLZ.

Eslicarbazepine

Mechanism of Action

Eslicarbazepine (ESL) is the S-enantiomer of licarbazepine, the primary and active metabolite of oxcarbazepine (see below) which in turn is a derivative of carbamazepine. Licarbazepine was formerly called MHD or monohydroxy derivative (of oxcarbazepine) and later renamed. ESL is available as a prodrug, eslicarbazepine acetate, which after entering the body is rapidly converted to ESL. Like CBZ, ESL blocks voltage-gated sodium channels. In contrast to CBZ, it does not affect the fast inactivation state but enhances slow inactivation of the sodium channel, thereby reducing sustained repetitive neuronal firing. In vitro experiments have also shown that ESL reduces NMDA currents but only at supratherapeutic concentrations. A ligand-binding study did not support the hypothesis that ESL binds to the NMDA receptor nor any other ligand-operated receptors, ion channels, or transporters (Ambrosio et al. 2002).

Clinical Use

ESL is currently licensed as adjunctive treatment of focal-onset epilepsies in adults.

Ethosuximide

Mechanism of Action

Ethosuximide (ESM) is a succinimide and, although not a barbiturate, chemically similar to phenobarbital (Fig. 4). However, its main MOA is not related to GABA. Instead, ESM blocks low-threshold T-type calcium channels (LTCCs) which are predominantly expressed in the hypothalamus. The hypothalamus plays a crucial role in the generation of the typical 3-Hz spike-and-wave discharges seen in the EEG during absence seizures. The exact kind of interaction of ESM with LTCCs is not known. Moreover, not all studies found an effect of ESM on neuronal calcium currents. ESM has also been found to block non-inactivating sodium currents in thalamocortical neurons (White et al. 2007). This potential dual effect on thalamic and thalamocortical neurons may explain ESMs strong efficacy in treating absence seizures. It has also been hypothesized that ESM acts on GHB- and dopamine-mediated neurotransmission and increases nitric oxide release. However, experimental support for these hypotheses is scarce. ESM is poorly fat-soluble, and it is assumed that its site of action is on the outer side of the neuronal cell membrane. There are other succinimides like mesuximide and phensuximide, but they are rarely used.

Clinical Use

ESM is a first-line agent to treat absence seizures. Interestingly, and in contrast to all other AEDs, ESM is clinically effective exclusively in this type of seizures. This supports the notion of a selective action on hypothalamic neurons.

Felbamate

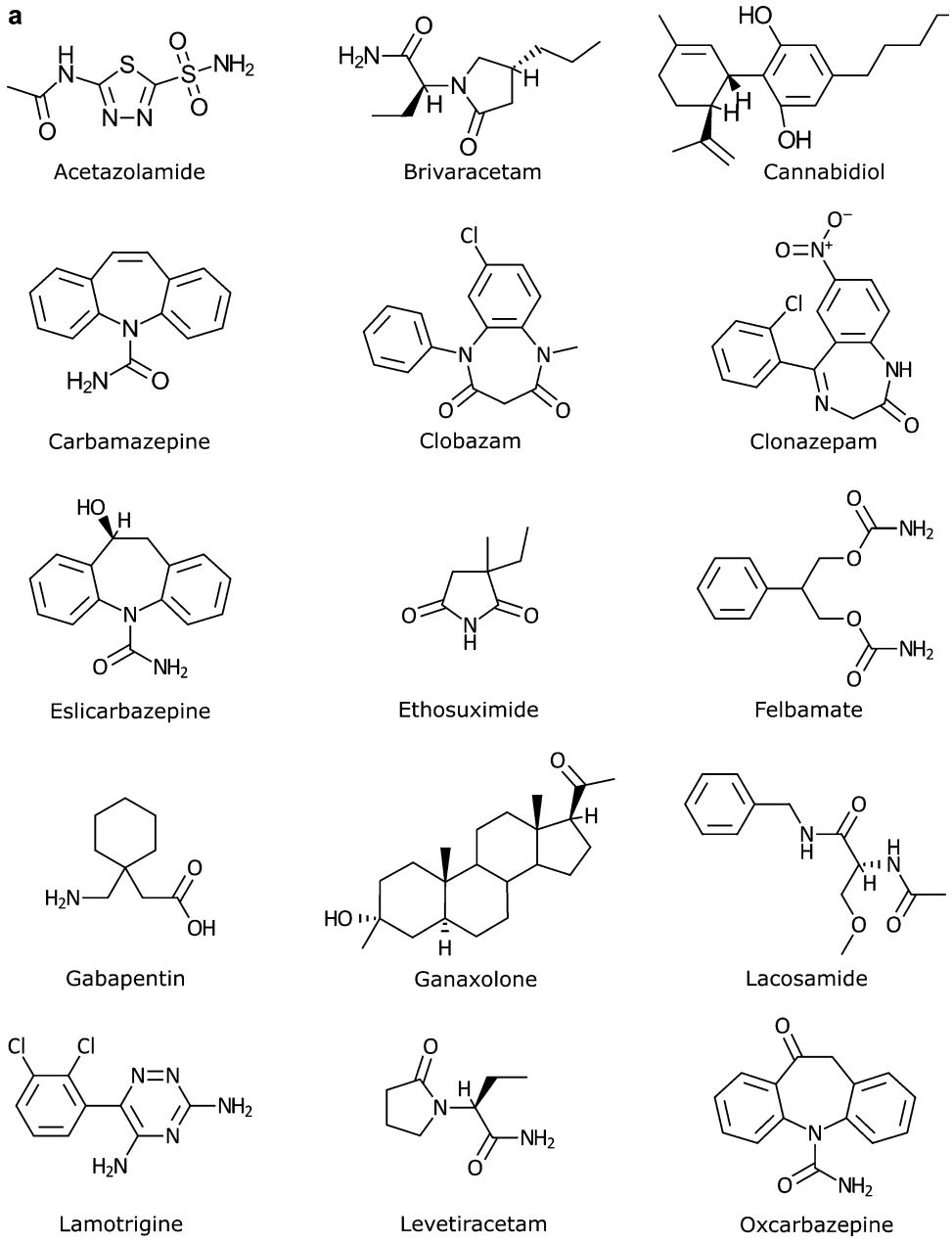
Mechanism of Action

Felbamate (FBM) is chemically related to meprobamate. It prolongs the duration of GABA-evoked chloride currents in cortical and hippocampal neurons. Accordingly, FBM has shown clinical efficacy against absence seizures. The binding site at the $GABA_A$ receptor is most likely distinct from the benzodiazepine binding site and the barbiturate binding site as FBMs effect is not blocked by flumazenil, and FBM does not affect the barbiturate-induced potentiation of GABA-mediated chloride currents. FBM also acts as a NMDA receptor antagonist, presumably through

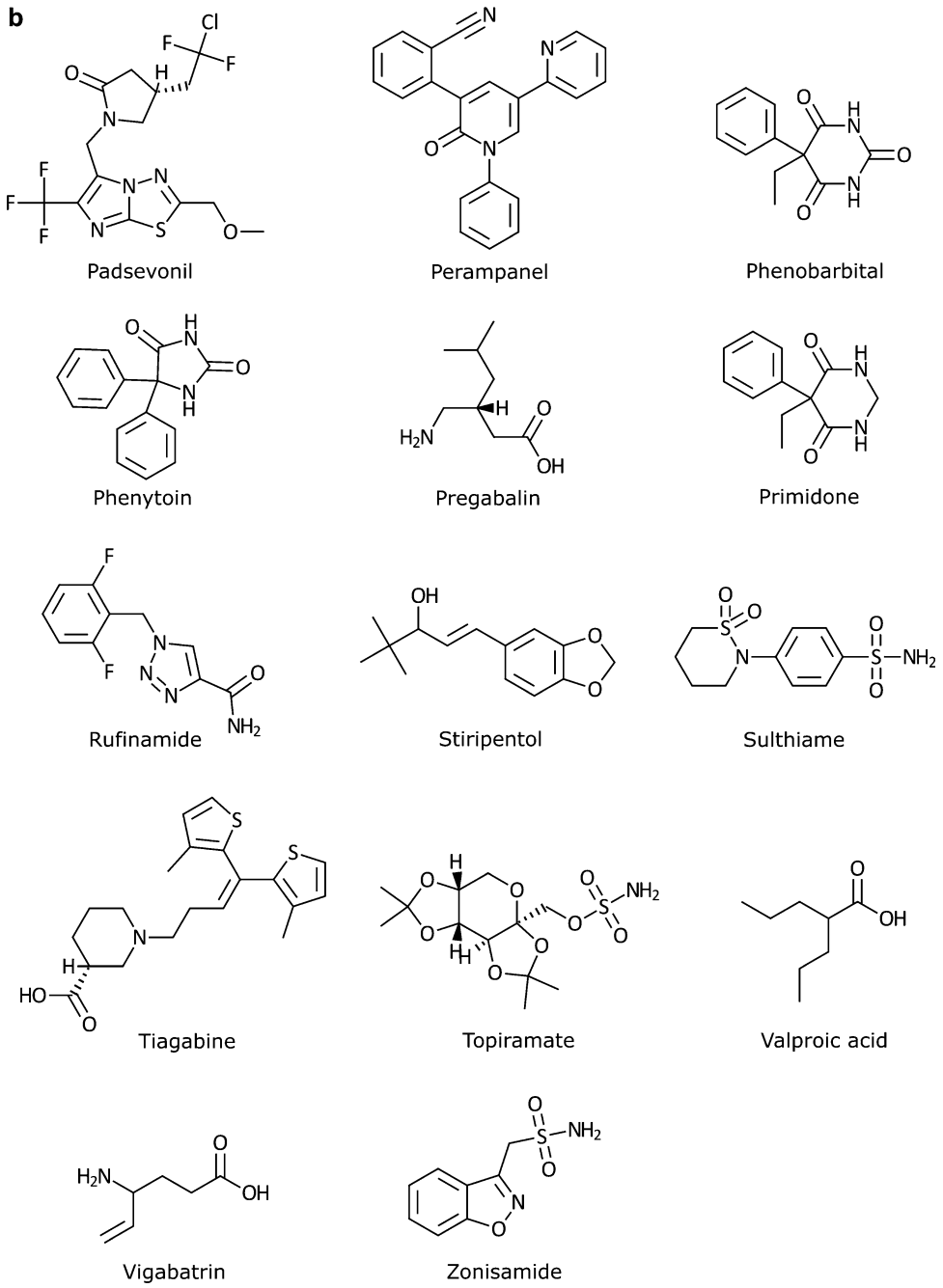
interaction at the glycine binding site. Like CBZ, FBM blocks voltage-dependent sodium channels by binding to them in their inactive state. Finally, FBM blocks voltage-dependent high threshold calcium currents (White et al. 2007).

Clinical Use

FBM has shown efficacy in all kinds of epilepsy including severe forms like Dravet-syndrome or Lennox-Gastaut syndrome. However, its clinical use is limited because it, like its sibling



Antiepileptic Drugs, Fig. 4 (continued)



Antiepileptic Drugs, Fig. 4 The chemical structures of antiepileptic drugs discussed in this chapter

meprobamate, may induce aplastic anemia with a mortality rate around 30%. In addition, FBM may induce severe hepatic failure. The manufacturer recommends using FBM only in those patients who do not respond well to other treatments and whose epilepsy is so severe that the risk of developing aplastic anemia or liver failure is outweighed by the potential benefit of felbamate treatment.

Gabapentin

Mechanism of Action

Gabapentin (GBP) is one of several compounds chemically engineered as GABA analogues (others, e.g., pregabalin and vigabatrin). The idea was to create antiepileptic drugs that mimic GABA. Indeed, GBP showed moderate antiepileptic efficacy in clinical studies. Its MOA, however, is not related to an action on GABA receptors. Instead, GBP exerts its effects by binding to the $\alpha_2\delta$-subunit at presynaptic L-, N-, and P-/Q-type voltage-gated calcium channels, thereby reducing presynaptic excitation and, consequently, neurotransmitter release into the synaptic cleft. This appears to reduce the release of all major neurotransmitters including the neuropeptides substance P and calcitonin gene-related peptide which are involved in nociception. It has long been known that GBP exerts synergistic effects together with glutamate antagonists in chronic pain states, and recently it has been reported that one of the two $\alpha_2\delta$ isoforms, $\alpha_2\delta$-1, physically interacts with NMDA receptors (Zhou et al. 2018). Such $\alpha_2\delta$-NMDA receptor coupling is of significance not only for pain signaling but also for memory and learning, and it has indeed been reported that GBP therapy is associated with a decline in memory, executive function, and attention. In addition, GBP may exert analgesic effects through reduction of protein kinase C and by suppressing transient receptor potential (TRP) ion channels like TRPA1 (Kukkar et al. 2013). There are also data suggesting that GBP may increase GABA levels in the brain by modulating glutamic acid decarboxylase and other enzymes involved in the synthesis of GABA, but these effects most likely play only a minor role in mediating GBP's clinical effects.

Clinical Use

GBP has only moderate efficacy as an antiepileptic drug. It is licensed for the treatment of focal seizures with and without secondary generalization, but, owing to its excellent analgesic properties, it is mainly used as a therapeutic agent in various neuropathic pain conditions, e.g., polyneuropathy or postherpetic neuralgia, usually in combination with other analgesics. It is also used to treat essential tremor.

Ganaxolone

Mechanism of Action

Ganaxolone (GNX) is a synthetic, 3-beta-methylated analog of the endogenous neurosteroid brexanolone (also known as allopregnanolone) which is derived from progesterone. The methyl group prevents the molecule from being converted back to progesterone which could induce hormonal adverse effects. GNX enhances GABA-evoked chloride currents by allosteric modulation of $GABA_A$ receptors at a binding site different from benzodiazepines (Greenfield 2013). In contrast to benzodiazepines, GNX also acts on extrasynaptic $GABA_A$ receptors (Chuang and Reddy 2019). The latter two properties of GNX may have benefits particularly in the treatment of status epilepticus that did not respond to first-line treatment with benzodiazepines alone.

Clinical Use

Although effective in animal models of epilepsy and early phase I and II studies, GNX showed no better efficacy than placebo in a randomized, placebo-controlled clinical trial in treatment-refractory patients with focal epilepsy. The manufacturer has initiated pivotal studies with ganaxolone in children with rare epilepsy syndromes like CDKL5 deficiency disorder or PCDH19-related epilepsy and is currently conducting studies in refractory status epilepticus.

Lacosamide

Mechanism of Action

Lacosamide (LCM) is a synthetic, "functionalized" amino acid. While naturally occurring L-amino acids (except cysteine) are S-enantiomers, LCM's biological activity has been mainly attributed to its R-enantiomer. LCM acts on voltage-gated sodium channels. Unlike other sodium channel blockers like CBZ or lamotrigine but similar to ESL, LCM enhances the slow

inactivation of these ion channels. The exact molecular target and mechanism of action have so far not been identified, although some data suggest an interaction with the batrachotoxin-binding site of the sodium channel (Errington et al. 2006). As a second MOA, LCM modulates the activity of the collapsin-response mediator protein 2 (CRMP2) which is involved in controlling neuronal plasticity (Wilson and Khanna 2015). This may represent a neuroprotective and antiepileptogenic MOA. The nature of the interaction of LCM with CRMP2 remains unclear. No other MOAs have been demonstrated so far. Radioligand binding studies and electrophysiology experiments suggest that LCM does not act at other ion channels or any major neurotransmitter receptor nor does it inhibit synaptic transmitter reuptake.

Clinical Use

LCM is licensed as monotherapy and adjunctive treatment of focal onset epileptic seizures in children and adults. It has also shown efficacy in stopping status epilepticus. In addition, it has been used to treat neuropathic pain.

Lamotrigine

Mechanism of Action

Lamotrigine (LTG) was originally developed with the intention to design a folic acid antagonist since it was (then) believed that folic acid antagonism was an antiepileptic MOA. In fact, LTG is an effective AED while being only a weak inhibitor of dihydrofolate reductase. Its supposed main MOA is the blockade of presynaptic, voltage-dependent sodium channels, thereby reducing synaptic neurotransmission. It is thought that this predominantly affects excitatory transmitters such as glutamate and aspartate. In addition, LTG also blocks L-, N-, and P- but not T-type calcium channels (White et al. 2007). This may account for its effectiveness in treating absence seizures. Besides, LTG is an antagonist at 5-HT_3 receptors. While other 5-HT receptors are G protein-coupled, the 5-HT_3 receptor is a ligand-gated ion channel (sodium, potassium, and calcium ions). Experimental data suggest that it is involved in promoting anxiety and seizure activity. LTG is also an effective treatment in bipolar disorder, particularly with depressive symptoms. It is not clear how it exerts this effect. Several hypotheses exist: LTG is an agonist at σ-(sigma) receptors, a characteristic it shares with several antidepressants, e.g., citalopram or fluoxetine, and other psychotropic compounds. In vitro experiments suggest that it also acts as an antagonist at $GABA_A$ receptors in the amygdala, a brain structure involved in emotions and fear. Finally, there is evidence that LTG dampens the NMDA receptor-mediated arachidonic acid cascade, thereby interfering with arachidonic acid-derived prostanoids like PGE_2 that promote neuroinflammation (Ramadan et al. 2012). At present, this effect on the arachidonic acid metabolic cascade is the only MOA known to be shared by all mood stabilizers (i.e., lithium, CBZ, LTG, and valproate), which supports the notion that this MOA plays a major role in treating bipolar disorder.

Clinical Use

LTG is used to treat epileptic seizures of focal or generalized onset in children and adults, either in monotherapy or as add-on treatment. It is also effective as a mood stabilizer and is licensed for the prophylactic treatment of bipolar disorder. Besides, there is extensive off-label use for a variety of other affective and schizoaffective disorders, mainly those that are primarily characterized by depressive symptoms.

Levetiracetam

Mechanism of Action

Levetiracetam (LEV) is a derivative of piracetam, a compound mainly used as nootropic drug since the 1960s. It is generally assumed that LEV exerts its antiepileptic effects through an interaction with the synaptic vesicle protein SV2A, a protein involved in calcium-dependent exocytosis of neurotransmitters. The way of LEV's molecular interaction with SV2A has not been fully elucidated, but it is assumed that transmitter exocytosis is

downregulated either via reduced calcium inward currents or other mechanisms. Indeed, it has been demonstrated that LEV inhibits presynaptic transmembrane calcium currents, probably through an (as yet unidentified) intracellular mechanism (Vogl et al. 2012). However, LEV also increases brain concentrations of GABA, interacts with the $GABA_A$ receptor, and diminishes glutamatergic excitation by modulation of NMDA and AMPA receptors as well as upregulation of glial glutamate transporters (Cortes-Altamirano et al. 2016). Data also suggest that LEV leads to neuronal hyperpolarization by opening potassium channels and that it acts on serotonergic and α_2-adrenergic signaling paths as well as μ-opioid receptors. It is not clear how much each of these mechanisms contributes to its clinical effects.

Clinical Use

LEV is used as a first-line treatment in monotherapy or as add-on treatment of both focal and generalized epileptic seizures. It is also used as a treatment of status epilepticus. Small studies suggest some efficacy in migraine and hyperalgesia. Based on experimental as well as clinical studies in humans, LEV has also been suggested as a neuroprotective agent.

Oxcarbazepine

Mechanism of Action

Oxcarbazepine (OXC) is the 10-keto analog of CBZ. While it is pharmacologically active and thus per definition not a prodrug, its plasma half-life is only 1–4 h as it is rapidly converted to its main (and active) metabolite, licarbazepine (10-hydroxy-carbamazepine), which has a much longer plasma half-life and therefore is considered the active form of OXC. OXC and licarbazepine block voltage-dependent sodium channels in a manner similar to CBZ. In parallel to CBZ, licarbazepine also decreases glutamatergic neurotransmission by modulating NMDA receptor function. OXC but not licarbazepine also potentiates $GABA_A$ receptor currents. In addition, licarbazepine blocks high-voltage-dependent N-type calcium channels and increases a hyperpolarizing potassium current (Ambrosio et al. 2002).

Clinical Use

The clinical spectrum of efficacy mimics CBZ; hence, it is used alone or in combination for the treatment of focal onset seizures with or without secondary generalization in children and adults. OXC appears to be somewhat better tolerated than CBZ although allergic cross-reactivity exists. It is not widely used in psychiatric conditions, though.

Padsevonil

Mechanism of Action

Padsevonil (PSL) is a newly developed AED that shares with BRV and LEV an oxygen-substituted pyrrolidine ring but looks otherwise chemically unrelated. In contrast to BRV and LEV, it binds to all known isoforms of the SV2 vesicle protein, i.e., SV2A, SV2B, and SV2C. As with BRV and LEV, the exact mechanism of this interaction is not fully understood. In addition, PSL acts as a partial agonist at the $GABA_A$ receptor through the benzodiazepine binding site (Wood et al. 2020). Experimental studies so far did not provide evidence for an interaction with other molecular targets like ion channels, other receptors, transporters, or enzymes.

Clinical Use

PSL is currently under clinical investigation regarding its safety, drug interaction potential, and efficacy as a treatment for focal seizures in adults.

Perampanel

Mechanism of Action

Perampanel (PER) is a selective, noncompetitive, allosteric antagonist at the AMPA receptor, thereby reducing glutamate-evoked calcium currents and, hence, excitatory postsynaptic

potentials (Ceolin et al. 2012). It has been demonstrated that PER has no effect on NMDA or kainate receptors. No other MOAs have been established so far for this drug.

Clinical Use

PER is a used as an adjunctive AED for the treatment of focal seizures with or without secondary generalization and primarily generalized tonic-clonic seizures.

Phenobarbital

Mechanism of Action

Phenobarbital (PB) acts as a GABA agonist through allosteric modulation of the $GABA_A$ receptor. Its binding site and mode of action are distinct from those of the benzodiazepines. While benzodiazepines increase the opening frequency of the chloride channel, PB does not affect the frequency but prolongs the duration of the channel's open state (White et al. 2007). In vitro, PB blocks sodium currents only in supratherapeutic concentrations and exerts weak antagonism at the AMPA receptor and no effect on calcium channels or NMDA receptors.

Clinical Use

PB was the first chemically synthesized compound used as an AED. Its antiepileptic properties were discovered by serendipity when it was observed that agitated psychiatric patients who also had epilepsy experienced a marked reduction in seizure frequency when they were given PB for sedation. PB is a broad-spectrum AED with efficacy in all types of seizures except absences. However, owing to its marked sedative properties, its drug interaction potential, and the availability of alternative AEDs, PB is no longer a favorite choice for maintenance treatment of epilepsy in high-income countries. It is still among the first-line drugs for the treatment of benzodiazepine-refractory status epilepticus. Because of its effectiveness and low cost, it is classified as an essential drug by the WHO and frequently used in low-income countries. PB is also used as treatment of various acute withdrawal syndromes (opiates, alcohol, etc.) including neonatal abstinence syndrome. In very high doses, it is used in euthanasia. Veterinarians use PB to treat epilepsy in animals.

Phenytoin

Mechanism of Action

Phenytoin (PHT, diphenylhydantoin) was developed with the intention to find an AED with less sedating properties than PB. In fact, its chemical structure is identical to PB's except in the 5-position where PB's ethyl group has been replaced by a second phenyl ring (Fig. 4). This change gives PHT a completely different pharmacological profile. While it, like PB, enhances $GABA_A$ receptor-mediated chloride currents, PHT also prolongs the inactive state of voltage-gated sodium channels and blocks L- and T-type calcium channels, thereby reducing sustained repetitive neural firing (Delorenzo and Sun 2002). It further stabilizes the neuronal cell membrane by increasing the activity of Na^+-/K^+-ATPase which leads to a hyperpolarization of the cell membrane. Intracellularly, PHT interferes with the calcium-calmodulin complex which affects phosphorylation steps in various enzyme systems, and it diminishes the activity of second-messenger systems like cAMP and cGMP. It is not clear whether these effects are direct ones or consequences of PHT's membrane-stabilizing actions. Finally, data suggest that PHT affects release and reuptake of various neurotransmitters including norepinephrine, glutamate, and GABA. The clinical relevance of these latter effects remains to be determined.

Clinical Use

PHT was the first AED without pronounced sedative effects and is still one of the most effective drugs to treat tonic-clonic seizures and status epilepticus. Like PB though, its use is declining in high-income countries due to its side effects, drug interaction potential, and the availability of equally effective AEDs with a better safety profile.

Pregabalin

Mechanism of Action

Like GBP, pregabalin (PGB) was designed as a GABA analog, and the two compounds are chemically similar. Its molecular MOAs resemble those of GBP, i.e., binding to the α2δ-subunit of presynaptic L- and P-/Q-type voltage-gated calcium channels (McKeage and Keam 2009). Sparse documentation suggests weak GABAergic effects, presumably through increased GABA synthesis, although this is not thought to be of clinical relevance. Like GBP, PGB does not bind to $GABA_A$ or $GABA_B$ receptors nor does it affect GABA reuptake or breakdown. Another parallel to GBP is its ability to reduce cognitive performance, presumably via interfering with the α2δ-NMDA receptor complex.

Clinical Use

Like GBP, PGB is only moderately effective as an AED and has thus been mainly marketed as a treatment for chronic pain. Despite being two to four times more potent than GBP as an analgesic, PGB offers no major advantage over GBP, but its pharmacokinetics are somewhat more favorable. In analogy to GBP, treatment of chronic pain states constitutes its main area of use. Moreover, it is used as an anti-anxiety drug, in restless legs syndrome, and in several other off-label indications. PGB can allegedly induce highs and euphoria, and it has a documented potential for drug abuse. Its prescription has therefore been restricted in several countries.

Primidone

Mechanism of Action

Primidone (PRM) or desoxyphenobarbital is a derivative of PB; the only difference is that one of PB's three carbonyl groups has been replaced by a methyl group (Fig. 4). However, PRM is biotransformed to PB and another (much less potent) active metabolite, phenylethylmalonamide (PEMA). Data on PRM's MOA are lacking, mainly because it has long been believed that it is only a prodrug for PB. However, PRM has antiepileptic properties by itself and has in some studies even shown stronger therapeutic efficacy than PB. It has been speculated that PRM's MOA involves blockade of sodium or calcium channels. This speculation is justified since another PB derivative, ethosuximide (ESM), acts via blockade of calcium channels. In an animal model of essential tremor that uses genetically $GABA_A$ receptor deficient mice, PRM showed significant reduction of tremor-like symptoms (Hedera et al. 2013). This indicates at least one MOA that does not involve $GABA_A$ receptors. In human studies, a significant reduction in tremor can be seen already 1 h after the first administration, i.e., long before relevant serum concentrations of PB are achieved. Taken together, there is sufficient reason to postulate that PRM is not a prodrug.

Clinical Use

PRM is effective against focal and generalized tonic-clonic seizures. Due to its adverse effect profile, mainly mediated through its metabolite PB, it is only a treatment of second or third choice. PRM is also used as first-line treatment of essential tremor, as an alternative to propranolol.

Rufinamide

Mechanism of Action

Rufinamide (RUF) is a triazine and, as such, structurally similar to lamotrigine. RUF prolongs the inactive state of voltage-gated sodium channels, thus limiting the spread of neuronal excitation. In light of its broad spectrum of efficacy, both in animal models of different forms of epilepsy and clinically, this MOA alone may not fully explain its clinical profile. In vitro studies have demonstrated an antagonistic effect of RUF on the metabotropic (= G protein-coupled) glutamate receptor 5 (mGluR5), but only at comparably high concentrations. RUF exerts neuroprotective effects against kainic acid (KA)-induced neuronal damage in the hippocampus of rats and mice. It has not been elucidated whether this is mediated

through KA receptor antagonism or other MOAs (Striano et al. 2018). Treatment with RUF produced improved cognitive function and increased neurogenesis in the hippocampus of aged gerbils through upregulation of insulin-like growth factor 1 (IGF-1), its receptor (IGF-1R), and phosphorylated cAMP-response element-binding protein (p-CREB). These actions may also contribute to RUF's neuroprotective effects.

Clinical Use

RUF has received orphan drug status for the adjunctive treatment of seizures associated with Lennox-Gastaut syndrome (LGS), a severe form of epilepsy that comprises different kinds of seizures. It appears to possess particular efficacy against atonic seizures (drop attacks) that often occur with LGS.

Stiripentol

Mechanism of Action

Stiripentol (STP) is chemically unrelated to other AEDs. It is an allosteric modulator of both synaptic and extrasynaptic subtypes of the $GABA_A$ receptor, increasing both the opening frequency and opening time of the chloride channel. Its binding site at the receptor is distinct from that of benzodiazepines and neurosteroids. Some data suggest that it may act at the barbiturate binding site. In addition, STP inhibits neuronal lactate dehydrogenase (LDH). This leads via intermediate steps to reduced levels of ATP which in turn triggers ATP-sensitive potassium channels in the cell membrane to open. The resulting potassium efflux leads to membrane hyperpolarization and reduced excitability (Nickels and Wirrell 2017). STP is the only AED known to act through this MOA.

Clinical Use

STP is mainly used for the treatment of Dravet syndrome, a severe form of epilepsy in children. Preliminary clinical experience also suggests efficacy in the treatment of benzodiazepine-refractory status epilepticus, most likely due to its action on extrasynaptic $GABA_A$ receptors.

Sulthiame

Mechanism of Action

Sulthiame (STM) contains a sulfonamide moiety. In parallel with other AEDs that share this characteristic, STM acts as an inhibitor of carbonic anhydrase (CA; for detailed description of this MOA, see acetazolamide). It is also a moderate blocker of voltage-dependent sodium channels, thereby reducing the generation of repetitive action potentials (Madeja et al. 2001). There are also data suggesting that STM increases regional GABA concentrations in the brain, while decreasing glutamate levels. It is not clear how exactly STM exerts these effects; they may be consequences of CA inhibition.

Clinical Use

STM has traditionally been used to treat focal and secondarily generalized tonic-clonic seizures, but it has reportedly also some efficacy in myoclonic seizures and infantile spasms. It is an AED of first choice in benign focal epilepsy with centrotemporal spikes (BECTS).

Tiagabine

Mechanism of Action

Tiagabine (TGB) has a unique MOA as it is the only AED known to act by binding to the GABA transporter GAT-1. This interaction reduces the uptake of extracellular GABA which results in increased GABA concentrations in the synaptic cleft and prolonged GABA-mediated inhibitory postsynaptic potentials (Greenfield 2013).

Clinical Use

TGB is effective as add-on treatment of focal onset seizures. It is also used off-label in anxiety and panic disorder. Its use is limited because of unfavorable pharmacokinetics and its alleged ability to aggravate certain seizure types including

status epilepticus. TGB has also been associated with new onset seizures and status epilepticus in patients without epilepsy.

Topiramate

Mechanism of Action

Topiramate (TPM) is a sulfonamide with a heterotricyclic ring substituent containing fructose. It has several MOAs. Being a sulfonamide, it inhibits carbonic anhydrase (for details, see acetazolamide). It also blocks voltage-operated sodium channels, leading to reduced repetitive firing. TPM inhibits excitatory postsynaptic potentials evoked by glutamate through an interaction with both NMDA and kainate/AMPA receptors. It enhances chloride currents through allosteric modulation of $GABA_A$ receptors via a binding site that is distinct from benzodiazepines. In addition, it blocks high-voltage-activated calcium channels (White et al. 2007). In animal experiments, TPM has shown neuroprotective effects by reducing oxidative stress, inflammation, and apoptosis. It is not clear whether this is mediated by one or more of the mentioned MOAs or by other MOAs as TPM presumably also modulates α_2-adrenergic, dopaminergic, and other signaling pathways like CREB and BDNF (Motaghinejad et al. 2017).

Clinical Use

TPM is effective against all seizure types except absences. Owing to its safety profile, mainly its negative cognitive effects, it is a drug of second choice. It is also used to treat essential tremor and alcohol dependence and to prevent migraine. Since weight loss is among its adverse effects, it is sometimes used as a weight loss agent.

Valproic Acid (Valproate)

Mechanism of Action

Valproic acid (VPA) is a short chain, branched fatty acid (chemically: dipropyl acetic acid). It is commonly named after its anion, valproate. VPA's antiepileptic properties were discovered by serendipity when it was used as a solvent to test new antiepileptic drug candidates. VPA is traditionally described as a GABAergic drug because it increases brain levels of GABA. The exact molecular mechanism underlying this effect is unknown. Inhibition of GABA degrading enzymes such as GABA transaminase and succinate-semialdehyde dehydrogenase as well as inhibition of the reuptake of GABA from the synaptic cleft has been suggested. Further MOAs of VPA are the blockade of voltage-gated sodium channels and T-type calcium channels as well as enhancement of potassium currents through M-channels. VPA also reduces excitatory neurotransmission by glutamate (via NMDA receptors) and aspartate (Kay et al. 2015). Besides, VPA is an inhibitor of histone deacetylase (HDAC). HDAC plays a major role in the epigenetic regulation of gene expression. In a genetic rat model of absence epilepsy, chronic treatment with VPA significantly reduced the later development of absence epilepsy and increased brain histone acetylation, paralleled by reduced HDAC1 and HDAC3 expression (Citraro et al. 2020). This suggests that HDAC inhibition by VPA may contribute to its antiepileptic efficacy and, more importantly, may prevent the development of epilepsy itself.

Clinical Use

VPA is a first-line, potent AED that is effective against all types of epileptic seizures and status epilepticus. It is also used as a mood stabilizer in bipolar disorder including acute manic episodes and in the prophylactic treatment of migraine. The clinical significance of VPA's HDAC-inhibiting properties is currently under investigation, mainly in oncology and neurodegenerative disorders.

Vigabatrin

Mechanism of Action

Chemically, vigabatrin (VGB) is a GABA molecule with a vinyl substituent at the γ-carbon

atom; hence, it is also named γ-vinyl-GABA (Fig. 4). Its similarity to GABA may explain its MOA which is inhibition of the GABA-degrading enzyme, GABA-transaminase (GABA-T). This inhibition is irreversible, and while the plasma half-life of VGB is only 5–7 h, elevated GABA concentrations in human cerebrospinal fluid have been measured for 1 week and longer after the last VGB dose. This duration is defined by the re-synthesis rate of GABA-T. Hence, VGB is an example of a so-called hit-and-run drug. There is also sparse evidence that VGB may modulate the conversion of α-ketoglutarate to glutamate *and* vice versa by inhibiting glutamate dehydrogenase (Ben-Menachem 2011). The relevance of this for the clinical effects of VGB is unclear. There is at present no evidence for other MOAs of VGB although it induces irreversible visual field defects by damaging the retina in up to 30–50% of patients. Several explanations for this adverse effect including GABA toxicity and taurine depletion have been suggested, but the exact mechanism remains to be determined.

Clinical Use

VGB is effective in the treatment of adults with partial seizures with impaired consciousness and in children with infantile spasms. Because of its safety profile, it is not a first-choice AED.

Zonisamide

Mechanism of Action

Like other AEDs that contain a sulfonamide moiety, zonisamide (ZNS) is an inhibitor of carbonic anhydrase (for details of this MOA, see acetazolamide). In addition, it acts as an inhibitor of voltage-dependent sodium and T-type calcium ion channels and exerts GABAergic effects through increased GABA release and inhibition of presynaptic GABA reuptake via GAT-1. ZNS is also an inhibitor of monoamine oxidase B (MAO-B). These multiple MOAs may explain its broad-spectrum clinical efficacy (Reimers and Ljung 2019).

Clinical Use

ZNS has shown efficacy against all types of epileptic seizures including absences. It is used alone and in combination with other AEDs. Because of its adverse effect profile, particularly cognitive impairment, it is used as a second-line drug. It is also licensed for the treatment of Parkinson's disease.

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Antigout Drugs

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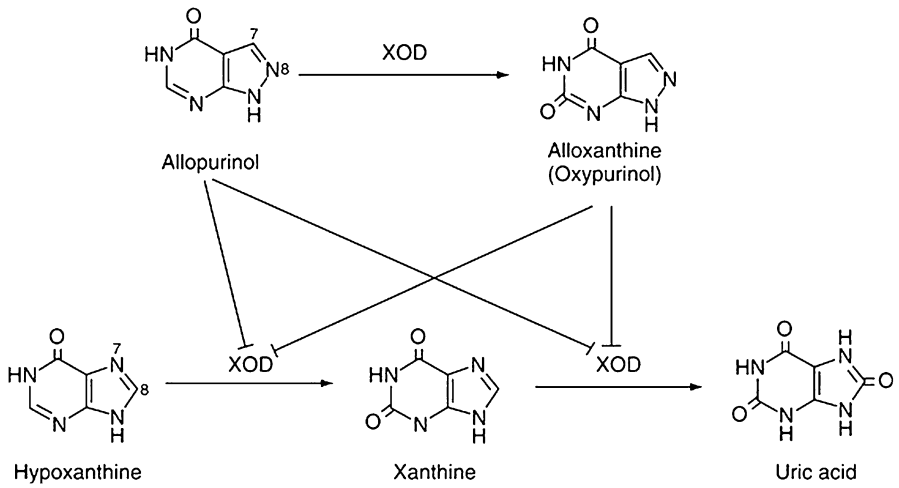
Synonyms

Drugs for the treatment/management of gout and/or hyperuricemia

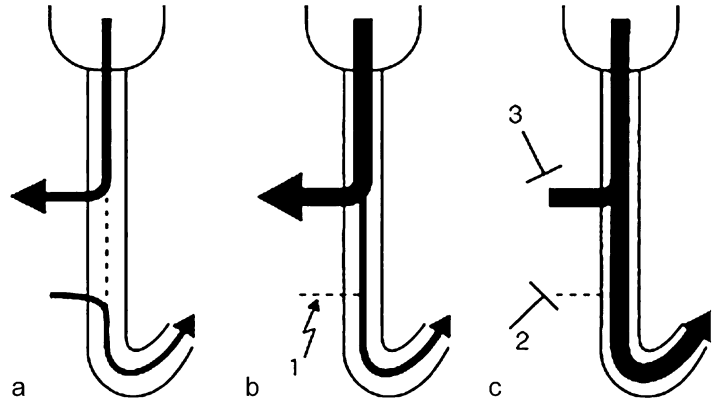
Definition

Pathophysiology and Clinical Manifestations of Gout

Uric acid is the end product of purine catabolism in man. Purines originate from food and the degradation of nucleic acids and nucleotides. Xanthine oxidase (XOD) is the key enzyme in purine degradation. XOD converts hypoxanthine to xanthine and xanthine to uric acid, respectively (Fig. 1). Uric acid is filtered in the glomerulus of the kidney, is almost completely absorbed in the proximal tubules, and secreted more distally (Fig. 2). At physiological pH (<7.4), uric acid exists predominantly in its ionic form (urate). At lower pH, the fraction of uric acid molecules (protonized form) increases. This is important because uric acid possesses a lower solubility than urate. Thus, a decrease in pH, as it occurs in inflammed tissue and in the tubules, facilitates the formation of uric acid crystals, which are the initial cause of gout. Of importance for therapeutic intervention is the fact that xanthine and hypoxanthine are more soluble than uric acid. Specifically, by preventing uric acid formation through XOD inhibition, the excretion of xanthine and hypoxanthine increases, and the risk of uric acid crystal formation decreases. An increase of the serum uric acid concentration above 416 µmol/L is referred to as hyperuricemia and is associated with an increased risk of uric acid crystal formation and acute attacks of gouty



Antigout Drugs, Fig. 1 Xanthine oxidase-catalyzed reactions. Xanthine oxidase converts hypoxanthine to xanthine and xanthine to uric acid, respectively. Hypoxanthine and xanthine are more soluble than uric acid. Xanthine oxidase also converts the uricostatic drug allopurinol to alloxanthine. Allopurinol and hypoxanthine are isomers that differ from each other in the substitution of positions 7 and 8 of the purine ring system. Although allopurinol is converted to alloxanthine by xanthine oxidase, allopurinol is also a xanthine oxidase inhibitor. Specifically, at low concentrations, allopurinol acts as a competitive inhibitor, and at high concentrations it acts as a noncompetitive inhibitor. Alloxanthine is a noncompetitive xanthine oxidase inhibitor. XOD: xanthine oxidase



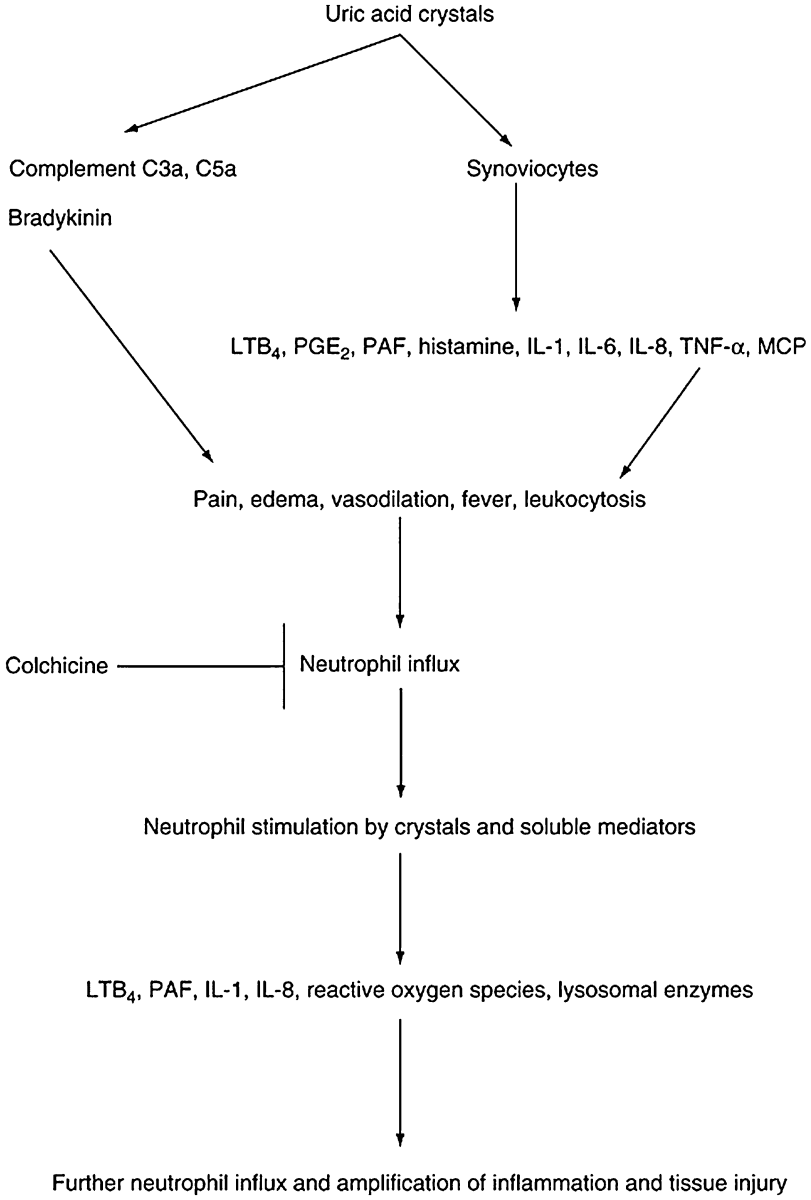
Antigout Drugs, Fig. 2 Reabsorption and secretion of uric acid in the proximal renal tubulus. (**a**) Normal situation. Uric acid is completely reabsorbed in the proximal segment of the renal tubulus and secreted more distally. (**b**) Situation in untreated hyperuricemia. In most genetically caused cases of gout, uric acid secretion is defective (*1*). (**c**) Situation in hyperuricemia under treatment with uricosouric drugs (*2*); inhibition of uric acid secretion by uricosuric drugs at low doses (*3*). Inhibition of uric acid secretion and reabsorption by uricosuric drugs in therapeutic doses. The inhibition of uric acid secretion with low doses of uricosuric drugs can further increase blood levels of uric acid and induce attacks of acute gouty arthritis

arthritis. With a serum uric acid level of 535 μmol/L, the annual incidence of gouty arthritis is 4.9–5.7%.

Hyperuricemia can have genetic causes or acquired causes. A defect of hypoxanthine-guanine phosphoribosyl transferase is the cause of Lesch–Nyhan syndrome, resulting in increased uric acid production. Among the genetically caused defects, impaired renal uric acid secretion is a very common cause of gout. Myeloproliferative diseases, a purine-rich diet (e.g., meat, beer, beans, peas, oatmeal, or spinach), obesity, and alcoholism are common causes of acquired hyperuricemia and result from increased uric acid production. Renal diseases and the application of certain drugs such as the tuberculostatic drug pyrazinamide, thiazide diuretics, loop diuretics, or acetylsalicylic acid at doses of up to 1–2 g/day and the immunosuppressant cyclosporin A are acquired causes of impaired uric acid secretion.

Gout is the consequence of hyperuricemia and is caused by uric acid deposits in joints, tendons, bursae, kidney, and urinary tract. In the USA, the prevalence of gout is $<1\%$ for all ages and both sexes. The prevalence of gout is higher in men than in women and exceeds 5% in men ≥ 65 years. These epidemiologic data are important for drug therapy since older patients are more sensitive to side effects of antigout drugs than younger patients. In the initial stage, gout is characterized by asymptomatic hyperuricemia. In the second stage, the disease manifests itself by acute gouty arthritis. The third (intercritical) stage is asymptomatic, and the fourth stage is characterized by progressive uric acid deposits in joints, tendons, bursae, kidney, and urinary tract (tophus formation). Uric acid deposits result in the deformation and loss of function of joints and recurrent episodes of urate lithiasis. Uric acid deposits in the kidney and urate lithiasis can ultimately result in renal failure.

Figure 3 illustrates important pathophysiologic events leading to acute gouty arthritis. Once the concentration of uric acid exceeds its solubility, uric acid crystals form in the synovial fluid of joints. Subsequently, the uric acid crystals are phagocytosed by synoviocytes that form the inner cell layer of joints. Next, synoviocytes release numerous mediators of inflammation including leukotriene B_4 (LTB_4), prostaglandin E_2 (PGE_2), platelet-activating factor (PAF), histamine, interleukins (ILs) 1, 6, and 8, and tumor necrosis factor-α that in conjunction with products of the complement cascade (C5a and C3a) and kinins (bradykinin) induce an

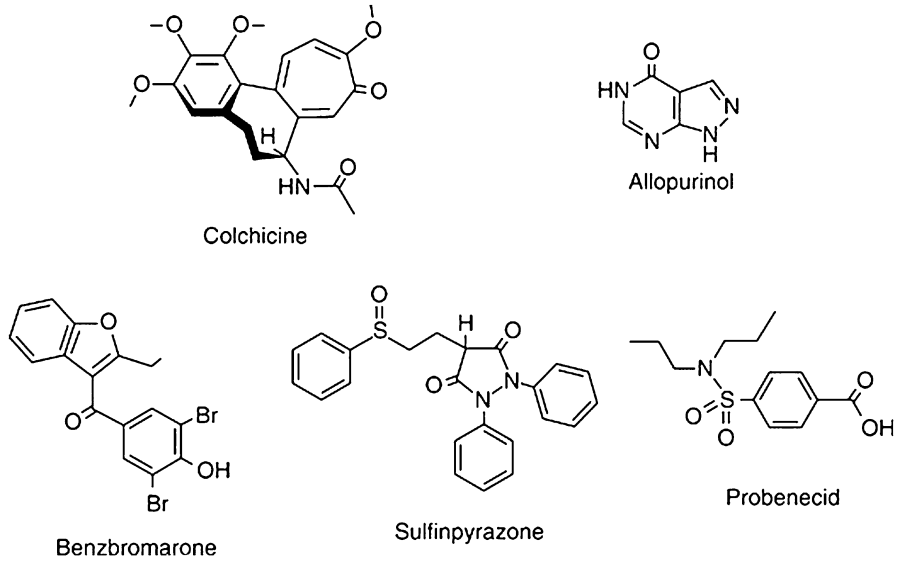


Antigout Drugs, Fig. 3 Important pathophysiologic events in acute gouty arthritis. Uric acid crystals activate the complement cascade, the formation of kinins, and the release of various mediators of inflammation from synoviocytes that phagocytose uric acid crystals. The combined action of the released mediators induces a strong inflammatory reaction that is further enhanced by neutrophils. Neutrophils migrate along a concentration gradient to loci in which C5a, LTB $_4$, PAF, and IL-8 are produced (chemotaxis). Accordingly, C5a, LTB $_4$, PAF, and IL-8 are also referred to as chemoattractants. Neutrophils phagocytose uric acid crystals. Upon exposure to uric acid crystals and chemoattractants, neutrophils release various mediators of inflammation, reactive oxygen species, and lysosomal enzymes. The concerted effects of all these compounds amplify the inflammatory reaction even further. Colchicine interrupts the vicious cycle of inflammation predominantly by inhibiting neutrophil chemotaxis. IL-1, interleukin 1; IL-6, interleukin 6; IL-8, interleukin 8; LTB $_4$, leukotriene B $_4$; MCP, monocyte chemoattractant protein; PAF, platelet-activating factor; PGE $_2$, prostaglandin E $_2$; TNF-α, tumor necrosis factor-α

inflammatory response. Moreover, LTB $_4$, PAF, C5a, and IL-8 attract polymorphonuclear leukocytes (neutrohpils). Neutrophils migrate into affected joints along a concentration gradient of these inflammatory mediators (chemotaxis). Accordingly, LTB $_4$, PAF, C5a, and IL-8 are also referred to as chemoattractants. Once present in joints, neutrophils phagocytose uric acid crystals. Uric acid crystals and chemoattractants trigger the release of cytotoxic lysosomal enzymes, NADPH oxidase-catalyzed formation of reactive oxygen species, LTB $_4$ formation, and the release of other proinflammatory molecules from neutrophils. The latter molecules attract additional neutrophils and mononuclear phagocytes. Moreover, neutrophils generate lactate that decreases the pH within the joint and further accelerates uric acid crystal formation. Oxygen radicals and lysosomal enzymes cause damage to tissues. Thus, the presence of uric acid crystals in joints triggers a vicious cycle, resulting in an extremely painful inflammation. A typical localization of acute gouty arthritis is the first metatarsal joint of the foot (podagra). The diagnosis of acute gouty arthritis is confirmed by the detection of urate crystals in the joint or tophus.

Antigout Drugs

Figure 4 shows the structures of commonly employed antigout drugs. The treatment of acute gouty arthritis aims at rapidly reducing the pain and inflammatory reaction Emmerson 1996; Frampton 2015; Khanna et al. 2014; Lange et al. 2001; Schlesinger and Schuhmacher 2001). This aim can be achieved by treatment with colchicine. In addition, nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, and adrenocorticotropic hormone (ACTH) can be used to treat acute gouty arthritis. However, since NSAIDs and glucocorticoids are used in numerous other commonly occurring inflammatory conditions, they are not per se considered specific antigout drugs. Glucocorticoids can be given systemically (orally, intramuscularly, or intravenously) or locally into afflicted joints. The long-term goals of gout treatment are the prevention of acute gouty arthritis, the prevention of urate lithiasis and renal failure, and the resorption of existing uric acid deposits in



Antigout Drugs, Fig. 4 Structures of commonly used antigout drugs. Colchicine is an alkaloid from the autumn crocus *Colchicum autumnale* and inhibits tubulin polymerization. Allopurinol is an isomer of xanthine and inhibits uric acid formation (uricostatic drug). Benzbromarone, sufinpyrazone, and probenecid are uricosuric drugs and inhibit uric acid reabsorption in the proximal tubulus of the kidney

the joints and urinary tract. The long-term therapy aims at reducing the serum concentration of uric acid below 357 μmol/L. Therapy with the uricostatic drug allopurinol and the uricosuric drugs benzbromarone, sulfinpyrazone, or probenecid can accomplish the long-term goals. These drugs are well tolerated in most patients. Uricostatic and uricosuric drugs can be combined. Additionally, low doses of colchicine can be used to prevent the occurrence of acute gouty arthritis. However, as is unfortunately often the case with classic diseases, there are only few well-conducted clinical studies assessing the clinical efficacy and safety of antigout drugs.

Mechanism of Action

Colchicine

Colchicine is an alkaloid from the autumn crocus *Colchicum autumnale.* Colchicine binds to the cytoskeletal protein tubulin and, thereby, prevents microtubule formation. As a result, colchicine inhibits neutrophil chemotaxis and the influx of these cells into areas containing uric acid crystals (Fig. 3). Colchicine also inhibits neutrophil phagocytosis. As a result, colchicine interrupts the vicious cycle of inflammation in gouty arthritis. However, because of its mechanism of action, colchicine is most effective only when given in the early stages of gouty arthritis, that is, within 24 h. Otherwise, the inflammatory reaction may be too advanced. Specifically, colchicine is effective in >90% of patients when given within the first few hours after the start of the attack, but after 24 h, the responsiveness decreases to 75%. Given the very significant side effects of colchicine, it is absolutely crucial to initiate colchicine therapy as early as possible.

Allopurinol

Allopurinol is an analog of hypoxanthine and is converted to alloxanthine by XOD. Both allopurinol and hypoxanthine inhibit XOD (Fig. 1). Alloxanthine is a noncompetitive inhibitor of XOD as is allopurinol at high concentrations. At low concentrations, allopurinol is a competitive inhibitor of XOD. As a result of XOD inhibition, the formation of the poorly soluble uric acid is reduced, whereas the formation of the more soluble metabolites hypoxanthine and xanthine is increased. Because of the good solubility of hypoxanthine and xanthine, formation of hypoxanthine/xanthine crystals is a rare complication of allopurinol treatment. Another consequence of XOD inhibition is the accumulation of the precursor of xanthine, inosine. Inosine inhibits the key enzyme of de novo purine synthesis, phosphoribosyl-pyrophosphate amidotransferase. The allopurinol metabolite allopurinol ribonucleotide also inhibits phosphoribosyl-pyrophosphate amidotransferase. Inhibition of purine biosynthesis contributes to the antihyperuricemic effects of allopurinol.

Uricosuric Drugs

Depending on the dose applied, uricosuric drugs inhibit tubular reabsorption and tubular secretion of uric acid in the kidney differentially (Fig. 2). At low (subtherapeutic) doses, uricosuric drugs inhibit uric acid secretion without inhibiting reabsorption. Therefore, low doses of uricosuric drugs can actually increase serum levels of uric acid and trigger acute attacks of gouty arthritis. At higher, that is, therapeutic doses, uricosuric drugs inhibit both tubular secretion and tubular reabsorption. Since inhibition of tubular reabsorption is quantitatively more important than inhibition of tubular secretion, the net effect is an increased renal elimination of uric acid. In order to avoid formation of uric acid crystals in the kidney and urinary tract, it is important that the pH of the urine is kept >6.0. This goal can be achieved by the oral administration of potassium sodium hydrogen citrate, sodium bicarbonate, or acetazolamide. In addition, it is mandatory that the patient drinks at least 3 L per day to avoid formation of uric acid crystals.

New Drugs

Febuxostat is a highly potent mixed-type inhibitor of XOD with Ki values in the 0.5–3 nM-range. Febuxostat displays high selectivity relative to a large number of other purine- and pyrimidine-metabolizing enzymes.

Canakinumab is a humanized monoclonal antibody neutralizing IL-1. By this mechanism, canakinumab exerts an anti-inflammatory effect.

Clinical Use (Including Side Effects)

Colchicine

Daily doses of 3–8 mg (6–8 times 0.5–1.0 mg) are used for the treatment of acute gouty arthritis. For prophylaxis, daily doses of 0.5–1.5 mg are used, but the use of colchicine for prophylaxis is controversial. The side effects of colchicine are very significant. About 80% of the patients experience gastrointestinal problems including nausea, vomiting, and diarrhea. The antimitotic effects of colchicine can result in thrombocytopenia, agranulocytosis, hair loss, and azoospermia. In the central nervous system, confusion, ascending paralysis, respiratory failure, and seizures have been reported. These side effects can be explained by the fact that intact microtubules are essential for proper transport functions in neuronal axons. Moreover, colchicine can cause myopathy. Because of the significant side effects, many physicians prefer to treat acute gouty arthritis with NSAIDs or glucocorticoids. Although colchicine is a classic antigout drug, colchicine can also be used to treat other inflammatory diseases including amyloidosis, Dupuytren's contracture, Behcet's syndrome, vasculitis, various forms of hepatic cirrhosis, pulmonary fibrosis, pericarditis, and various inflammatory diseases of the skin. Colchicine is extensively metabolized through the hepatic cytochrome CYP 3A4. Accordingly, inhibitors of CYP 3A4 such as diltiazem, gestodene, grapefuit juice, ketoconazole, and macrolide antibiotics prolong and enhance the pharmacological (and toxic) effects of colchicine. Drugs that are inactivated via CYP 3A4 such as steroid hormones, lidocaine, midazolam, quinidine, terfenadine, nifedipine, and verapamil can also prolong colchicine action. Because of its antimitotic effects, colchicine should not be used in pregnant women.

Allopurinol

The daily dose of allopurinol is 300–600 mg. In combination with benzbromarone, the daily allopurinol dose is reduced to 100 mg. In general, allopurinol is well tolerated. The incidence of side effects is 2–3%. Exanthems, pruritus, gastrointestinal problems, and dry mouth have been observed. In rare cases, hair loss, fever, leukopenia, toxic epidermolysis (Lyell syndrome), and hepatic dysfunction have been reported. Allopurinol inhibits the metabolic inactivation of the cytostatic drugs azathioprine and 6-mercaptopurine. Accordingly, the administered doses of azathioprine and 6-mercaptopurine must be reduced if allopurinol is given simultaneously.

Uricosuric Drugs

Benzbromarone The daily dose of benzbromarone is 50–200 mg. In combination with allopurinol, the benzbromarone dose is reduced to 20 mg. Benzbromarone is well tolerated. Rare side effects are headaches, gastrointestinal problems, and exanthems.

Probenecid The daily dose of probenecid is 0.5–3.0 g. Probenecid is well tolerated, and there are few serious side effects. In less than 10% of the treated patients, gastrointestinal disturbances, hypersensitivity, and skin reactions occur.

Sulfinpyrazone The daily dose of sulfinpyrazone is 200–400 mg. The side effects of sulfinpyrazone are comparable with those of probenecid. A potential therapeutic advantage of sulfinpyrazone in patients with coronary heart disease and thromboembolic diseases is its inhibitory effect on platelet aggregation.

Inflammation

New Drugs

Febuxostat The daily dose of febuxostat is 80–120 mg. The drug is used in chronic gout in cases in which allopurinol is ineffective or not tolerated. Febuxostat causes vomiting, diarrhea, headache, and allergic reactions. There is an ongoing discussion whether the use of febuxostat is associated with an increased cardiovascular risk.

Canakinumab Canakinumab is a "biological" and has to be injected subcutaneously. The drug is used in acute gout in cases in which NSAIDs, colchicine, or glucocorticoids are ineffective or not tolerated. Canakinumab can cause allergic reactions at the injection site and infections of the respiratory or urinary tract.

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Antihyperglycaemics

► Glucose-Lowering Drugs Other than Insulin

Antihypertensive Drugs

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Synonyms

Antihypertensives; Blood pressure lowering drugs

Definition

Reducing blood pressure by pharmacological means reduces cardiovascular morbidity and mortality rates. Benefits include protection from stroke, coronary events, heart failure, progression of renal disease, progression to more severe hypertension, and, most importantly, mortality from all causes. Owing to the complexity of the pathogenesis of hypertension, antihypertensive drugs are directed against a variety of pharmacological targets in various cell types in different organs involved in blood pressure control.

Mechanism of Action

The fundamental mechanisms involved in blood pressure control have been outlined in chapter "► Blood Pressure Control." In addition to direct neuronal modulation of arterial pressure, two neurohumoral systems, i.e., the sympathetic nervous system and the renin–angiotensin–aldosterone system (RAS), play a pivotal role in blood pressure control. Both systems are always either directly or indirectly affected by treatment with any antihypertensive drug. The most important antihypertensive agents for clinical use can be categorized into eight different drug classes (Table 1, Fig. 1).

Angiotensin-Converting Enzyme (ACE) Inhibitors

The core of the RAS pathway involves the conversion of angiotensinogen to angiotensin I (Ang I) by renin followed by its conversion to angiotensin II (Ang II) by ►angiotensin-converting enzyme (ACE). This class of drugs inhibits competitively the activity of ACE to decrease the availability of Ang II at both angiotensin type 1 (AT1) and angiotensin type 2 (AT2) receptors in the blood and tissues including the vasculature, kidney, heart, and brain (Paul et al. 2006; Arendse et al. 2019). Ang II, the main effector of the RAS, is a potent vasoconstrictor and promotes aldosterone synthesis and secretion, thus increasing sodium and water reabsorption and increasing blood pressure. Blocking the production of Ang II results in vasodilation of small resistance arteries, reduction in total peripheral resistance, and blood pressure lowering while preserving cardiac output. ACE inhibitors blunt the expected increase in sympathetic nervous activity typically seen after vasodilation, hence preventing the increase in heart rate despite blood pressure lowering (Benowitz 2015). Their use is not associated with postural hypotension, presumably due to a reset of baroreceptor function.

Nevertheless, ACE inhibitors could only partially block the RAS due to production of Ang II by alternative non-ACE enzymatic pathways, such as chymase which can attenuate the blood pressure lowering effect of ACE inhibitors during chronic therapy (Arendse et al. 2019). In this respect, other

Antihypertensive Drugs, Table 1 Common side effects, contraindications, and clinical application of antihypertensive drugs

Class of drug		Side effects	Selected contraindications	Clinical use
Primary agents				
ACE inhibitors		Cough, hyperkalemia, angioedema	Pregnancy, hyperkalemia, bilateral renal artery stenosis, history of ACE inhibitor-associated angioedema	First line
ARBs		Hyperkalemia	Pregnancy, hyperkalemia, bilateral renal artery stenosis	First line
Beta-blockers		Fatigue, hyperglycemia, bronchospasm, weight gain bradycardia, sinoatrial or atrioventricular block, dyslipidemia, vivid dreams	Asthma, bradycardia, high-grade sinoatrial or atrioventricular block	First line[a]
CCBs	DHP	Edema, headache, flushing, tachycardia, gingival hypertrophy	Tachyarrhythmia, heart failure (HFrEF, class III or IV), preexisting severe leg edema	First line
	Non-DHP	Bradycardia, sinoatrial or atrioventricular block, gingival hypertrophy	High-grade sinoatrial or atrioventricular block, bradycardia, severe left ventricular dysfunction (LV ejection fraction <40%), constipation	First line[b]
Diuretics	Thiazide and thiazide-like	Hyperuricemia, hyperglycemia, hyperlipidemia, hypokalemia, dyslipidemia, metabolic alkalosis	Gout, metabolic syndrome glucose intolerance, hypercalcemia, hypokalemia, pregnancy	First line
Secondary agents				
Diuretics	Loop diuretics	Ototoxicity, hypovolemia, hyperuricemia, hypomagnesemia	Gout, metabolic syndrome glucose intolerance, pregnancy	If GFR < 30 ml/min/1.73 m^2
Potassium-sparing diuretics	MRA	Hyperkalemia, gynecomastia (spironolactone > eplerenone)	Severe renal impairment (GFR < 30 mL/min/1.73 m^2), hyperkalemia	Resistant hypertension[c]
	ENaC inhibitors	Hyperkalemia, metabolic acidosis	Severe renal impairment (GFR < 30 mL/min/1.73 m^2), hyperkalemia	Hypokalemia, resistant hypertension (amiloride)
Alpha-blockers		Orthostatic hypotension, rapid drop of blood pressure after first dose, dizziness, fluid retention, incontinence	History of orthostatic hypotension	Resistant hypertension, men with prostatic disease
Centrally acting sympatholytics	α_2-receptor agonists	Somnolence, dry mouth, rebound hypertension	Severe depression	Resistant hypertension, last line
	Imidazoline-receptor agonists			

(continued)

Antihypertensive Drugs, Table 1 (continued)

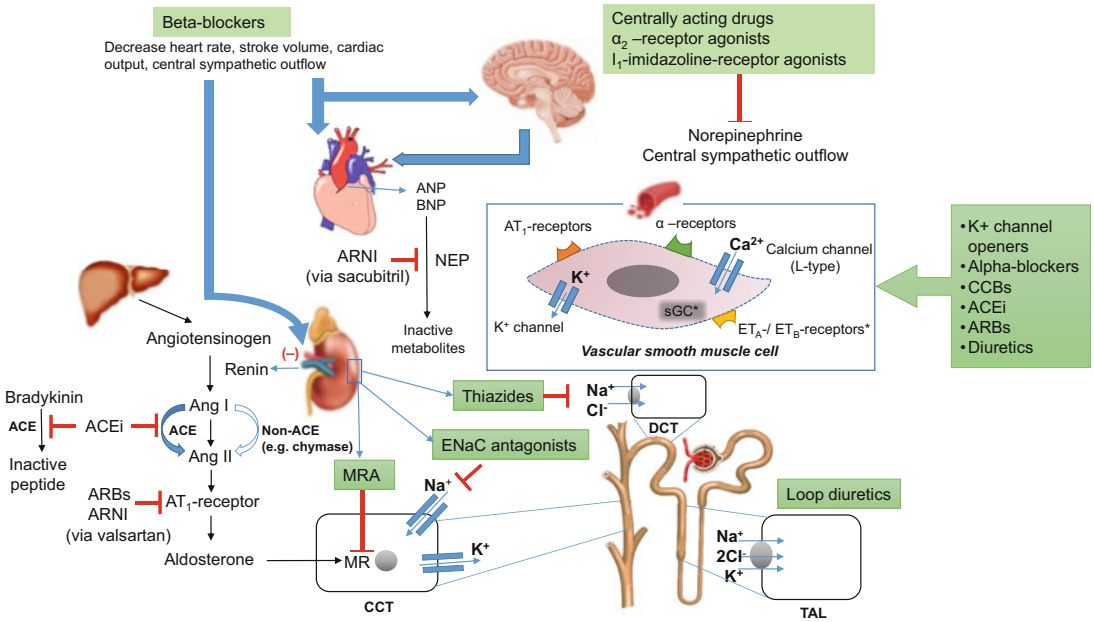
Class of drug		Side effects	Selected contraindications	Clinical use
Vasodilators	Hydralazine	Fluid retention, lupus-like reactions	Severe tachycardia, heart failure with a high cardiac output	Only in rare cases, last line
	Minoxidil	Hirsutism, ECG changes, pericardial effusion	Pheochromocytoma	Only in rare cases, last line

ACE angiotensin-converting enzyme, *ARBs* angiotensin receptor blockers, *CCBs* calcium channel blockers, *ENaC* epithelial sodium channels, *GFR*, glomerular filtration rate, *HFrEF* reduced ejection fraction heart failure, *LV* left ventricular, *MRA* mineralocorticoid receptor antagonists

[a]Preferred in certain conditions (Fig. 2)

[b]Not in combination with beta-blockers

[c]Spironolactone as preferred drug in resistant hypertension (Fig. 2)



Antihypertensive Drugs, Fig. 1 Site of action of different classes of antihypertensive drugs. Antihypertensive drugs are directed against a variety of pharmacological targets in various cell types in different organs involved in blood pressure control. The most important targets in the brain, heart, vasculature (vascular smooth muscle cells), and the kidney (nephron) are shown. Non-ACE-dependent conversion of angiotensin I (Ang I) to angiotensin II (Ang II) may occur independent from ACE due to the activity of other enzymes in different tissues such as chymase in the heart; (−) indicates inhibition. *ACEi* ACE inhibitors, *ANP* atrial natriuretic peptide, *ARB* angiotensin receptor blocker, *ARNI* angiotensin receptor–neprilysin inhibitor, *BNP* B-type natriuretic peptide, *CCBs*, calcium channel blockers, *CCT* cortical collecting duct, *DCT* distal convoluted tubule, *ENaC* epithelial sodium channel, *MR* mineralocorticoid receptor, *MRA* mineralocorticoid receptor antagonist, *NEP* neprilysin, *TAL* thick ascending limb of the loop of Henle, *sGC* soluble guanylate cyclase. *Indicates targets for novel antihypertensive drug development

hemodynamic mechanisms may be important. The accumulation of kinins, including bradykinin, secondary to inhibition of ACE (known also as kininase II) that promotes vasodilation has been suggested to contribute to the overall antihypertensive effect of ACE inhibitors (Arendse et al. 2019). In addition, ACE is responsible for the degradation of angiotensin (1–7) which is an important part of

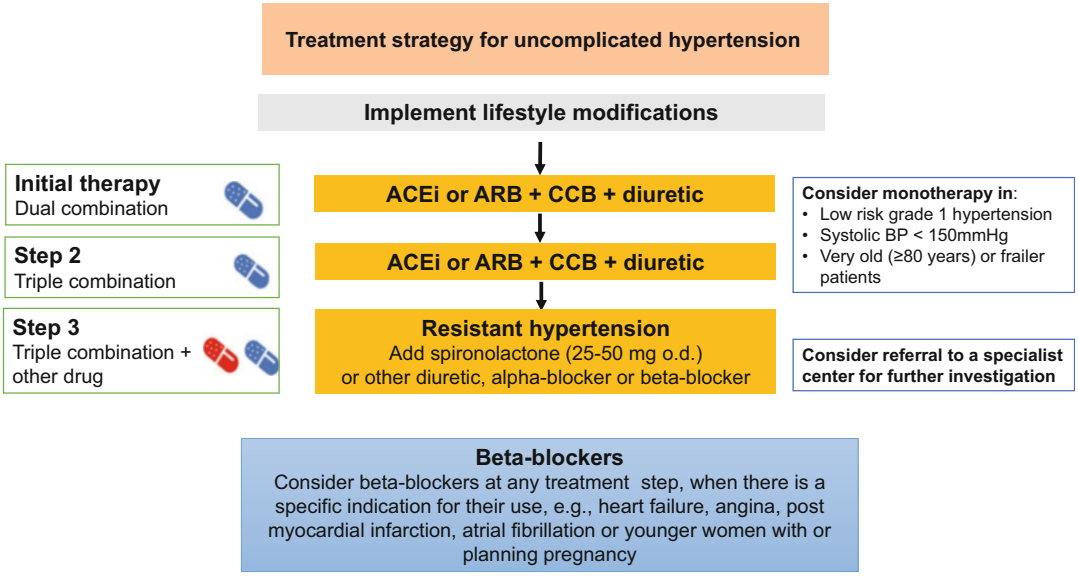
the counter regulatory pathway within the RAS. As a result, ACE inhibitors may increase the plasma concentration of the vasodilator peptide angiotensin (1–7) (Arendse et al. 2019). Independent of its hemodynamic effects, Ang II mediates a variety of adverse processes related to cell growth, inflammation, and vascular remodeling which contribute to hypertensive target organ damage. Consequently, the inhibition of tissue ACE (Paul et al. 2006) plays an important role for the prevention and regression of hypertension-mediated organ damage (Arendse et al. 2019).

Angiotensin Receptor Blockers (ARBs)

In contrast to ACE inhibitors, ARBs attenuate the effects of Ang II on vascular tone by directly blocking AT1 receptors, thereby promoting reduction in the total peripheral resistance with no effect on either the cardiac output or heart rate. At the same time, they exert the same favorable effects on cell proliferation and inflammation protecting against target organ damage. Their direct interaction at the receptor level overcomes some of the shortcomings of ACE inhibitors, namely, the reactive increase in renin and Ang I levels and the production of Ang II by non-ACE pathways (Arendse et al. 2019). The absence of accumulation of kinins that may be responsible for some of the beneficial effects of ACE inhibitors but also their side effects, especially cough as well as angioedema, is another difference. Due to their high selectivity, ARBs do not interact with AT2 receptors whose functions tend to oppose actions of the AT1 receptor and exhibit vasodilatory and antiproliferative effects upon stimulation by Ang II (Arendse et al. 2019; Paul et al. 2006). During chronic therapy with RAS blockers, the feedback inhibition of renin release is suppressed leading to reactivation of the RAS cascade and Ang II production. The latter may interact with unblocked AT2 receptors enhancing vasodilation and further blood pressure reduction. The combination of vasopeptidase inhibitors with ARBs is a novel approach to promote natriuresis in addition to vasodilation by simultaneous inhibition of neutral endopeptidase (NEP) and the RAS. This results in more pronounced blood pressure lowering than that exerted by either approach alone. NEP inhibitors increase the availability of vasodilatory peptides such as atrial (ANP), B-type (BNP), and C-type (CNP) natriuretic peptides (Volpe 2014). A novel antihypertensive agent LCZ696 is the first angiotensin receptor–neprilysin inhibitor (ARNI), consisting of the neprilysin inhibitor sacubitril in combination with valsartan, and has been shown effective in treatment of hypertension (Ruilope et al. 2010) although approval and market access for this compound was achieved for the treatment of heart failure (McMurray et al. 2014).

Beta-Blockers

Beta-blockers constitute a heterogeneous group of antihypertensive agents that competitively antagonize the effects of catecholamines at adrenergic beta-adrenoceptors. Beta-blockers differ in their receptor selectivity and the presence of additional intrinsic sympathomimetic activity (ISA) whereby an agent partially stimulates the receptor. Beta-blockers with ISA are generally not recommended anymore since partial agonism failed to prove significant clinical benefits (Benowitz 2015). The mechanisms by which beta-blockers lower blood pressure are still not fully elucidated, likely involving several modes of action. A short-term mechanism is the reduction of cardiac output through their negative chronotropic (heart rate lowering) and inotropic effects. However, this is not the primary blood pressure lowering mode of action because the ensuing baroreceptor stimulation would increase peripheral resistance. Cardiac output usually falls only acutely but returns to normal during chronic treatment, while long-term lowering of blood pressure occurs because of late lowering of peripheral vascular tone. Thus, the hemodynamic hallmark of chronic established hypertension, which is an increased peripheral resistance in the majority of cases, is also normalized by beta-blockers. Other possible mechanisms include inhibition of renin secretion from the kidneys, decrease in plasma Ang II and catecholamines levels, and central reduction in sympathetic outflow reducing vasomotor tone (Laurent 2017). In addition, prejunctional beta



Antihypertensive Drugs, Fig. 2 Synopsis of the treatment strategy for uncomplicated hypertension. The drug treatment strategy shown represents the core treatment strategy for patients with uncomplicated hypertension according to the 2018 European guidelines (Williams et al. 2018). This strategy is also appropriate for most patients with hypertension-mediated organ damage, cerebrovascular disease, diabetes, peripheral artery disease or the elderly

receptors may also be stimulated which leads to a decline in norepinephrine release and hence adrenergic drive. Currently used beta-blockers are usually classified into three different classes: (i) nonselective beta-blockers that block both beta-1 and beta-2 receptors (e.g., propranolol); (ii) beta-1 selective or *cardioselective* beta-blockers (e.g., atenolol, bisoprolol, and metoprolol) that preferentially block beta-1 receptors; and (iii) beta-blockers with additional vasodilatory effects (e.g., carvedilol and nebivolol). Of interest, beta-selectivity of the available beta-blockers for clinical use is poor (Baker 2005). Thus, when used in usual dosages, they block both beta-1 and beta-2 receptors. The additional vasodilatory effect elucidated by carvedilol is based on its capacity to block adrenergic alpha-1 receptors in the arteries, while the vasodilatory action of nebivolol results from nitric oxide release possibly due to activation of beta-3 receptors (Ignarro 2008). The latter two drugs could be therefore preferred as antihypertensive drugs, because of this vasodilatory effect and their improved tolerability and metabolic profile which has been also linked to this action (Ayers et al. 2012). Finally, beta-blockers exhibit a wide variation in their pharmacokinetics and their lipophilic or hydrophilic properties. Lipophilic agents such as labetalol, metoprolol, pindolol, and propranolol have relatively short plasma half-lives, cross blood–brain barrier, and exhibit wide interindividual variability compared to more hydrophilic agents, e.g., atenolol.

Calcium Channel Blockers

Calcium channel blockers (CCBs) inhibit the influx of extracellular calcium through binding to the L-type voltage-gated Ca^{2+} channels located on the vascular smooth muscle, cardiac myocytes, and cardiac nodal tissue (sinoatrial and atrioventricular nodes). The inhibition of inward calcium flux causes relaxation of vascular smooth muscle cells mainly in the arterial bed and promotes vasodilation and lowering of blood pressure. In cardiac muscle, they reduce cardiac contractility and slow conduction velocities in cardiac nodal tissue. All available CCBs are vasodilators acting primarily by reducing the total

peripheral resistance to reduce blood pressure (Laurent 2017). They are a heterogeneous group of compounds that vary in their structure as well as their vascular and cardiac selectivity. They can be divided into two different main classes (dihydropyridines and non-dihydropyridines) that work on different sites within the L-channel and hence produce different effects in the heart and vasculature. The dihydropyridine (DHP) (e.g., nifedipine and amlodipine) exhibit less cardiac depressant properties. Therefore, DHP CCB, particularly rapid- and short-acting agents, may induce unpredictable blood pressure decline and reflex tachycardia due to baroreflex stimulation. Short-acting dihydropyridine formulations have, therefore, been modified to slow-release formulations (in some cases, e.g., for nifedipine) or replaced by longer-acting ones to overcome these disadvantages and to improve the tolerability and safety of DHP CCBs.

The non-dihydropyridine (non-DHP) type, further subdivided into benzothiazepines, such as diltiazem, and phenylalkylamines, such as verapamil, attenuate the increase in heart rate in response to blood pressure lowering or exercise due to their negative chronotropic (verapamil > diltiazem) effects. In addition, they are in parallel to beta-blockers' negative dromotropic drugs and can be therefore used as alternatives to beta-blocker therapy when heart rate control is needed, e.g., in patients with atrial fibrillation (Williams et al. 2018). CCBs have a high first-pass effect, and all compounds are substrate of cytochrome P450 3A4 (CYP3A4) and p-glycoprotein (P-gp) (Algharably and Kreutz 2019). The non-DHP CCB verapamil and diltiazem are, in addition, moderate to strong inhibitors of CYP3A4 and P-gp (Algharably and Kreutz 2019). Hence, unlike most of the other antihypertensive drugs, verapamil and diltiazem have a potential to cause pharmacokinetic drug–drug interactions with drugs that are substrates of CYP3A4 and/or P-gp, e.g., simvastatin.

Diuretics

All diuretics act primarily by impairing sodium reabsorption in the renal tubules, thus increasing its excretion. They differ in their mode of action that relates to their major site of action within the nephron. These differences determine their relative efficacy, as expressed in the maximal percentage of filtered sodium excreted, and determine their pharmacological effects as well as clinical indications for each type of diuretic (Sarafidis et al. 2010).

Thiazide (e.g., hydrochlorothiazide or bendroflumethiazide) and thiazide-like (chlorthalidone or indapamide) diuretics that are functionally related compounds are referred to sometimes only as thiazide diuretics in the literature (Sarafidis et al. 2010). They act by inhibiting the Na^+/Cl^- cotransporter in the distal convoluted tubule of the kidney, where only ~5–10% of filtered NaCl is reabsorbed (Tamargo et al. 2014). The increased water loss is secondary to the increased excretion of NaCl. Thiazides are less potent than loop diuretics that act proximally at the ascending limb of the loop of Henle (Sarafidis et al. 2010). Thiazide diuretics lower blood pressure through a triphasic mechanism: (i) a short-term (i.e., first 2–4 weeks) reduction in extracellular fluid volume followed by reduction of cardiac output which is counteracted by reactive rise in sympathetic activity, plasma renin, and subsequent transient increase in peripheral resistance, thus opposing the blood pressure lowering effect; (ii) long-term effects, where gradual reduction in peripheral vascular resistance due to sodium loss occurs while cardiac output and plasma volume return to pretreatment levels; and (iii) chronic antihypertensive effect reached after about 2 months due to a persistent reduction in peripheral resistance, while a new steady state of reduced total body sodium and blood pressure is established. Hydrochlorothiazide, chlorthalidone, and indapamide represent the most frequently used thiazide/thiazide-like diuretics with chlorthalidone being the longest-acting agent.

Loop diuretics exert their effects at the thick ascending limb of the loop of Henle, where about 30% of filtered Na^+ is reabsorbed by inhibiting ►$Na^+/K^+/2Cl^-$ cotransport (►Na+/K+/$2Cl^-$ cotransporter). Loop diuretics are more potent diuretics than thiazides, but they have shorter half-lives and induce more pronounced reflex stimulation of the sympathetic nervous activity and RAS, which attenuates their blood pressure lowering efficacy (Laurent 2017). In the kidney, loop diuretics maintain or increase even the glomerular filtration rate (GFR) by prostaglandin-

mediated dilation of the afferent arteriole and by blocking of the tubuloglomerular feedback mechanism (Sarafidis et al. 2010). For all diuretics, active secretion via an organic acid transporter in the proximal tubule is important to reach their luminal site of action. Their concentration at the site of action determines the diuretic response. Unlike thiazide diuretics, loop diuretics present a sigmoidal relation between the natriuretic response and the amount of diuretic reaching their site of action. A maximally effective dose can therefore completely block Na^+ reabsorption. However, they have no place in the routine management of hypertension in patients with normal renal function due to their shorter duration and profound effects on natriuresis and diuresis. Their use is reserved to patients with severe renal impairment (e.g., when GFR is <30 mL/min/1.73 m^2) or in patients with severe edematous disorders (i.e., cardiac failure, nephrotic syndrome) when control of volume status is needed.

Potassium-Sparing Diuretics

Potassium-sparing diuretics act primarily at the cortical collecting duct, where about 3% of the filtered Na^+ load is reabsorbed, producing weaker natriuretic effects compared to loop diuretics and thiazide diuretics that act more proximally in the nephron (Sarafidis et al. 2010). They block the action of the epithelial Na^+ channel (ENaC) either directly (ENaC inhibitors) or indirectly (mineralocorticoid receptor antagonists; MRA). The ENaC inhibitors (amiloride, triamterene) block their target in the luminal membrane of the collecting duct, independently of aldosterone. They reduce aldosterone-sensitive sodium-potassium exchange and increase urinary sodium excretion coupled with relative potassium-sparing effect. Mineralocorticoid receptor antagonists (e.g., spironolactone and eplerenone) competitively inhibit the binding of aldosterone to the nuclear mineralocorticoid receptors in collecting duct cells and render them transcriptionally inactive for the synthesis of ENaC and subunits of the Na^+K^+-ATPase pump (Sarafidis et al. 2010).

Although ENaC accounts only for a minor fraction of sodium exertion, this channel plays a major role in maintaining sodium and potassium homeostasis as well as in blood pressure control.

Additional Antihypertensive Drugs

These agents are not generally recommended for the routine treatment of hypertension, but rather used as add-on therapy in patients with resistant hypertension or used in hypertensive patients with specific comorbid conditions responsive to them.

Alpha-Blockers

Prazosin, terazosin, and doxazosin are alpha-blockers available for the treatment of hypertension, although prazosin due to its short half-life (3–4 h) is no longer preferred. They selectively block α_1-adrenoceptors in the vasculature, leading to vasodilation in both resistance and capacitance vessels and lowering of blood pressure. By selective blocking of α_1-receptors, local feedback control mechanisms involving prejunctional α_2-receptors within the neurovascular junction are spared, thus reducing the risk of reflex sympathetic activation and cardiac stimulation during chronic treatment. Their effect on blood pressure is more prominent in the upright position or during exercise. On the long term, peripheral resistance is reduced with little or no change in cardiac output, heart rate, and cardiac index (Laurent 2017).

Centrally Acting Sympatholytic Agents

These agents interfere with sympathetic outflow from vasomotor centers in the brainstem but allow these centers to retain or even increase their sensitivity to baroreceptor control. This type of drugs can be classified according to their relative affinities to α_2- and/or imidazoline (I_1) receptors (Benowitz 2015). The most commonly used drugs are clonidine, moxonidine, and rilmenidine, all of which are considered as a mixed α_2- and I_1-agonist; clonidine has a higher affinity for α_2- and moxonidine/rilmenidine for I_1-receptors (Prichard and Graham 2000). Clonidine lowers blood pressure by decreasing both cardiac output and total peripheral resistance. Methyldopa, on the other hand, is a selective α_2-agonist. After metabolic conversion, methyldopa decreases sympathetic outflow and lowers blood pressure by reducing peripheral vascular resistance with a variable reduction in heart rate and cardiac output (Benowitz 2015).

Vasodilators

The direct vasodilator minoxidil works by opening potassium channels in vascular smooth muscle cells in arterioles, which leads to K^+ efflux and hyperpolarization. The latter inhibits Ca^{2+} influx through voltage-gated calcium channels. The mechanisms of action of hydralazine are not fully understood but may ultimately involve an altered calcium balance in vascular smooth muscles via an unidentified primary target (Knowles et al. 2004). Another possible mechanism may involve enhancing nitric oxide release from the vascular endothelium by increasing nitric oxide synthase expression (Xu et al. 2017). Blood pressure is lowered due to vasodilation but since the heart is not directly affected, direct vasodilators lead, when used alone, to a significant neurohumoral activation of both the sympathetic nervous system and RAS resulting in increases in heart rate and force of contraction as well as fluid retention.

In addition to these classes of antihypertensive drugs, several new drug classes involving targets inside and outside the RAS system are currently being developed at either the preclinical or clinical stage (Kreutz and Algharably 2016). Some additional novel drugs with blood pressure lowering potential are already in clinical use for the treatment of hypertension (esaxerenone; a novel nonsteroidal MRA) or other associated diseases such as pulmonary arterial hypertension (riociguat; a sGC activator) or heart failure (sacubitril; a neprilysin inhibitor).

Clinical Use

The mainstay in the management of arterial hypertension is the implementation of healthy lifestyle, and several lifestyle changes are recommended in current guidelines that may be sufficient to delay or prevent the need for drug therapy (Williams et al. 2018; Whelton et al. 2018). Nevertheless, most patients will require drug therapy in addition to lifestyle measures to achieve optimal blood pressure control. Despite subtle differences between international guideline recommendations, there are still five major (first line) drug classes that are recommended for drug treatment of hypertension (Williams et al. 2018; Whelton et al. 2018). These classes include ACE inhibitors, ARBs, beta-blockers, CCBs, and diuretics (thiazides and thiazide-like diuretics). This rationale is based on evidence from placebo-controlled studies that demonstrated their efficacy to reduce cardiovascular events, morbidity, and mortality (Williams et al. 2018). These studies also demonstrated that this benefit predominantly derives from their blood pressure lowering effect without significant differences among the five classes. However, when compared with other remaining four classes, beta-blockers are considered to be less effective in the prevention of stroke (Williams et al. 2018; Whelton et al. 2018; Wright et al. 2018). Consequently, in some guidelines they are not considered as first-line agents anymore. In the 2018 European guidelines of the European Society of Cardiology and European Society of Hypertension for the management of arterial hypertension, they are still considered as first-line agents, but their use in uncomplicated hypertension is limited. Accordingly, their use is preferred in patients with specific indications (Fig. 2). The backbone in the recommended core drug treatment strategy is the use of an ACE inhibitor or an ARB, while their combination is not recommended, because dual inhibition with both ACE inhibitors and ARB causes more harm than benefit. Thus, either RAS blocker in combination with a CCB or a thiazide/thiazide-like diuretic can be used as dual combination therapy for the initiation of treatment in the majority of patients. To increase adherence to therapy, the use of single-pill combinations is encouraged. A summary of important side effects and absolute or relative contraindications for the different antihypertensive drug classes is shown in Table 1. Although there is no consensus between treatment goals among guideline recommendations (Williams et al. 2018; Whelton et al. 2018), systolic and diastolic blood pressure should be lowered below systolic 140–130 and diastolic 80 mmHg in most patients. If blood pressure control is not achieved, the use of triple therapy by combining one RAS blocker with a CCB and diuretic is recommended. About 10% of patients exhibit resistant hypertension, i.e., they cannot be controlled with the recommended triple therapy. In this condition low-dose spironolactone is the

preferred drug for additional treatment, based on a randomized, double-blind, crossover trial (Williams et al. 2015).

As an alternative for drug treatment, interventional therapies such as renal denervation and baroreflex activation have been recently developed for the treatment of arterial hypertension (Williams et al. 2018; Schmieder et al. 2018). Additional novel non-pharmacologic interventional approaches include carotid body ablation and arteriovenous fistula placement, but renal denervation represents the most advanced approach (Schmieder et al. 2018). The long-term safety and efficacy of this method and its value on top or as an alternative for drug treatment therapy of hypertension remain to be established.

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Antihypertensives

► Antihypertensive Drugs

Anti-integrins

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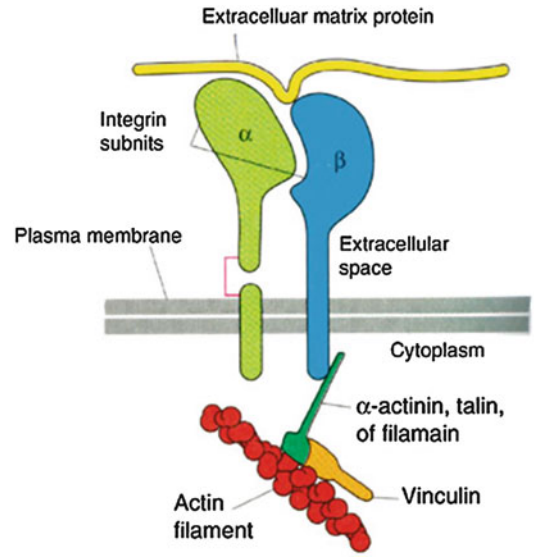
Definition of Integrin

Integrins are heterodimeric proteins of the plasma membrane that are critical to cell-cell interactions and to interactions of the cell with extracellular matrix (ECM) proteins. These physical interactions are critical to individual cell migration paths and multicellular tissue structure. There are 24 or more integrins, each composed of one of 18 monomeric alpha subunits and one of 8 beta subunits (Seguin et al. 2015). Each heterodimeric integrin binds to at least one ECM protein. Such interactions result in a conformational change in the integrin ("activation") (Arnout 2002; Arnaout et al. 2007; Ginsberg 2014). The physical changes in the extracellular integrin component generate intracellular signals via induced changes in the cytoplasmic components ("feet") of the integrins (Fig. 1). These cytoplasmic extensions in cancer cells of the integrin change their physical relationships with components of signal transduction pathways to regulate downstream functions such as the cell cycle and mechanisms of apoptosis (Davis et al. 2016). Specific integrins may act on blood vessel cells to modulate angiogenesis (Eliceiri and Cheresh 1998).

Alterations in the conformation of the large extracellular component of the integrin expose (or obscure) receptor sites for other large molecule ligands in the extracellular space or, as is now



Anti-integrins, Fig. 1 Diagrammatic representation of integrin inside-out signaling and integrin activation, which require conformational switch from bent-closed or extended-closed to extended-open confirmation

known, of extracellular small molecule ligands. As discussed below, such ligands include thyroid hormone analogues (Bergh et al. 2005; Davis et al. 2016) or steroid or steroid-like molecules (Lin et al. 2009).

Anti-integrin

The concept that the substantial extracellular domain of integrins may be manipulated by naturally occurring factors such as ECM proteins – for example, fibronectin, vitronectin, and von Willebrand factor – prompted investigators to search for pharmacologic ligands that might alter normal cell function or the functions of cancer and endothelial cells. Such protein ligands are designated **anti-integrins**. Protein ligands of integrins may contain similar short amino acid sequences, for example, Arg-Gly-Asp (RGD), and such short peptides can serve as anti-integrins (Danhier et al. 2012; Reardon et al. 2008). Another category of anti-integrins is antibodies developed to bind to specific regions of an integrin to modify its function, for example, interactions with the adjacent plasma membrane proteins (Byron et al. 2009). The interactions targeted by anti-integrins may be between integrins and extracellular matrix proteins or between integrins and cell surface proteins of adjacent cells (cell-cell interactions). Finally, anti-integrins may be small molecule integrin ligands, such as thyroid hormone analogues, that block the activity of the specific thyroid hormone (L-thyroxine, T4) at its discrete cell surface receptor on integrin αvβ3 (Cheng et al. 2010). In contrast, RGD peptides may bind to multiple integrins at sites of different overall structure that recognize the RGD sequence. Integrin antagonists demonstrated a wide range of therapeutic applications in thrombosis, vascular restenosis, cancer, asthma, allergy, and inflammatory diseases (Desgrosellier and Cheresh 2010; Dotan et al. 2020; Raab-Westphal et al. 2017).

In addition to affecting functions of integrins, anti-integrins may also serve as drug delivery tools (Li et al. 2016; Sudha et al. 2017a; Sun et al. 2017) or as imaging agents (Danhier et al. 2012) for cancers or inflamed tissues expressing integrins.

Basic Characteristics of Anti-integrins and Mechanisms of Anti-integrin Action

The protein-ligands of integrins, such as ECM proteins, often contain the RGD sequence, and RGD peptides have anti-integrin activity. Another strategic approach to the identification of anti-integrin ligands has been to determine whether naturally occurring small molecules, such as nonpeptide hormone analogues (Davis et al. 2013), might bind to specific sites on specific integrins under physiological circumstances to modify integrin-based cell functions. An example of the use of this strategy was the description of the pro-angiogenic action of thyroid hormone, T4 (Mousa et al. 2014; Davis et al. 2015), initiated at a previously unrecognized hormone receptor on integrin αvβ3. Removal of a single iodine from T4 occurs in vivo to yield tetraiodothyroacetic acid (tetrac) and resulted in an anti-angiogenic molecule that eradicated the pro-angiogenic activity of T4 in endothelial cells (Mousa et al. 2014; Stryker et al. 2019). This topic is reviewed in more detail in the specific anti-integrin section below on αvβ3.

Anti-integrin products may be directed at specific integrin monomers or at an intact heterodimer. The RGD peptide model and the thyroid hormone analogue and resveratrol anti-integrin molecules require heterodimeric integrin structure to construct the discrete receptor sites that may be formed at conjunctions of the specific monomers in a heterodimer. Antibody anti-integrins may be directed at specific facets of monomers.

As this chapter describes in more detail below, integrin αvβ3 also contains discrete sites that specifically bind dihydrotestosterone (DHT) and resveratrol that are unrelated to the thyroid hormone analogue receptor. DHT may act via its receptor on the integrin to stimulate the proliferation of breast cancer cells (Lin et al. 2009) and thus is not anti-integrin. In contrast, resveratrol is an anti-integrin, blocking the proliferation of a wide variety of cancer cells. Among the qualities of resveratrol in tumor cells is interference with the PD1/PD-L1 immune checkpoint (Lin et al. 2019).

In addition to αvβ3, several other integrins have been the foci of anti-integrin development. Integrin-focused drug development has been

substantial for possible application to inflammatory states, such as inflammatory bowel disease (Dotan et al. 2020; Park and Jeen 2018; Sabino et al. 2019; Shah et al. 2017). Integrins other than αvβ3 have also been linked to cancer (Seguin et al. 2015; Desgrosellier and Cheresh 2010; Raab-Westphal et al. 2017). Certain integrins have been implicated in ophthalmologic diseases and have been considered targets for anti-integrins (Gonzalez-Salinas et al. 2018). Finally, there are thrombotic conditions in which anti-integrins have been mentioned as possible therapeutic interventions (Mousa et al. 2010; Davis et al. 2018a; Mousa et al. 2018; Davis et al. 2018b). Because normal platelets bear αvβ3 that originated in the plasma membrane of the megakaryocyte, thyroid hormone action on platelet aggregation via αvβ3 might be a source of hypercoaguability (Mousa et al. 2010). Anti-integrin treatment might be a strategy in such patients.

Activity of anti-integrins may be modified by the state of the activation (conformation) of the highly plastic heterodimeric integrin (Ginsberg 2014). That is, activation state/conformation change may alter the accessibility of binding sites for ligands on the extracellular domain of integrins. Small molecules such as thyroid hormone analogues alter activation state (Leith et al. 2017) and physical factors may also modify the conformation of integrins. X-irradiation is an example of a factor that may rapidly activate integrin αvβ3 (Leith et al. 2018). Thyroid hormone analogues that interact with the same integrin will also affect the interactions of the integrin with ECM proteins, such as vitronectin (Davis et al. 2015) and thus change the motility of cells that anti-integrins may be designed to affect.

Therapeutic Potential of Anti-integrins

Integrins are involved in and modulate cell-cell interactions, interactions of ECM proteins and cells, motility of a large variety of cells, tissue structure, and the state of the cytoskeleton. Pharmaceutics directed at integrins are under study in a wide variety of clinical states. These include inflammation (Arnaout 2016; Kourtzelis et al. 2017) – such as that of the intestinal tract (Catalan-Serra and Brenna 2018), in the vascular system (Huang and Frangogiannis 2018; Edwards and Bix 2019) and joints (Morshed et al. 2019) – cancer biology (Lavergne et al. 2017; Raab-Westphal et al. 2017; Leith et al. 2018; Mousa et al. 2018; Davis et al. 2019) and angiogenesis (Mousa et al. 2014; Davis et al. 2015; Duro-Castano et al. 2017; Guerrero and McCarty 2018).

We have pointed out above that chemical modification of anti-integrin molecules to enable the transporting of anti-cancer drugs allows the anti-integrin, when bound to cancer cell integrin, to unload specific chemotherapeutic agents at the cancer site (Sudha et al. 2017a, b; Sun et al. 2017).

Against this background, it is understandable that a preclinical and limited clinical anti-integrin literature has emerged. The most substantial literature involves application of anti-integrins to inflammatory bowel disease, neuronal disorders, thrombosis, cancer, and cancer-associated complications, as discussed below.

Specific Anti-integrins

Alpha Integrins

Alpha 1 Beta 1 Integrin

Short chain disintegrin obustatin demonstrated high affinity and specificity for the α1 β1 Integrin with potent anti-angiogenesis activity (Marcinkiewicz et al. 2003).

Alpha 2 Beta 1 Integrin (Very Late Activating Antigen 2, VLA2)

A key role for α2β1 integrin in cell adhesion, cell motility, angiogenesis, stemness, and immune/blood cell regulations and its implication in cancer has been demonstrated (Adorno-Cruz and Liu 2018).

Alpha 3 Beta 1 Integrin

The α3β1 integrin expressed in human breast cancer cells and its participation in the degradation

and phagocytosis of the extracellular matrix has been shown (Coopman et al. 1996).

Alpha 4 Beta 1 Integrin (Very Late Activating Antigen 4, VLA4)

The α4 integrin-dependent leukocyte trafficking promotes cognitive impairment in multiple sclerosis (MS), Alzheimer's disease (AD), and other neuropathological disorders, which suggests that blocking α4 integrins might offer a new therapeutic strategy in MS, AD, and other neuronal diseases. The FDA-approved humanized monoclonal antibody against the cell adhesion molecule α4-integrin, namely natalizumab, is indicated for the improvement of disability and reduction of relapse rate in MS patients (Mazdeh et al. 2018; Engelhardt and Kappos 2008; Li et al. 2018; Manocha et al. 2018; Dattoli et al. 2018; Pietronigro et al. 2019).

Alpha 4 Beta 1/Beta 7 Integrin

Natalizumab is also FDA approved for inflammatory bowel disease (IBD), namely Crohn's disease, but has limited risk. In contrast, vedolizumab α4β7 integrin antagonist is approved for IBD with fewer systemic adverse effects versus natalizumab (Park and Jeen 2018).

Alpha 5 Beta 1 Integrin

Integrin subunit α5 (ITGA5) often combines with ITGB1 to form integrin α5β1, which serves as a receptor for cell differentiation, cell development, migration, angiogenesis, and invasion of cancer cells or bacteria (Kim et al. 2000; Cue et al. 2000; Mostafavi-Pour et al. 2018; Ren et al. 2009). The emergence of integrin α5β1 expression was found to be associated with tumor progression in lung cancer and other cancers (Rivera et al. 2017).

Beta 2-Integrin

Beta2-integrins are complex leukocyte-specific adhesion molecules, which are essential for leukocyte trafficking and immunological processes such as neutrophil phagocytosis, T cell activation, and ROS production and are implicated in various inflammatory and immune diseases (Fagerholm et al. 2019).

Beta 3 Anti-integrins

Alpha IIb/β3 Integrin – Integrin αIIbβ3, Glycoprotein IIb/IIIa (GPIIb/IIIa)

Platelet integrin αIIbβ3 (GPIIb-IIIa) binds to fibrinogen and fibrin, and novel antagonists were developed and evaluated as effective anti-thrombotic in various settings (Hantgan et al. 2007; Mousa et al. 1996; Mousa et al. 1998, 1999; Mousa and Ahmad 2007). There are already three FDA approved intravenous GPIIb/IIIa receptor inhibitors including eptifibatide, tirofiban, and abciximab used in patients with acute coronary syndrome undergoing percutaneous coronary intervention (Capodanno et al. 2019; Podolnikova et al. 2014). Eptifibatide and tirofiban are specific GPIIb/IIIa inhibitors, while abciximab cross-reacts with αvβ3 and α2β1 integrins. Hence, abciximab might reduce restenosis, myocardial infarct size, inhibit adhesion of monocytes, and impact the inflammatory response. In that regard, potent small molecule high affinity αvβ3 integrin antagonists demonstrated anti-restenosis efficacy in various preclinical models (Bishop et al. 2001; Srivatsa et al. 1997). Additionally, anti-angiogenesis efficacy was demonstrated in a retinal neovascularization model with small molecule αvβ3 antagonists (Luna et al. 1996; Santulli et al. 2008).

Alpha v Beta 3 Integrin

Tetrac blocks the actions of vascular growth factors, such as fibroblast growth factor (FGF2), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) (Mousa et al. 2014). Thus, tetrac can be designated a naturally occurring anti-integrin with anti-angiogenic properties.

Tetrac and chemically modified tetrac also have anti-cancer properties (Bharali et al. 2013; Mousa et al. 2018; Rajabi et al. 2019), but the growth of noncancer cells appears to be unaffected by tetrac (Davis et al. 2016). This reflects the generous expression of integrin αvβ3 by cancer cells compared with nonmalignant cells (except for endothelial cells). High affinity and specific αvβ3 antagonists were developed and evaluated in various settings (Helluin et al. 2000;

Kerr et al. 2000, 2001, 2002; Mousa 2005; Mousa and Mohamed 2005).

Alpha v Beta 6 Integrin

Integrin αvβ6 is exclusively expressed in epithelial cells and it activates transforming growth factor-β1 (TGF-β1) to modulate innate immune surveillance in lungs, skin, and gastrointestinal tract to maintain epithelial stem cell quiescence. The expression of αvβ6 integrin and its activation of TGF-β1 are associated with organ fibrosis and cancer. Therefore, αvβ6 integrin might serve as an attractive target for cancer therapy, imaging, and fibrosis (Koivisto et al. 2018). Additionally, β6 expression activates multiple systems involved in tumor lesions and cancer metastasis and its overexpression correlated with reduced patient survival in renal carcinoma and perhaps other cancer (Cantor et al. 2015).

Neurotransmitter Receptors and Integrin

Endothelial cells, as key cells for the angiogenesis process, express several nonneuronal nicotinic acetylcholine receptors (AChRs). In endothelial cells, alpha7 AChR stimulation indirectly triggers the activation of the integrin αvβ3 receptor and an intracellular MAP kinase (ERK) pathway that mediates angiogenesis. The intracellular mechanisms by which alpha7 AChR activation mediates angiogenesis were examined by our group (Arias et al. 2009).

Future directions should focus on these different interfaces between neurotransmitter receptors as well as hormones and integrins function, which might serve as novel therapeutics as highlighted in this brief overview.

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Anti-obesity Drugs

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Synonyms

Weight-loss therapies; Weight-management pharmacotherapy

Definition

Obesity is characterized by the accumulation of body fat resulting from excess energy intake over energy expenditure for a prolonged period of time. A commonly used assessment to define obesity is the body mass index (BMI), measured as a person's body weight in kilograms divided by the squared height in meters (kg/m^2). Obesity is defined by a BMI of $\geq$30 kg/m^2, and overweight is defined by a BMI of 25–29.9 kg/m^2. Obesity increases the risk for comorbidities such as type 2 **diabetes** and cardiovascular disease. Treatment with anti-obesity drugs is recommended for obese patients with BMI $\geq$30 kg/m^2 or BMI $\geq$27 kg/m^2 with existing comorbidities and aims to reduce body weight by $\geq$5%, an effect that was shown to be beneficial for reducing comorbidities and high glucose or high triglyceride profiles. The anti-obesity drugs currently approved for long-term weight management act primarily as appetite suppressants on the central nervous system, and one agent leads to reduced fat absorption from the intestines.

Mechanism of Action

In the healthy state, energy intake, driven by food consumption and nutrient intestinal absorption, counterbalances energy expenditure driven by thermogenesis, metabolism, and physical activity. Obesity results from an imbalance when energy intake exceeds energy expenditure over a prolonged period of time. The chronic surplus of energy is stored as triglycerides, the most condensed energy form, in adipose tissue. Environmental factors, such as the general availability of high-calorie food and limited need for physical exercise favor the development of obesity. Genetic factors that predispose to weight gain further contribute to it. While the different components affecting energy balance in theory give rise to various pharmacotherapy approaches, the development of an anti-obesity drug has yet to overcome the high hurdle of being efficacious and safe. It should result in a reduction of the fat mass while saving lean body mass and help to maintain the reduced weight. To account for counter-regulatory effects and the redundancy of mechanisms affecting energy intake and homeostasis, the concept of "polytherapies" – i.e., combination agents that are designed to target more than one biological mechanism – is becoming stronger.

Reflecting these challenges, there has been a long history of weight-loss therapies, and most of them had to be withdrawn from the market due to safety concerns. Those include sheep-derived thyroid hormone or 2,4-dinitrophenol, which reduce weight by increasing energy expenditure. Likewise, sibutramine, a combined **serotonin** and norepinephrine reuptake inhibitor, was more recently withdrawn from the market, as patients with preexisting cardiovascular conditions exhibited increased cardiovascular events. The currently approved anti-obesity drugs (or drug combinations) affect primarily energy intake as explained in more detail below.

Reduction of Energy Intake: Decreasing Fat Absorption

Inhibition of the absorption of fat (triglycerides) in the gastrointestinal lumen represents the most

efficient approach for reduction of caloric intake, as triglycerides are the most condensed energy stores. Ingested triglycerides are split by lipases into free fatty acids and monoglycerides in the intestinal lumen before crossing into the epithelial cells of the intestinal wall and resynthesis to triglycerides.

Orlistat

Orlistat is a hydrogenated derivative of lipstatin, a natural occurring lipase inhibitor of bacterial origin. The agent inhibits the activity of the pancreatic lipase by covalently binding to a serine residue in the active site of the enzyme, thereby the hydrolysis of triglycerides into absorbable free fatty acids and monoglycerides is reduced, which results in diminished fat absorption by ca. 30% (Table 1).

Reduction of Energy Intake: Decreasing Food Intake

Food intake and **appetite control** are influenced by complex mechanisms including central effects in the brain as well as cross talk with signals from the periphery. There are four drugs (or drug combinations), i.e., lorcaserin, phentermine/topiramate, naltrexone/bupropion, and liraglutide which promote weight loss primarily by mediating anorexigenic effects (reducing food intake) as explained in more detail below (and for review see Müller et al. 2018; Bray et al. 2018; Patel 2015).

Lorcaserin

Lorcaserin, a benzazepine derivative, is a selective **serotonin** (5-hydroxytryptamine [5-HT]-2C receptor agonist (Table 1). The 5-HT_{2C} receptor has been shown to be expressed (not exclusively)

Anti-obesity Drugs, Table 1 Drugs approved by the Food and Drug Administration (FDA) for long-term pharmacotherapy of obesity and their mechanism of action. EMA European Medicines Agency; 5-HT_{2C}-R = 5-hydroxytryptamine 2C receptor; GABA = γ-aminobutyric acid; GLP-1-R = glucagon-like peptide-1 receptor. ER extended release; SR sustained release. With the exception of liraglutide which is administered by subcutaneous (sc) injection, the drugs are administered as tablets

Generic name	Trade name (year of approval by FDA)	Mechanism of action	Dosage
Orlistat	Xenical® (1999) Alli® over-the-counter, (2007)	Inhibitor of pancreatic lipase reduces availability of fatty acids for absorption from intestinal lumen	120 mg, three times daily 60 mg, three times daily
Lorcaserin	Belviq® (2012) Not approved by EMA	Serotonin 5-HT_{2C}-receptor agonist acts centrally to reduce food intake	10 mg, twice daily (or 20 mg extended release)
Phentermine/ Topiramate ER	Qsymia® (2012) Not approved by EMA	Phentermine: sympathomimetic, increases norepinephrine levels in hypothalamus, resulting in anorexigenic effects Mechanism of topiramate to reduce body weight unclear may involve central GABA receptor modulation	3.75 mg/23 mg daily (14 days) 7.5 mg/46 mg (thereafter), max. 15 mg/92 mg
Naltrexone SR/ Bupropion SR	Contrave® (2014)	Naltrexone: antagonist of opioid receptors (μ-opioid receptor) that prevents the autoinhibition of hypothalamic POMC neurons which have anorexigenic effects Bupropion: agent that enhances the activity of POMC neurons	8 mg/90 mg daily
Liraglutide	Saxenda® (2014)	GLP-1 receptor agonist, acts on centrally expressed GLP-1 receptors	Up to 3 mg sc injection daily (starting with 0.6 mg/day)

in brain areas connected to eating behavior. Lorcaserin is assumed to reduce appetite and increase satiety by binding to $5HT_{2C}$ receptors on pro-opiomelanocortin (POMC) neurons in the hypothalamus which are known to promote anorexigenic effects. 5-HT_{2C}-deficient mice are hyperphagic and develop obesity, insulin resistance, and impaired glucose tolerance. Previous, unselective 5-HT agonists which were used as anti-obesity drugs, fenfluramine and dexfenfluramine, had been withdrawn due to cardiac valvulopathy likely mediated by their binding to $5HT_{2B}$ receptors expressed on the heart valve. Treatment with lorcaserin, also referred to as the third-generation 5HT agonist, did not show this issue over 2 years, in line with its selectivity profile.

Phentermine/Topiramate Extended Release (ER)

Phentermine/topiramate is a combination drug. Phentermine is a nonselective stimulator of synaptic norepinephrine (and of serotonin and dopamine) levels. This sympathomimetic drug is among the earliest pharmacological agents used for weight management. It is currently approved as monotherapy only for short-term (<12 week) treatment of obesity due to safety concerns about cardiovascular risk and abuse potential. The combination has lower dose of phentermine than usually prescribed for phentermine alone. It supposedly acts as an appetite suppressant by increasing norepinephrine levels in the central nervous system as the main mechanism (Table 1). The dopamine levels are only slightly increased, and their contribution to the anorectic effect is less clear.

Topiramate, a sulfamate-substituted monosaccharide derived from D-fructose, is commonly used to treat **epilepsy** and migraine, and was approved later on for weight management as the clinical trials showed effects on body weight. Topiramate is assumed to selectively decrease CNS neuronal activity by inhibiting certain neuronal calcium and sodium channels and modulating central glutamate and γ-aminobutyric acid (GABA) receptors to mediate the effects in epilepsy and migraine. The mechanism by which topiramate regulates body weight is not yet well understood, but may also involve modulation of GABA receptors (Table 1). The weight loss achieved with the combination is greater than treatment with the single agents alone.

Naltrexone Sustained-Release (SR)/Bupropion SR

This combination drug is a pairing of a known **opioid** receptor antagonist (naltrexone) with a known norepinephrine and dopamine reuptake inhibitor (bupropion) (Table 1). Bupropion alone is approved for depression and smoking cessation. This agent shares similar structural design and CNS actions as the sympathomimetic diethylpropion which is being used for short-term treatment of obesity. As an inhibitor of the reuptake of norepinephrine and dopamine, bupropion likely produces anorexigenic effect via combined dopaminergic and noradrenergic effects on POMC neurons in the arcuate nucleus of the hypothalamus. There is some evidence of additional centrally mediated effects on energy expenditure. Naltrexone alone is approved for treatment of alcohol and opioid dependence. It is an opioid receptor antagonist with high affinity for the μ-opioid receptor. It has appetite suppressant effects likely due to disrupting autoinhibition of POMC neurons. In combination with bupropion, it further enhances the activity of POMC neurons. Research suggests that the combination leads to improved food craving control and reduced overeating episodes.

Liraglutide

Liraglutide is a glucagon-like peptide (GLP-1) receptor agonist with 97% homology to human GLP-1. The peptide hormone GLP-1 is released by the small intestines in response to food ingestion and enhances pancreatic insulin production and secretion, while glucagon release is inhibited. While the natural GLP-1 has a very short half-life of 1–2 min and is cleaved by dipeptylpeptidase IV, modifications obtained for liraglutide resulted in extended circulating half-life of ca. 13 h. Liraglutide was originally approved for treatment of type 2 **diabetes** mellitus and was later on, since it showed ability to reduce body weight in the trials, approved for obesity treatment (at higher dose). Liraglutide exerts its effects to reduce body weight likely by a combination of influences on energy intake and

energy homeostasis. Consequences exerted on feeding behavior are likely mediated by acting on GLP-1R expressed in brain areas. In more detail, GLP-1 was shown to directly activate POMC/CART neurons and indirectly inhibit, via GABAergic transmission, the **neuropeptide Y**/agouti-related peptide (NPY/AgRP) neurons, which collectively results in signals that reduce food intake. It has been suggested that the delay in gastric emptying and the effect to slow gut motility comprises also part of the weight-loss mechanism of action of liraglutide.

Clinical Use

Obesity has reached an epidemic level not only in developed but also in developing countries. In 2016, the World Health Organization estimated that more than 1.9 billion adults were overweight and of these over 650 million were classified as obese (WHO 2018). Percentage wise, 39% of adults were overweight, and 13% were obese. The USA accounts for the highest global incidence, with a recent survey indicating that 35% of men and 40% of women in the USA were obese. Pharmacotherapy is generally recommended as the second-line treatment for obesity after lifestyle modifications. As obesity is a chronic disease, it requires long-term therapy. There are currently five drugs (with two combination) approved by the FDA for long-term (more than 6 months) anti-obesity therapy (Table 1). They are indicated for patients with a BMI $\geq$30 kg/m^2 or $\geq$27 kg/m^2 with an obesity-related comorbidity, such as type 2 **diabetes** mellitus, hypertension, or dyslipidemia, and they are used as an adjunct to lifestyle modifications. The treatment of obesity aims at a sustained loss of 5–10% of body weight, which has been shown to reduce the risk of obesity-associated comorbidities. It should be noted that, in addition, the FDA approved before 1960 the sympathomimetic agents benzphetamine, diethylpropion, phendimetrazine, and phentermine for short-term ($<$12 weeks) treatment of obesity. The clinical use and clinical trial data for the currently approved anti-obesity drugs as well as their side effects are summarized below and were compiled from Patel 2015, Bray et al. 2018, and Andrew et al. 2019.

Orlistat, marketed as Xenical®, has been approved by the FDA for obesity management already in 1999 (Table 1). There have been numerous clinical trials with orlistat. In a 4-year clinical trial in obese patients in conjunction with lifestyle intervention, the mean weight loss was significantly greater with orlistat (5.8 kg) than with placebo (3 kg). Orlistat was shown to have beneficial effects on associated comorbidities, as shown in clinical trials in obese patients with cardiovascular risks, where blood pressure was reduced. Lipid profiles were also improved, with an especially strong effect to reduce LDL-C levels that may be due to its mechanism of action. Furthermore, the XENDOS trial showed that glycemic control was ameliorated with orlistat. Of note, orlistat is the only drug approved by the FDA for weight management in obese adolescents.

While orlistat itself is minimally absorbed, the main unwanted effects of orlistat are attributable to its mode of action, as non-digested fat remains in the intestinal lumen and can cause steatorrhea (fatty stools), flatulence and fecal incontinence. These effects are associated with a high-fat meal, and therefore a low-fat diet is recommended. As absorption of fat-soluble vitamins may be hampered, supplementation of these vitamins is recommended.

Lorcaserin, marketed as Belviq®, has been approved in 2012 (Table 1). This agent has been investigated in three major phase 3 clinical trials. In the 1 year BLOOM trial, obese patients treated with lorcaserin lost a significantly greater percentage (5.8%) of body weight from baseline compared to placebo-treated patients (2.2%, $p > 0.001$) and achieved in absolute numbers a body weight reduction by 5.8 kg for the drug-treated versus 2.2 kg in the placebo-treated patients. Weight loss was similar in the other two clinical trials in obese and overweight adults. The trials also showed improvements in cardiovascular risk factors in line with the effect to reduce body weight. Furthermore, lorcaserin treatment of

obese or overweight patients with type 2 **diabetes** mellitus resulted in significant improvements in glycemic control.

The most common adverse events reported with lorcaserin are headache, dizziness, fatigue, and nausea. In contrast to the former unselective 5HT agonists, lorcaserin treatment did not show cardiac valvulopathy in clinical trials so far. This drug is not approved for treatment of obesity by the European health authority.

Phentermine/topiramate ER, marketed as Qsymia®, has been approved by the FDA in 2012 (Table 1). The efficacy of phentermine/topiramate ER in patients with overweight and obesity has been demonstrated in two phase 3 clinical trials and one extension trial. As an example, in the CONQUER trial, overweight or obese patients (BMI of 27–45 kg/m^2) with $\geq$2 comorbidities were treated with placebo, phentermine 7.5 mg/topiramate 46 mg, or phentermine 15 mg/topiramate 92 mg for 1 year. The patients lost 1.2% of baseline body weight in the placebo group, compared to 7.8% and 9.8% in the drug-treated groups, respectively ($p < 0.0001$ vs. placebo). There were significant improvements in cardiometabolic parameters for the combination treatment at the higher dose. In an extension trial, following 2 years of treatment, patients on the combination treatment lost body weight in a similar range, showed greater improvements in cardiometabolic parameters as well as reduced progression to type 2 **diabetes** mellitus compared with placebo.

The most common adverse reactions reported with phentermine/topiramate ER use include paresthesia, dizziness, dysgeusia, insomnia, constipation, and dry mouth. This combination drug is not approved for treatment of obesity by the European health authority.

Naltrexone SR/bupropion SR, marketed as Contrave®, is another combination drug (Table 1). Four one year trials were conducted to evaluate the safety and efficacy. In the COR-I and COR-II trials, obese patients or patients with BMI of $\geq$27 kg/m with one comorbidity were treated with naltrexone/bupropion at 360 mg/32 mg or placebo, resulting in placebo-adjusted weight loss between 2.5% and 5.2% of initial body weight. As weight loss occurred, glucose tolerance and lipid profiles improved.

The most common side effects are nausea, constipation, headache, dizziness, insomnia, and dry mouth. Abuse potential has not been reported. Slight effects to increase blood pressure and heart rate were observed, which may be due to the effect of bupropion. While they seem clinically insignificant, further studies are needed to assess the safety of naltrexone/bupropion in the cardiovascular disease population.

Liraglutide, trade name Saxenda®, is administered as a subcutaneous injection (Table 1). It is marketed at a lower dose under Victoza for treatment of type 2 **diabetes** mellitus. The effects of Liraglutide in the obese population were addressed in the SCALE program. In a clinical trial with 1-year treatment, the weight loss was 7.8 kg at the maximal dose of 3 mg daily liraglutide versus 2.0 kg in the placebo-treated group. Furthermore, in a different setup with an initial period of losing weight due to low-calorie diet, weight loss was 6.8 kg (and 6.2% weight loss relative to baseline weight) in the drug-treated patients, while placebo-treated patient did not show an additional significant weight reduction. Clinical trials in patients with cardiovascular comorbidities or type 2 **diabetes** mellitus showed an improvement of the respective parameters.

The most common adverse events reported included nausea, vomiting, diarrhea, constipation, and dyspepsia. Liraglutide as monotherapy has a low risk for hypoglycemia, but should not be taken with another antidiabetic agent.

Cross-References

- ▶ Adipokines
- ▶ Antiepileptic Drugs
- ▶ Appetite Control
- ▶ Diabetes Mellitus
- ▶ Ghrelin
- ▶ Glucose-Lowering Drugs Other than Insulin
- ▶ Neuropeptide Y
- ▶ Opioid Systems
- ▶ Serotoninergic System

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Antioxidants

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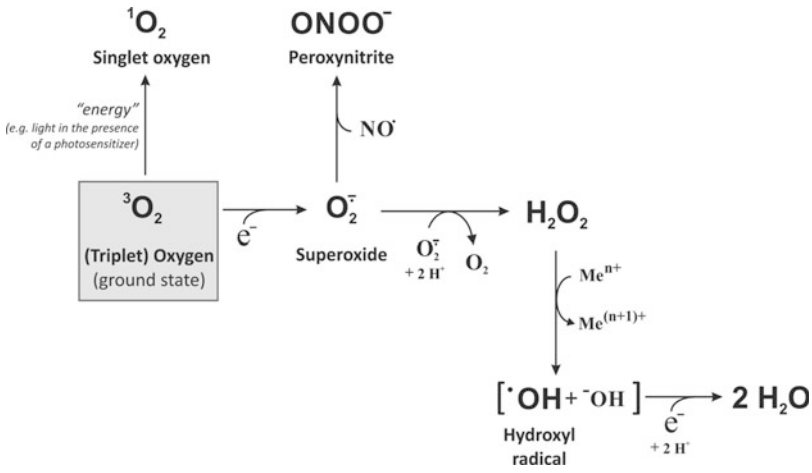
Introduction

Definitions: Oxidants, Antioxidants, Free Radicals, ROS

Antioxidants are molecules characterized by function rather than common structural motifs. Their common feature is the capability of acting "anti"-oxidants in biological systems. Owing to the exposure of tissues to high concentrations of physically dissolved molecular oxygen as well as to nitrogen-containing biomolecules, the oxidants, usually implied as being opposed by antioxidants, are oxygen- and/or nitrogen-derived reactive species generated in biological systems (Fig. 1). There is, of course, a plethora of other biologically relevant reactive species and oxidants, including sulfur-centered radicals or chlorinated species. Referring to all of these using the frequently employed acronym ROS ("reactive oxygen species"), as we will in this entry, is, therefore, strictly speaking, incorrect. It has, however, the advantage that two inconsistencies are being avoided: if the compounds shown in Fig. 1 were referred to simply as "oxidants" or "free radicals," this would neglect the fact that not all of these compounds are necessarily oxidants (such as the superoxide anion, which may act as reductant in biological systems) or radicals (such as the non-radical reactive species H_2O_2, $ONOO^-$, HOCl).

ROS may be generated endogenously and upon exposure to exogenous stimuli. Endogenous generation occurs wherever electrons are passed along in the presence of oxygen, such as in the mitochondrial respiratory chain or during xenobiotic metabolism (Klotz and Steinbrenner 2017; Kehrer and Klotz 2015). It may be catalyzed by dedicated enzymes, including oxidases that reduce oxygen to generate superoxide or hydrogen peroxide, and endogenous generation of ROS may occur as (by)product of an interaction of other ROS – such as peroxynitrite, which is generated through a combination of superoxide and nitrogen monoxide radicals, or hydroxyl radical, which may be generated by a metal ion-induced reduction of peroxides (Fenton reaction). Exogenous stimuli eliciting the endogenous generation of ROS include physical stimuli such as UV radiation or ionizing radiation and toxins.

Just as, chemically, ROS are not necessarily oxidants, the opposing antioxidants not necessarily act as reductants in a biological setting: in fact, oxidants can evoke an adaptive cellular response, for example, by stimulating transcription factors, and therefore serve one of the four commonly found antioxidative strategies (Sies 1993; Klotz 2015). These strategies include (i) the prevention of oxidant formation, (ii) interception of reactions between oxidants and their molecular targets, (iii) repair of molecules damaged by oxidants, and (iv) adaptation, through stress-induced signaling, by upregulating endogenous antioxidant defenses.



Antioxidants, Fig. 1 Reactive oxygen species derived from molecular oxygen by energy or electron transfer. Me, metal ion

Antioxidative Strategies: Endogenous Antioxidants and Pharmacological Approaches

Endogenous antioxidants may be categorized roughly by molecular mass. High-molecular-mass antioxidants would therefore include proteins, mostly enzymes, whereas low-molecular-mass (lmm) antioxidants would refer to widely known antioxidants such as vitamins C or E. A second level of distinction would be the extent to which the respective lmm compound is soluble in water. Examples of both proteins and hydrophilic as well as lipophilic lmm antioxidants are listed in Table 1.

Regarding the aforementioned antioxidative strategies, they can be illustrated using antioxidant proteins/enzymes as examples:

(i) *Prevention*: storage proteins, such as ferritin, or chelating proteins, such as metallothioneins or even serum albumin, may be considered antioxidants with respect to their capability of preventing the Fenton reaction from occurring: chelation of redox-active metal ions, such as copper ions, may prevent peroxide reduction to generate the hydroxyl radical. Prevention by enzyme inhibition is certainly another approach: oxidases such as xanthine oxidase or NADPH oxidase may be targeted by inhibitors, thereby attenuating superoxide and peroxide generation.

Pharmacologically, chelation therapy is treatment of choice in diseases accompanied by elevated transition metal ion levels, such as Wilson's disease (WD). Pathological copper accumulation in WD patients causes deterioration of tissues, in part due to oxidative reactions elicited by copper ions, resulting in membrane peroxidation and damage. Copper chelation therapy, employing chelators such as D-penicillamine, triethylenetetramine, or tetrathiomolybdate, is, therefore, standard therapy. In addition zinc salts sometimes are being used to induce endogenous metallothionein production, again to enhance copper chelation capacity. Oxidase inhibition would be achieved using pharmacologically active compounds such as the antihyperuricemic allopurinol, an inhibitor of xanthine oxidase.

(ii) *Interception*: Direct scavenging of ROS is achieved by dedicated enzymes. Superoxide and hydrogen peroxide as well as alkyl peroxides are substrates of enzymes (see Table 1), with superoxide disproportionation ("dismutation") being catalyzed by superoxide dismutases (SOD) and peroxide reduction to water or the respective alcohol catalyzed by peroxidases.

Pharmacologically, lmm enzyme mimetics have been synthesized and developed for decades, but only few have been successful beyond in vitro studies. In line

Antioxidants, Table 1 Categories of antioxidants and examples (not exhaustive)

Non-enzymatic	Enzymes
a) Low-molecular-mass (lmm)	Superoxide dismutases
Hydrophilic	Cytosolic Cu,Zn-SOD (SOD1)
Vitamin C (ascorbic acid)	Mitochondrial Mn-SOD (SOD2)
Urate/uric acid	Extracellular SOD (EC-SOD; Cu,Zn-dependent)
Glutathione	Peroxidases
Lipoic acid	Heme peroxidases (e.g., catalase)
	Glutathione peroxidases (GPx; selenium-containing and non-selenium-containing)
Lipophilic	Peroxiredoxins
Vitamin E (tocopherols, tocotrienols)	Reductases
	Glutathione disulfide reductase
Carotenoids	Glutaredoxins
Ubiquinone	Thioredoxins, thioredoxin reductases
	Phase I/phase II enzymes
b) Proteins	NAD(P)H:quinone oxidoreductase-1 (NQO1)
Metal ion-binding proteins	Glutathione S-transferases (GSTs)
Metallothioneins	Repair enzymes
Serum albumin	Methionine sulfoxide reductases
	8-Oxoguanine glycosylase-1 (OGG1)

with three types of SOD being present in humans (Cu, Zn-SOD, Mn-SOD, EC-SOD; see Table 1), with Cu or Mn ions at the active site, copper- or manganese-based SOD mimetics have been around for decades, yet only used in in vitro studies so far. Peroxidase-mimetic compounds were also developed, including Fe- or Se-based compounds, including the glutathione peroxidase mimetic, ebselen, which was developed after glutathione peroxidase was demonstrated to be a selenoenzyme. Ebselen, in the 1980s and 1990s, was tested as a drug candidate in the treatment of diseases associated with elevated ROS levels, such as brain ischemia and stroke. Although oral administration of ebselen was found to be safe for humans in a clinical trial with Japanese patients suffering from acute ischemic stroke, ebselen never progressed beyond clinical trials (for review, see Steinbrenner et al. 2016).

(iii) *Repair*: Oxidative damage elicited upon exposure of biomolecules to ROS (i.e., under condition of insufficient interception) may be reversed by dedicated enzymes, including methionine sulfoxide reductases (reducing oxidized protein-bound methionine sulfoxides), thioredoxins (reducing protein disulfide bonds), phospholipid-hydroperoxide glutathione peroxidase (GPx4, reducing oxidized lipids), and OGG1 (8-oxoguanine glycosylase-1, recognizing and excising oxidized guanine moieties in oxidatively damaged DNA, thereby initiating DNA base excision repair). Moreover, cellular structures damaged beyond repair, such as oxidized proteins and membrane components, are degraded in a targeted approach; this also constitutes a part of the cellular antioxidative defense (Grune et al. 1997; Jung et al. 2009; Korovila et al. 2017).

Pharmacologically, the repair of oxidative damage does not appear to be a major area of interest. As an exception, consumer products may be mentioned that use DNA repair enzymes in order to repair sun-induced DNA damage. In fact, liposomal preparations of DNA repair enzymes, including OGG1, were described as potent means of lowering the extent of skin cell DNA damage.

(iv) *Adaptation*: ROS may stimulate transcription factors, including Nrf2 or FOXO transcription factors (Gille et al. 2019), to elicit a transcriptional response that results in upregulation of antioxidant enzymes. Enzymes upregulated upon stimulation of Nrf2 include glutathione biosynthetic enzymes, quinone reductases, heme oxygenase-1 (resulting in production of bilirubin), and phase II enzymes, such as glutathione S-transferases (Klotz and Steinbrenner 2017). FOXO transcription factors also

regulate the expression of genes encoding superoxide dismutases, peroxidases (such as catalase), and others (for review, see Klotz et al. 2015).

Pharmacologically, compounds known to stimulate Nrf2 signaling are being used as drugs, including fumaric acid esters, such as dimethyl fumarate (DMF), which is approved for the treatment of certain forms of multiple sclerosis and for the treatment of psoriasis. DMF, as an electrophile and Michael acceptor, will stimulate Nrf2 signaling through interaction with cysteine residues of the Nrf2 interaction partner Keap-1. The same reaction will occur between DMF and glutathione, leading to glutathione depletion, which in vitro causes cell death and may be responsible for side effects of DMF use (Satoh and Lipton 2017). To what extent the therapeutic effect of DMF relies on its activating Nrf2 is not entirely resolved (Schulze-Topphoff et al. 2016). It should be mentioned that an adaptive response to ROS can also be stimulated by exercise and physical stimuli, including cold exposure (Siems et al. 1999; Powers et al. 2011).

Mechanism of Action of Low-Molecular-Mass Antioxidants

Following the above classification of antioxidative strategies and the ensuing broad definition of an antioxidant, the list of potential antioxidants seems overwhelming. Therefore, this entry will focus on pharmacologically relevant low-molecular-mass (lmm) antioxidants belonging to the second category, intercepting antioxidants. In fact, this is the category usually associated with an antioxidant: reacting with, scavenging, or quenching a reactive oxygen species.

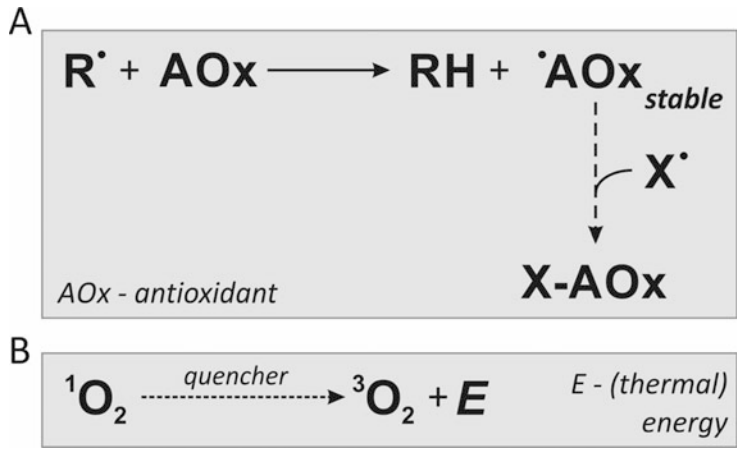
Endogenous and Exogenous lmm Antioxidants: Two Modes of Action

Numerous lmm antioxidants are endogenously formed, such as glutathione, uric acid, or bilirubin, breakdown products of purines and heme, respectively, or are both endogenously generated and provided from exogenous sources, such as lipoic acid or ubiquinone. Others are exclusively derived from exogenous sources, such as vitamins. These compounds are present in the relevant in vivo compartments at significant (at least micromolar) concentrations.

The first major mechanism of antioxidant action at the level of interception is reduction, i.e., the donation of (an) electron(s) to a recipient molecule. When interacting with a radicalic ROS (such as the hydroxyl radical or peroxyl radicals), transfer of a single electron would turn this radical into a non-radical, often stable, and less reactive compound. The remaining "antioxidant radical" is assumed to be a radical that is chemically relatively stable, providing time for selective reaction with a next antioxidant in a chain reaction, eventually leading to a stable radical molecule or a recombination of two radicals, forming an inert molecule (Fig. 2a). In the case of tocopherols, for example, the phenolic hydroxyl is the basis for the antioxidant activity of this molecule. Upon donation of an electron to a reaction partner, it forms the minimally reactive tocopheroxyl radical.

The potential of a chemical to reduce a prooxidant by electron transfer is governed by kinetic and thermodynamic parameters of the underlying redox equilibrium. Hence, redox potentials and concentrations of reaction partners in a given compartment define the likelihood of a successful scavenging reaction.

For example, melatonin (N-acetyl-5-methoxytryptamine) has been frequently suggested to be an effective antioxidant, avidly scavenging various ROS. Its reaction with the hydroxyl radical indeed occurs at a diffusion-controlled rate. However, considering the high reactivity of the hydroxyl radical, one is tempted to ask: which hydrogen-containing molecule's reaction with the hydroxyl radical does not occur at a considerable rate? And further considering the in vivo concentrations achieved by melatonin, which are usually not above nanomolar concentrations, and therefore several orders of magnitude lower than concentrations of other antioxidants in vivo, it is rather obvious that in vivo melatonin is unlikely to contribute to overall hydroxyl radical scavenging. Similar conclusions



Antioxidants, Fig. 2 Two modes of antioxidative action. (**a**) Reduction of a radical by an antioxidant, (**b**) quenching of an excited species, such as singlet oxygen

can be drawn with respect to the contribution of melatonin to scavenging of other ROS. So, whereas it seems unlikely that melatonin is an effective in vivo scavenger of ROS, it is indeed conceivable that it contributes to adaptive responses also at its nanomolar in vivo concentrations which are compatible with those of signaling molecules.

A second form of oxidant detoxification is through physical quenching, i.e., (stepwise) energy dissipation by reaction of a quencher molecule with an excited species. In essence, relaxation of electronically excited species will be induced, transferring energy to the quencher, which, in turn, dissipates this energy as vibrational (thermal) energy to the molecular environment (Fig. 2b). Among the lmm antioxidants found in vivo, carotenoids are prototypic quenchers, including provitamin A- (such as beta-carotene) and non-provitamin A-carotenoids (such as lycopene). They quench singlet oxygen ($^1O_2$) with very high efficiency in vitro. To what extent their quenching excited species such as $^1O_2$ significantly contributes to carotenoid antioxidative activity in humans is currently a matter of discussion (Bosio et al. 2013).

It should be mentioned here that, in principle, all antioxidants can also be pro-oxidants (as the reactions they are part of are redox equilibria). However, the demonstration of this effect is limited to in vitro test systems, since such concentration relations allowing for reversion of the reaction equilibrium can rarely be achieved in vivo.

Plant Secondary Metabolites

In addition to the abovementioned antioxidants, numerous plant secondary metabolites, such as plant polyphenols and many others, were reported as antioxidants. However, while indeed in vitro some of these act as scavengers of ROS, these compounds do not fulfill some important criteria that the above lmm antioxidants do. Not only is unmodified uptake unlikely in many of these compounds, as both microbiome and standard first-pass effect will lower concentrations of the original natural compound to be achieved in vivo. Also, a major advantage of the above lmm antioxidants is that many are constantly being recycled, including glutathione, as well as vitamins C and E, which may, to a certain extent, be recycled from their oxidized forms at the expense of cellular reducing equivalents. This is not always the case with plant secondary metabolites taken up by humans. Most importantly, however, at the concentrations usually achieved in vivo upon uptake of such compounds, and considering the much higher concentrations of well-known antioxidants in vivo, it is rather unlikely that interception of ROS-induced reactions contributes to any biological effect such plant secondary metabolites may have in humans. *NB*, the situation and significance of these metabolites in other biological materials, such as food products, juices, etc., is of course quite different from that in humans. There is (similar to the case made against and for melatonin above) a plethora of data suggesting that non-antioxidant activities are found with such compounds, including

interference with cellular signaling cascades (Klotz 2015). For example, several plant polyphenols act as kinase inhibitors, in part through competition with the ATP-binding site of kinases (Guerra and Issinger 2019).

Clinical Use (Incl. Side Effects)

The use of "antioxidants" in clinical practice is based upon the hypothesis that an underlying symptom, "oxidative stress," needs to be suppressed. Conceptually, "oxidative stress" is a situation in which the formation of ROS overwhelms the system's capacity of antioxidative defense, leading to cellular damage.

It is extremely difficult to determine whether a clinical situation is not only generally associated with but also mechanistically connected with oxidative stress in a concrete case. Therefore, antioxidants are sometimes used as a side therapy.

One of the major problems is, therefore, that the body's demand for antioxidants is not easy to assess (Grune and Berger 2007; Breusing and Grune 2010; Frijhoff et al. 2015). The determination of some vitamins has become standard clinical practice; however, conclusions as to the antioxidative capacity of the body cannot be drawn from that. For example, vitamin C levels could both reflect the antioxidative status or rather a nutritional habit. The same is true for other vitamins. The status of some trace elements, such as selenium, might give some insight into the endogenous levels of antioxidant selenoproteins, but such data have to be taken with a grain of salt as the distribution of trace elements is highly regulated and hierarchical. Many attempts have been made to assess oxidative tissue damage using various oxidation products, such as products of lipid peroxidation, protein, or DNA damage. However, none of these methods is currently suitable for use in clinical routine, predominantly owing to issues with pre-analysis stabilization of samples in clinical settings and the standardization between laboratories (Breusing et al. 2010; Weber et al. 2013; Augustyniak et al. 2015). Therefore, most of these methods are limited to single laboratory use and for clinical trials.

However, due to intensive research in this area, some diseases are now known to be likely connected with an increased demand on antioxidants. This includes atherosclerosis, some neurodegenerative diseases, chronic inflammation (e.g., rheumatoid diseases), and some cases of poisoning.

In general, most exogenous antioxidants are nutritional compounds, and even at pharmacological doses, they do not usually have serious side effects, often due to a very limited bioavailability.

More recently, however, the concept of "oxidative stress" was complemented by the concept of "oxidative eustress" (in contrast to the damaging "oxidative distress") (Sies et al. 2017). This is owing to the fact that a certain physiological steady-state ROS concentration, resulting from a steady flux of ROS generation and depletion, has signaling and regulatory effects in cellular metabolism and is required for normal cellular function and reactivity. Suppressing such redox regulation might result in dysregulation and cellular damage. Therefore, super-natural, high doses of oxidants might reduce the cellular response to physiological situations. The earlier belief that high doses of antioxidants (especially vitamins) are health promoting is, therefore, outdated. There are no indications that any high dosage of antioxidants in an otherwise healthy person has any positive effect. On the contrary, ongoing research and discussions suggest that consumption of super-natural, high antioxidant doses is not advisable.

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Antiplatelet Drugs

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Synonyms

Platelet aggregation inhibitors; Platelet inhibitors

Definition

Platelets play a central role in primary hemostasis. They are also important in pathological processes leading to thrombosis. Antiplatelet drugs are primarily directed against platelets and inhibit platelet activation by a number of different mechanisms. They are used for the prevention and treatment of thrombotic processes, especially in the arterial vascular system.

Mechanism of Action

Antiplatelet therapy is an important means in the prevention and treatment of thromboembolic artery occlusions in cardiovascular diseases. Platelets are discoid cell fragments, derived from megakaryocytes in the bone marrow that circulate freely in the blood. Under normal conditions they neither adhere to each other nor to other cellular surfaces. However, when blood vessels are damaged at their luminal side, platelets adhere to the exposed subendothelium. Adherent platelets release various factors (see below) that activate other nearby platelets resulting in the recruitment of more platelets at the site of vascular injury. The rapid formation of a "platelet plug" at sites of vascular injury is the main mechanism of primary hemostasis. This is followed by a strengthening of the primary thrombus due to the formation of fibrin fibrils by the coagulation cascade. Platelets also play an important role in pathological conditions since they can become activated on ruptured atherosclerotic plaques or in regions of disturbed blood flow. This in turn leads to thromboembolic complications that underlie common diseases such as myocardial infarction or thrombotic stroke.

Mechanisms of Platelet Activation

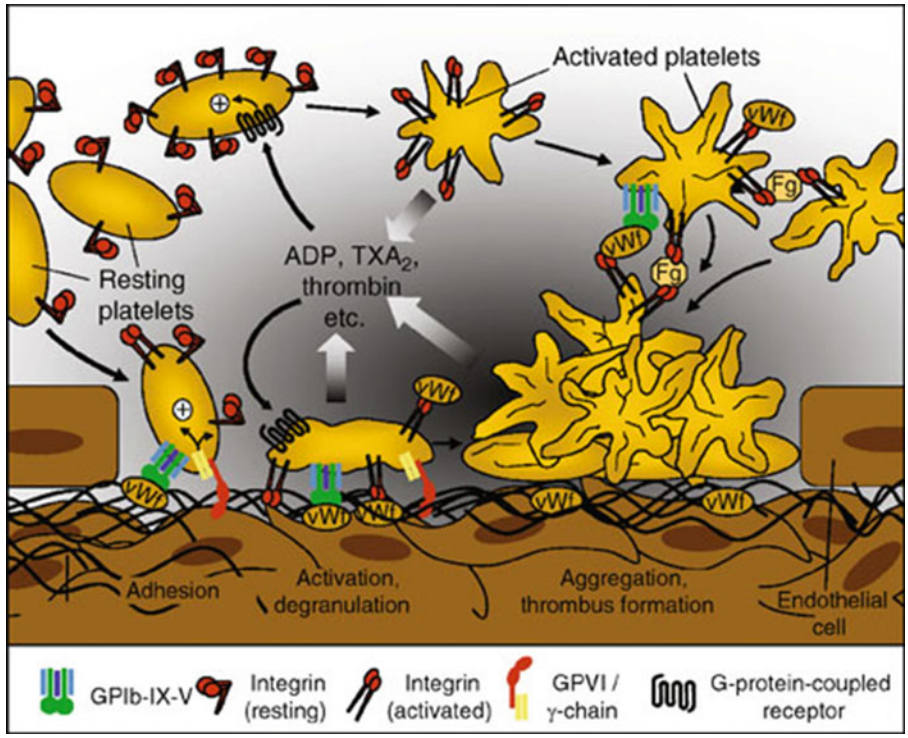
During the first phase of platelet activation, platelets adhere to extracellular matrix proteins of the subendothelium (see Fig. 1) (van der Meijden and Heemskerk 2019). Platelet adhesion is initially mediated by von Willebrand factor (vWf) which after binding to subendothelial collagen changes its conformation and interacts with the platelet receptor complex glycoprotein Ib-IX-V (GPIb-IX-V). This interaction brings platelets in contact with the subendothelium but does not result in a stable interaction. A stable adhesion of platelets is induced by the extracellular matrix protein collagen which via the platelet-specific receptor glycoprotein VI (GPVI) leads to an activation of platelets. This in turn results in the activation of several integrins like integrin α2β1 and αIIbβ3 which then mediate the firm adhesion of platelets to collagen, fibronectin, or laminin exposed at the subendothelial surface.

The formation of a platelet aggregate requires the recruitment of additional platelets from the bloodstream to the injured vessel wall. This process is executed through a variety of diffusible mediators which act through G-protein-coupled receptors. The main mediators involved in this process are adenosine diphosphate (ADP), thromboxane A_2 (TXA_2), and thrombin (factor IIa). These mediators of the second phase of platelet activation are formed in different ways. While ADP is secreted from platelets by exocytosis, the release of TXA_2 follows its new formation in activated platelets. Thrombin can be formed on the surface of activated platelets (see Fig. 2).

Initially, activated platelets change their shape, an event immediately followed by the secretion of platelet granule contents (including ADP, fibrinogen, and serotonin) as well as by platelet aggregation. Aggregation of platelets is mediated by fibrinogen or vWf. They connect platelets by bridging complexes of glycoprotein IIb/IIIa (integrin αIIbβ3) on adjacent platelets, forming a platelet aggregate. Each platelet contains about 50,000–80,000 glycoprotein IIb/IIIa (GPIIb/IIIa) molecules on its surface. In order to bind fibrinogen and vWf, GPIIb/IIIa has to be converted from low-affinity/low-avidity state to a high-affinity/high-avidity state by a process described as inside-out signaling that is initiated during platelet activation (Fig. 2).

Acetylsalicylic Acid (Aspirin)

TXA_2 is produced by activated platelets via the sequential conversion of arachidonic acid by phospholipase A_2, cyclooxygenase-1 (COX-1), and thromboxane synthase. Similar to ADP, TXA_2 acts as a positive feedback mediator. In vascular endothelial cells, COX-1 is involved in the generation of prostacyclin (PGI_2), which inhibits platelet activation and leads to vasodilation. Low doses of acetylsalicylic acid (aspirin) have an antiplatelet effect by inhibiting the TXA_2 production by irreversibly acetylating COX-1 at serine-530 close to the active site of the enzyme

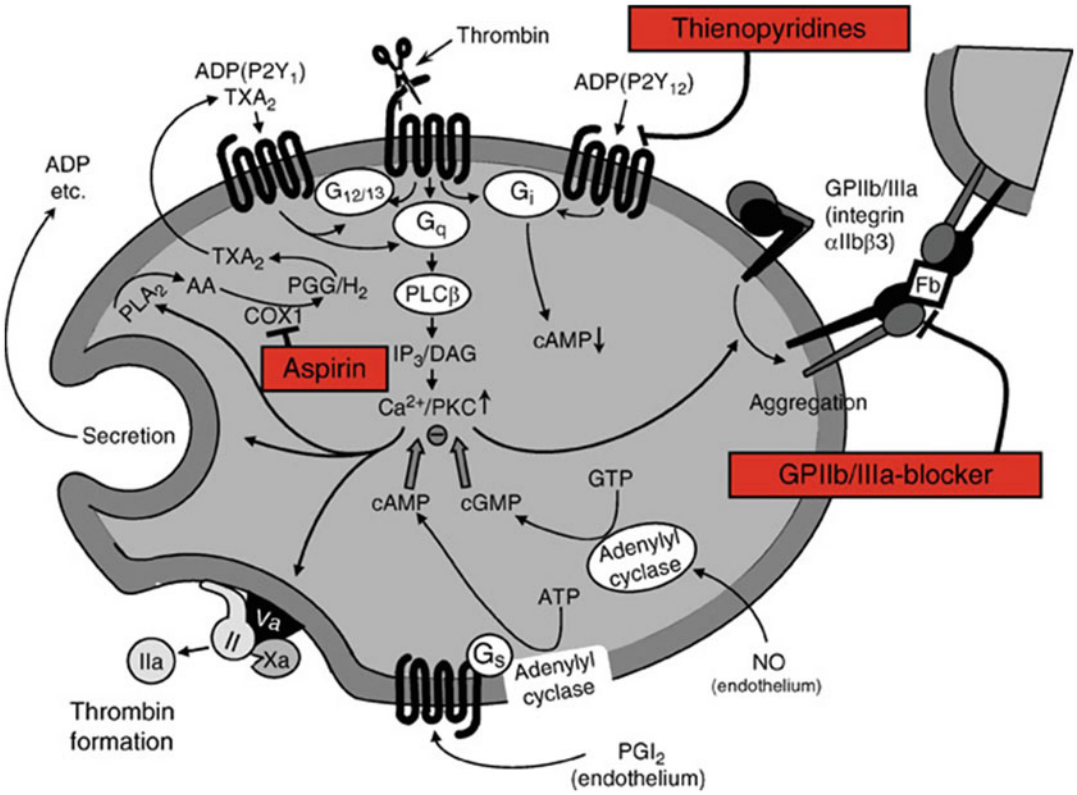


Antiplatelet Drugs, Fig. 1 Platelet adhesion, activation, aggregation, and thrombus formation on subendothelial surface at an injured blood vessel. After an injury of the vascular endothelium, the processes of primary hemostasis (platelet activation) as well as of secondary hemostasis (fibrin formation via the coagulation cascade) are triggered by a variety of stimuli. Shown are only the mechanisms of primary hemostasis. Platelets adhere via their receptor GPIb-IX-V and GPVI mediated by von Willebrand factor (vWf) or binding of extracellular matrix (especially collagen) to the subendothelium. Binding of collagen to GPVI initiates platelet activation resulting in integrin activation and the formation of a variety of diffusible mediators like ADP, thromboxane A_2 (TXA_2), as well as thrombin. These mediators initiate the second phase of platelet thrombus formation by recruiting platelets from the bloodstream into a growing platelet aggregate. The cross-linking of platelets primarily involves the activated integrin αIIbβ3 (GPIIb/IIIa) which can bind the bivalent ligands vWf and fibrinogen (Fb) resulting in the cross-linking of platelets

which interferes with the binding of the substrate arachidonic acid to the enzyme. This results in impaired platelet function for the rest of its life span (7–10 days). Anucleated platelets, in contrast to nucleated cells, are unable to de novo synthesize COX-1. The aspirin doses required for this antiplatelet effect are therefore considerably lower than those necessary to achieve inhibition of prostacyclin formation in endothelial cells or analgetic and antipyretic effects elsewhere in the body. Following oral administration of aspirin, platelets are exposed to a relatively high concentration of aspirin in the portal blood. This may further contribute to the relatively high sensitivity of platelets toward the action of aspirin. Most other tissues are partly protected from irreversible COX-1 inhibition by presystemic metabolization of aspirin to salicylate through esterases in the liver.

P2Y $_{12}$ Receptor Antagonists

ADP is released from activated platelets by the secretion of dense granules and acts through at least three receptors. These are the ionotropic purinoceptor $2X_1$ ($P2X_1$) and two G-protein-coupled receptors, the G_q-coupled purinoceptor $2Y_1$ ($P2Y_1$), and the G_i-coupled $P2Y_{12}$ receptor. The latter has also been termed $P2T_{AC}$ or $P2_{cyc}$ and is targeted by a group of antiplatelet agents. These include thienopyridines – such as clopidogrel and prasugrel. To become activated, clopidogrel and prasugrel require biotransformation by the hepatic CYP3A4 and CYP2C19



Antiplatelet Drugs, Fig. 2 Mechanisms of platelet activation, together with sites of drug action. Most platelet activators function directly or indirectly through G-protein-coupled receptors and induce several intracellular signaling pathways that eventually lead to secretion of granule contents, change of shape, inside-out activation of GPIIb/IIIa (integrin αIIbβ3), the exposure of factor Va, and subsequent formation of the prothrombinase complex which forms thrombin (factor IIa) as well as the activation of phospholipase A_2 (PLA_2). Activation of GPIIb/IIIa allows fibrinogen (Fb) or vWf to cross bridge adjacent platelets. The main pathway that leads to platelet activation involves the G_q/phospholipase C-β (PLC-β)-mediated formation of inositol 1,4,5 trisphosphate (IP_3) and diacyl glycerol (DAG). This in turn results in the release of Ca^{2+} from intracellular stores and the activation of protein kinase C (PKC) isoforms. Major inhibitors of platelet activation are the endothelium-derived mediators nitric oxide (NO) and prostacyclin (PGI_2). While NO via activation of guanylyl cyclase increases cGMP levels, PGI_2 via activation of a G_s-coupled receptor increases the levels of cAMP. Both cyclic nucleotides inhibit via different mechanism signaling processes involved in platelet activation. Aspirin blocks the conversion of arachidonic acid (AA) to prostaglandin G_2 and H_2 (PGG/H_2) by irreversibly inhibiting cyclooxygenase-1 (COX-1). Active metabolites of thienopyridines and other small molecules such as ticagrelor and cangrelor block ADP ($P2Y_{12}$) receptors on platelets, and GPIIb/IIIa blockers interfere with fibrinogen- and vWf-mediated platelet aggregation. TXA_2 stands for thromboxane A_2

enzymes into active metabolites. The active metabolites irreversibly modify the $P2Y_{12}$ receptor. Due to the requirement of the formation of active metabolites, thienopyridines have a delayed onset of action. Similar to the antiplatelet effects of aspirin, the effects of thienopyridines are long-lasting due to the irreversible inhibition of the $P2Y_{12}$ receptor. In contrast to thienopyridines, ticagrelor and cangrelor are no prodrugs, as they block the receptor directly in a reversible manner.

GPIIb/IIIa (Integrin-IIbβ3) Inhibitors

Most antiplatelet drugs only partially inhibit platelet activation. In contrast, blockers of GPIIb/IIIa interfere at the end of the pathway common to platelet aggregation. They prevent fibrinogen and vWf from binding to activated GPIIb/IIIa and can therefore completely inhibit platelet aggregation. The first GPIIb/IIIa antagonist developed was a hybrid human/murine monoclonal antibody. Its Fab fragment, termed abciximab, is clinically used and functions in a noncompetitive manner.

An alternative approach to block GPIIb/IIIa involves the use of peptides that mimic short protein sequences of fibrinogen or vWf. Several peptides (e.g., the cyclic heptapeptide eptifibatide) or nonpeptidic, low molecular weight compounds (e.g., tirofiban, lamifiban) have been developed and function as competitive antagonists (Table 1).

Others

The proteolytic enzyme thrombin is known to play a crucial role in the overall thrombotic event leading to both arterial and venous thrombosis by transforming fibrinogen into fibrin and by serving as a direct platelet activator. Thrombin exerts its effects on platelets via G-protein-coupled protease-activated receptors (PAR-1 and PAR-4 in human platelets). Thrombin-dependent receptor activation is achieved by cleaving an N-terminal extracellular peptide. Exposure of the newly generated N-terminal region functions as a tethered ligand for the receptor. Substances that directly bind to thrombin have been developed. The 65 amino acid long protein hirudin, originally isolated from the medical leech, *Hirudo medicinalis*, as well as related analogs have been recombinantly produced. They bind with the stoichiometry of 1:1 to thrombin and prevent its proteolytic action on fibrinogen as well as its binding to and the activation of PAR. The proteolytic activity of thrombin can be inhibited also by several small molecules such as argatroban and dabigatran. Since thrombin inhibitors primarily act by inhibiting thrombin-dependent fibrin formation, they are generally classified as anticoagulants.

Since platelets are the major source of TXA_2 production and action, inhibitors of thromboxane synthase and TXA_2 receptor (TP) antagonists are being developed. TXA_2 synthesis inhibitors may have some disadvantages as they lead to the accumulation of cyclic endoperoxides (e.g., PGH_2) that are themselves agonists at the TXA_2 receptor.

The major physiological inhibitors of platelet activation are endothelium-derived mediators like prostacyclin (PGI_2) which via a G_s-coupled receptor activates the formation of cAMP formation by adenylyl cyclase as well as nitric oxide (NO) which stimulates the formation of cGMP by activating guanylyl cyclase. NO-generating drugs like organic nitrates lead to platelet inhibition; however, their main effect is on the vascular smooth muscle. Dipyridamole can inhibit the degradation of cAMP by inhibition of cyclic nucleotide phosphodiesterase and has been used as an antiplatelet agent. However, its clinical usefulness is not clear.

Clinical Use

Due to the pivotal role of platelets in thrombus formation, especially in the arterial system, inhibition of platelet function has become a central pharmacological approach (Depta and Bhatt 2015; Franchi et al. 2015; McFadyen et al. 2018; Metharom et al. 2015). Antiplatelet drugs are given in order to prevent and treat thromboembolic diseases such as coronary heart disease, peripheral disease, and cerebrovascular disease. They have also revolutionized the procedures of invasive coronary interventions as they reduce the risk of restenosis and thrombosis.

Aspirin leads to maximal antithrombotic effects at doses much lower than required for other actions of the drug. Clinical trials have

Antiplatelet Drugs, Table 1 Pharmacological properties of GP IIb/IIIa inhibitors

	Abciximab	Eptifibatide	Tirofiban
Molecular weight (Da)	50,000	800	500
Integrin selectivity	αIIbβ3; αVβ3	αIIbβ3	αIIbβ3
Affinity for αIIbβ3 (K_D; nmol/l)	5	120	15
Plasma half life	0.5 h	2–2.5 h	2 h
Duration of action	12–24 h	2–2.5 h	2 h
Elimination	Proteolysis/renal	Mainly renal	Mainly renal

demonstrated that aspirin is maximally effective as an antithrombotic drug at daily doses of 75–160 mg. Higher doses have no advantage but increase the frequency of side effects, especially bleeding and upper gastrointestinal symptoms. Despite the development of various other compounds, aspirin has remained the gold standard for antiplatelet drugs due to its relative safety and extremely low cost. Several studies have demonstrated a beneficial role for aspirin as an adjunctive therapy in unstable angina and acute myocardial infarction. Mortality and disease progression were significantly reduced by low-dose aspirin treatment. Patients with a history of arterial thromboembolism including myocardial infarction, stroke, transient ischemic attack, or unstable angina were shown to benefit from low-dose aspirin treatment in several trials. The overall rate of mortality, as well as the occurrence of further vascular events, was reduced in these patients. The results of these studies led to the recommendation to use aspirin for secondary prevention of arterial thromboembolism. However, aspirin is not generally recommended for primary prevention of arterial thromboembolism. A possible beneficial effect, such as a decreased risk of nonfatal myocardial infarction, may outweigh the risk of hemorrhagic complications only in a population already at high risk of cardiovascular diseases but not in a population of average health. Aspirin may also be beneficial as a prophylactic agent to reduce the risk of deep venous thrombosis and pulmonary embolism. However, the effectiveness compared to existing therapies remains to be determined; anticoagulants are still the mainstay of treatment in these conditions.

$P2Y_{12}$ receptor antagonists are principally suited to treat conditions that respond to aspirin. In various trials, clopidogrel has been shown to be safe and similarly effective as aspirin. In patients at high risk for cerebrovascular events, thienopyridines seem to be somewhat more effective than aspirin in preventing serious vascular complications. $P2Y_{12}$ receptor antagonists may be used instead of aspirin when the latter is not tolerated. However, aspirin still remains the first choice in most cases due to its low cost, relative safety, and well-documented efficacy. $P2Y_{12}$ receptor antagonists can also be given in patients with acute coronary syndrome as well as during coronary interventions.

GPIIb/IIIa antagonists have to be administered parenterally. They can be used prophylactically during intracoronary interventions such as percutaneous transluminal revascularization with balloon angioplasty or intracoronary stenting, as well as to treat acute coronary syndromes like unstable angina and acute myocardial infarction. They are, however, usually not the first-choice therapy. The main complications are bleeding and thrombocytopenia. The bleeding risk appears to increase further with concomitant therapy with heparin at standard doses.

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Antiprotozoal Drugs

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Synonyms

Antiprotozoan chemotherapeutics; Protocidal drugs

Definition

Protozoa are unicellular eukaryotes and a subregnum of the animal kingdom. Some protozoa exhibit a parasitic life style and are pathogenic to humans, animals, and plants. An estimated 1.5 billion people suffer from protozoal infections, with malaria (*Plasmodium* spp.) alone causing an estimated 219 million clinical cases each year. Other examples of important human infectious diseases with protozoan etiology include toxoplasmosis (*Toxoplasma gondii*), African sleeping sickness (*Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*), Chagas disease (*Trypanosoma cruzi*), visceral, mucocutaneous, and cutaneous leishmaniasis (*Leishmania* spp.), amoebic colitis and liver abscess (*Entamoeba histolytica*), and lamblic enteritis (*Giardia lamblia*). Although there is a huge demand for antiprotozoal drugs, particularly drugs affordable by people living in developing countries, the incentives to develop such drugs are low because of an uncertain return of investments. Accordingly, there is a dearth of efficacious and safe drugs. Only in recent years has this problem been addressed through several private-public partnerships (Aerts et al. 2017). For instance, in 2012 a joint initiative by pharmaceutical companies, donors, endemic countries, and nongovernment organizations signed the London declaration on Neglected Tropical Diseases with the objective of controlling the 10 most important neglected infectious diseases of the tropics and subtropics. Another example includes the Drugs for Neglected Disease Initiative (http://www.dndi.org/), which has had a major impact on the treatment of African trypanosomiasis by fostering the development of nifurtimox-eflornithine combination therapy, fexinidazol as the first all-oral treatment (which was approved by the European Medicines Agency in 2018), and acoziborole as a single dose oral treatment (Drugs for Neglected Diseases Initiative (DNDi) 2018; Pierce et al. 2017). In the following chapter, important antiprotozoal drugs, including their modes of action, will be discussed.

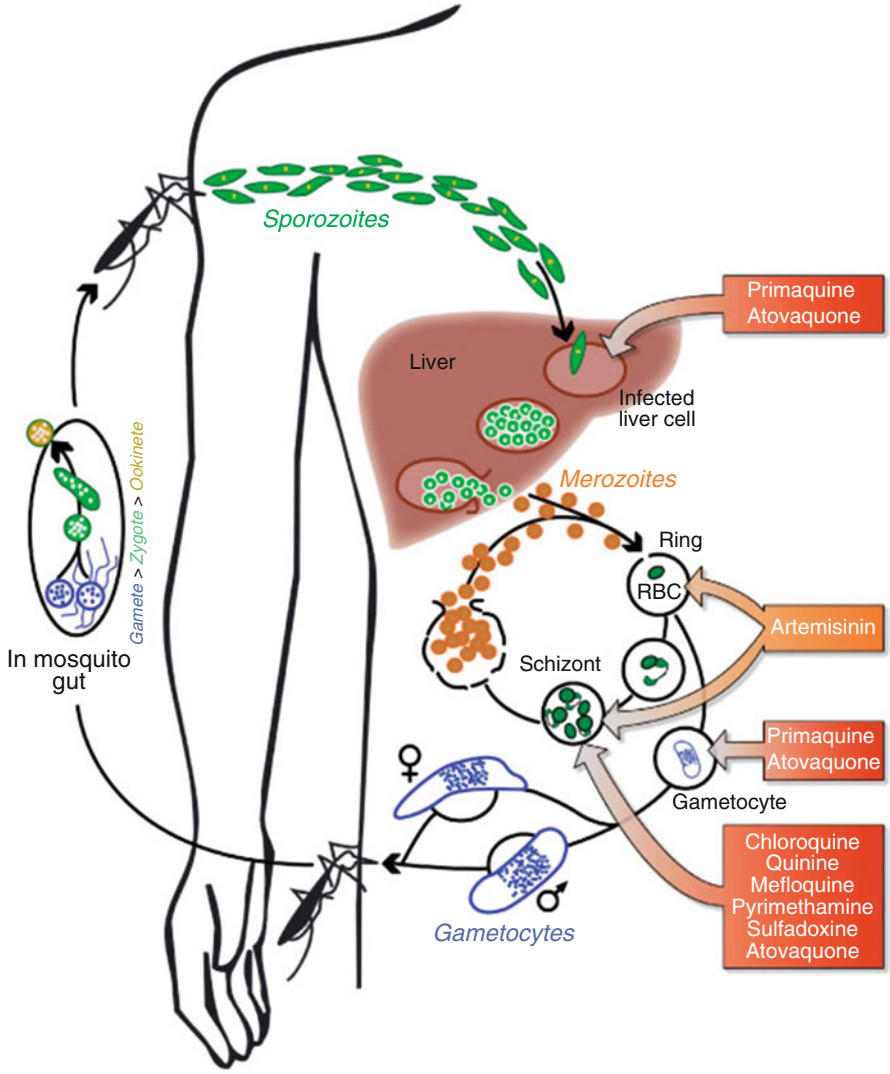
Antimalarial Drugs

Five different protozoa of the genus *Plasmodium* – *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi* – can cause malaria in humans. *P. falciparum* is the most virulent, being responsible for virtually all fatal malaria cases. Humans are infected by a feeding female Anopheles mosquito (Fig. 1). The clinical symptoms of malaria are associated with the development of the parasite within human red blood cells, whereas the liver stages remain asymptomatic. The following drugs (in alphabetical order) are currently in use for the treatment of malaria (Fig. 2) (White et al. 2014; World Health Organization (WHO) 2015).

Mechanism of Action

Amodiaquine, a Mannich base 4-aminoquinoline, eliminates blood stage parasites. Its mode of action is similar to that of chloroquine (see below) and there is some cross-resistance (Martin et al. 2018).

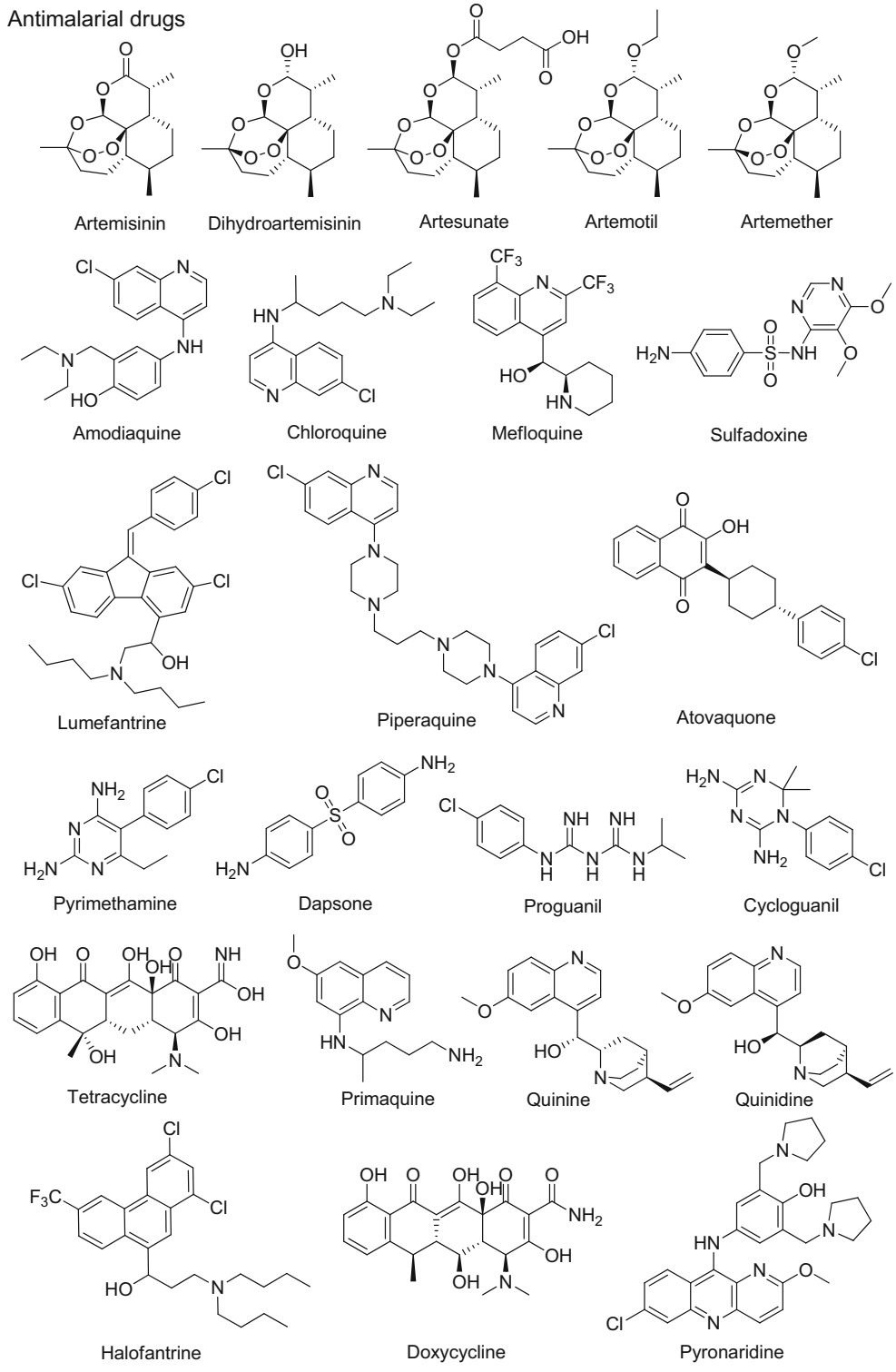
Artemisinin and its derivatives – artesunate, dihydroartemisinin, and arthemether – kill both asexual and sexual blood stages (Fig. 1). However, artemisinins are quickly eliminated from the body, resulting in parasite recrudescence, and are therefore combined with schizontocides that have a longer biological half-life, such as amodiaquine, lumefantrine, mefloquine, piperaquine, and sulfadoxine/pyrimethamine (White et al. 2014; World Health Organization (WHO) 2015). Artemisinin is a sesquiterpene lactone extracted from the leaves of *Artemisia annua*. Also known as qinghaosu, artemisinin has been used in China for the treatment of fever for more than 1000 years. Artemisinins are activated in the parasite via a mechanism that involves iron-catalyzed breakage of their endoperoxide bridge, resulting in carbon centered radicals that subsequently alkylate proteins and other biomolecules (Fig. 3). Damaged proteins are usually polyubiquitinated and targeted for degradation via the proteasome. However, the build-up of damaged and polyubiquitinated proteins during artemisinin exposure overextends the proteasome capacity, which, together with an ensuing stress response, leads to parasite death (Ross and Fidock 2019; Tilley et al. 2016). Artemisinin resistance has emerged at the Thai/Cambodian border in form of a delayed parasite clearance rate. Artemisinin resistance is associated with mutations in the kelch



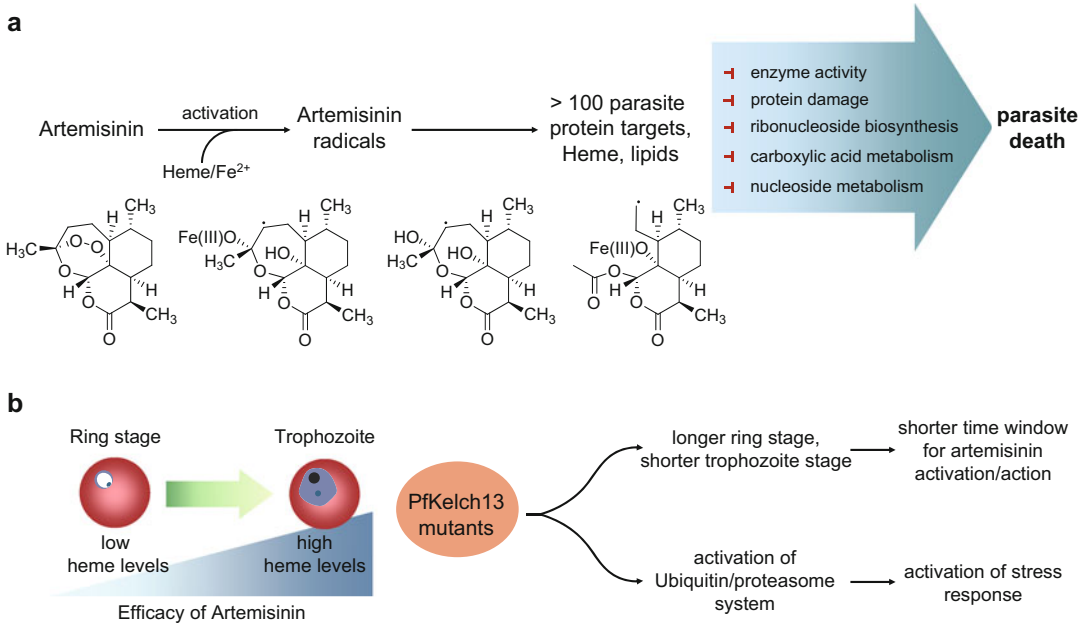
Antiprotozoal Drugs, Fig. 1 Life cycle of malaria parasites and site of action of different antimalarial drugs. Malaria is caused by protozoan parasites of the genus *Plasmodium.* Infected *Anopheles* mosquitoes transmit the parasite to humans during blood feeding. The infective stages are the sporozoites, which invade liver cells where they replicate to form merozoites. Upon rupture of the infected hepatocyte, merozoites are released into the blood stream where they infect erythrocytes. Within the erythrocyte, the parasites develop from ring stages to trophozoits and then to schizonts. When the infected erythrocyte finally ruptures, merozoites are released, which again invade erythrocytes. Some intraerythocytic ring stages develop to sexual stages (gametocytes). Gametocytes are taken up by feeding *Anophelines.* Within the mosquito, the parasites develop into gametes, zygotes, ookinets, and finally sporozoites. Primaquine and atovaquone are effective against liver forms of all *Plasmodia* including dormant stages of *P. vivax* and *P. ovale.* Primaquine also acts against gametocytes. Artemisinin destroys intraerythrocytic ring stage parasites and schizonts. The so-called schizonticidal drugs, chloroquine, quinine, mefloquine, pyrimethamine, and sulfadoxine, act against intraerythrocytic schizonts

propeller domain protein 13, which seems to play a role in ubiquitin-mediated protein degradation and oxidative stress response (Fig. 3) (Ross and Fidock 2019; Tilley et al. 2016).

Atovaquone, a hydroxynaphthoquinone, selectively inhibits the respiratory chain of protozoan mitochondria at the cytochrome bc1 complex (complex III) by mimicking the natural substrate,



Antiprotozoal Drugs, Fig. 2 Chemical structure of relevant antimalarial drugs



Antiprotozoal Drugs, Fig. 3 Proposed mode of action and mechanism of resistance of artemisinins. (**a**) Artemisinins are activated via reaction with ferrous iron, yielding carbon-centered radicals that subsequently alkylate proteins and other biomolecules. As a result, the intracellular processes that degrade damaged proteins via the proteasome are overpowered, leading to a break-down of metabolic pathways. (**b**) Artemisinins act on all asexual blood stages, although the efficacy increases with increasing amounts of heme levels. Artemisinin resistance is associated with mutations in the kelch propeller domain protein 13, which seems to play a role in ubiquitin-mediated protein degradation and oxidative stress response. In addition, artemisinin resistance involves a quiescence mechanism by which parasites remain in a ring-lake stage to outwait toxic drug concentration

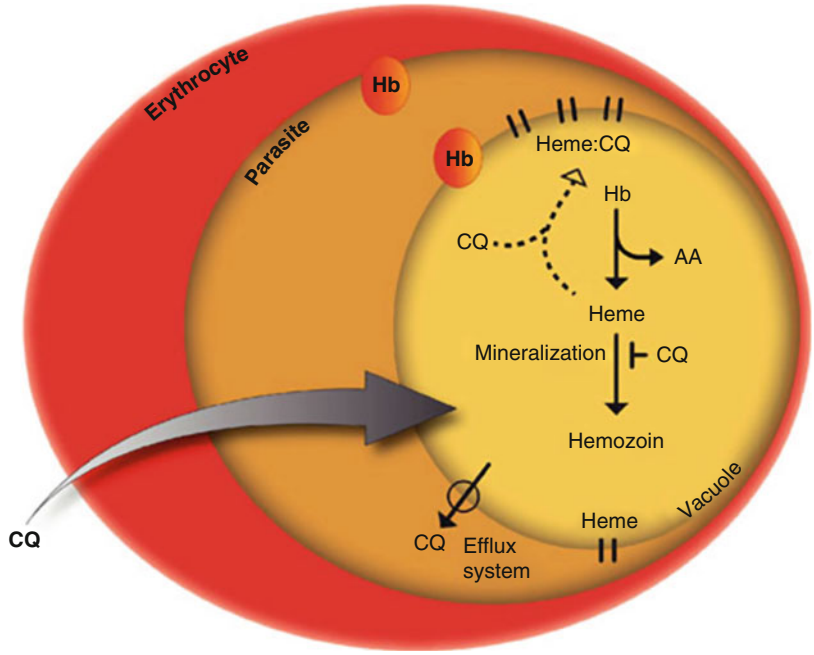
ubiquinone. Inhibition of cytochrome bc1 disrupts the mitochondrial electron transfer chain and leads to a breakdown of the mitochondrial membrane potential. Atovaquone is effective against all parasite stages in humans, including the liver stages.

Chloroquine, a 4-aminoquinoline, targets the intraerythrocytic stages of malarial parasites (Fig. 1). Its mode of action is intricately linked with the plasmodial heme metabolism (Fig. 4). During development within erythrocytes, *Plasmodia* feed on the host cell's hemoglobin, which is digested within an acidic food vacuole. Heme released during hemoglobin proteolysis is highly cytotoxic and perforates cellular membranes. Malaria parasites detoxify heme through biomineralization within their food vacuoles to insoluble and inert hemozoin (malaria pigment). Chloroquine, which accumulates in the food vacuole, prevents heme biomineralization by capping the growing biomineral and by forming complexes with heme, resulting in a build-up of heme and heme–chloroquine complexes. Chloroquine resistance is conferred by mutations in a food vacuolar transporter, termed the *P. falciparum* chloroquine resistance transporter (PfCRT), that expels drugs from the food vacuolar lumen (Fig. 4) (Ross and Fidock 2019).

Dapsone, an aromatic sulfone, is administered in combination with a proguanil derivative. Dapsone inhibits the plasmodial dihydropteroate synthase (DHPS) (Fig. 5).

Tetracycline and its derivative doxycycline are antibiotics widely used in the treatment of bacterial infections. They also exert an antimalarial activity. Tetracyclines inhibit the binding of aminoacyl-tRNA to the ribosome during protein synthesis.

Piperaquine, a bisquinoline, is a rapid acting blood schizontocide. It inhibits the heme



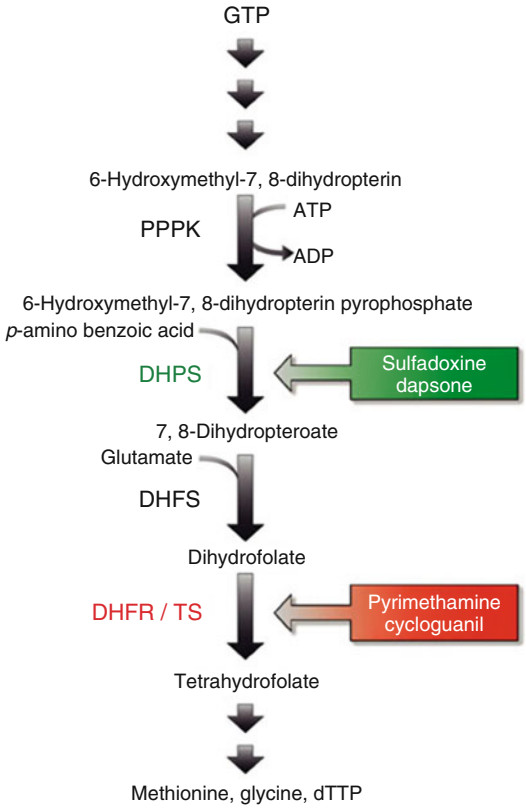
Antiprotozoal Drugs, Fig. 4 The antimalarial activity of chloroquine. Chloroquine's mode of action is associated with heme detoxification. During intraerythrocytic development, *P. falciparum* degrades hemoglobin down to amino acids (AA) in its acidic food vacuole. Heme released from hemoglobin (Hb) is toxic and destroys cellular membranes unless it is converted to an inert biomineral, termed hemozoin or malaria pigment. Chloroquine (CQ) binds to heme, thereby preventing its biomineralization. The build-up of membrane-lytic heme/chloroquine complexes kills the parasite. Chloroquine resistant parasites have acquired an efflux system that expels the drug from the food vacuole

detoxification pathway. Resistance to piperaquine is associated with distinct mutations in the chloroquine resistance transporter PfCRT (Ross and Fidock 2019).

Primaquine, an 8-aminoquinoline, eradicates the dormant stages (hypnozoites) of *P. vivax* and *P. ovale* from the liver. Its mode of action remains obscure.

Proguanil appears to have a dual activity. Part of it is metabolized to cycloguanil, which subsequently inhibits the protozaon dihydrofolate reductase/thymidylate synthase (DHFR/TS) (Fig. 5). Proguanil acts synergistically in combination with atovaquone by lowering the atovaquone concentration required to disrupt the mitochondrial membrane potential.

Pyrimethamine, cycloguanil, and sulfadoxine (sulfadiazine) are folate antagonists that interfere with the folic acid biosynthesis pathway in malarial parasites and other protozoa, including *T. gondii* (Fig. 5) (Ross and Fidock 2019). Folate is an essential precursor of the pyrimidine deoxythymidintriphosphate (dTTP) and the amino acids serine and methionine. Both protozoa and mammalian cells require folate for DNA and protein synthesis. However, protozoa can either synthesize dihydrofolate de novo or salvage folate precursors, whereas mammalian cells have no de novo dihydrofolate synthesis and must rely on dietary sources. By acting as an analogue of *p*-aminobenzoic acid, sulfadoxine (sulfadiazine) inhibits the DHPS, which then fails to convert dihydropteroate to hydroxymethyldihydropterin, resulting in a lack of dihydrofolate in the parasite. This mechanism does not affect the mammalian cells. Pyrimethamine and cycloguanil, the active metabolite of proguanil, act further down in the folic acid pathway by inhibiting the DHFR/TS enzyme complex. In mammalian cells, the DHFR and the TS are two independent enzymes.



Antiprotozoal Drugs, Fig. 5 Mode of action of folate antagonists in protozoa. Protozoa are capable of de novo synthesis of dihydrofolate, a precursor of thymidine, serine, and methionine. The 6-hydroxymethyl-7,8-dihydropterin pyrophosphate pyrophosphokinase (PPPK) and DHPS form one enzyme complex with two distinct active sites. The PPPK transforms GTP to 6-hydroxymethyl-7,8-dihydropterin and then to 6-hydroxymethyl-7,8-dihydropterin pyrophosphate. The product is linked to p-aminobenzoic acid (PABA) by DHPS, forming 7,8-dihydropteroate. Sulfadoxine exerts its activity by acting as a substrate analogue of PABA in this reaction. Glutamate is added to 7,8-dihydropterate by dihydrofolate synthase (DHFS) resulting in dihydrofolate. Dihydrofolate reductase (DHFR) and thymidylate synthase (TS) form a single enzyme complex with two enzymatically active sites. The catalytic site responsible for DHFR activity converts dihydrofolate into tetrahydrofolate. Pyrimethamine and cycloguanil exert their antimalarial activity by inhibiting the protozoal DHFR. The site of DHFR/TS responsible for TS activity converts tetrahydrofolate into deoxythymidintriphosphate (dTTP)

The protozoal DHFR/TS enzyme complex has a higher affinity for pyrimethamine and cycloguanil than does the human DHFR, which explains their high antiprotozoal activity. To avoid deficiency of folic acid in patients treated with antifolate antagonists, folinic acid should be administered. Pyrimethamine is a blood schizontocide and further acts against the liver stages (Fig. 1). Since resistance to pyrimethamine occurs rapidly due to single-point mutations within the DHFR/TS enzyme complex, pyrimethamine is only used in combination with sulfonamides for curative treatment (World Health Organization (WHO) 2015).

Quinine, an arylaminoalcohol, was the first antimalarial known to the Western world. It was originally produced from the bark of the cinchona tree and distributed as a powdery substance, which became known as Jesuits powder. Several potent antimalarials are derived from quinine, including lumefantrine, mefloquine, halofantrine, and quinidine, the dextrarotatory diastereoisomer of quinine. All arylaminoalcohols are believed to kill asexual blood stages by inhibiting heme detoxification in the parasite's food vacuole (Fig. 4), although the mechanism of action is not well understood.

Clinical Use

Malaria treatment has relied on a small number of drug classes: the aminoquinolines (such as chloroquine, amodiaquine, piperaquine); the aminoalcohols (mefloquine, halofantrine, lumefantrine); antifolates (sulfadoxine, pyrimethamine, proguanil); endoperoxides (e.g., artemisinin and derivatives); and the hydroxynaphtoquinone, atovaquone. Various degrees of resistance and geographic distributions of resistance phenotypes have developed for all classes of antimalarials (Martin et al. 2018; Ross and Fidock 2019). Chloroquine now fails in the treatment of falciparum malaria almost everywhere. Even resistance to artemisinin derivatives and artemisinin combination therapies have emerged and are threatening the efficacy of the current mainstay of malaria chemotherapy (Tilley et al. 2016). To prevent, or at least slow down, the emergence of resistance, compounds with different modes of action are being combined. The choice of drug, the route of drug application, and the

dose regimen depend on the type of infection and the severity of the disease. The primary objective of treating uncomplicated malaria is to cure the infection, whereas in severe malaria it is to prevent death.

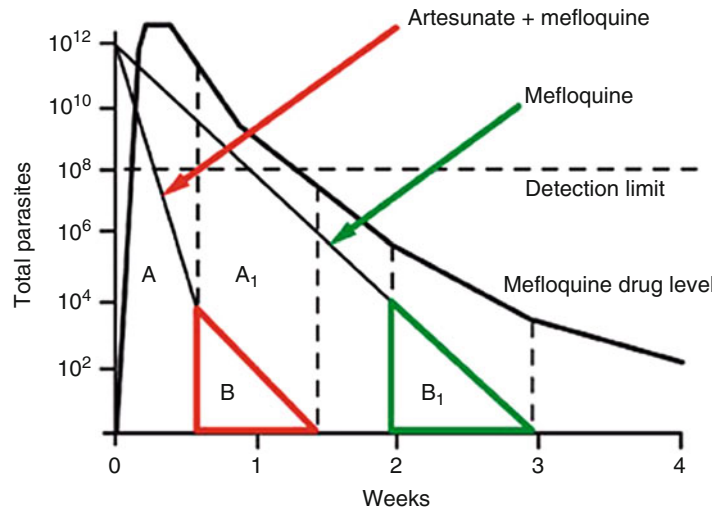
Severe Malaria

Artesunate is the treatment of choice for severe malaria in adults and children (World Health Organization (WHO) 2015). In severe malaria, rapid clearance of parasites is vital. Artesunate achieves this by damaging circulating ring stages, which are subsequently removed from circulation by the spleen. As a consequence, the parasite load rapidly declines by approximately four orders of magnitude within 48 h. Moreover, as the development of ring stages to the trophozoites is disrupted by artesunate, the progression of the disease is stopped. Trophozoites are the main pathological cause of falciparum malaria as they acquire cytoadhesive properties and sequester in the deep vascular bed of inner organs, including the brain, lung, and placenta, where they can cause hypoxia, impaired tissue perfusion, and the syndromes associated with cerebral and maternal malaria (White et al. 2014). Artesunate is water-soluble and is given by intravenous or intramuscular injection. In case artesunate is unavailable, artemether is preferred over quinine (World Health Organization (WHO) 2015). Artemether is applied intramuscularly.

Uncomplicated Malaria

The treatment of uncomplicated malaria seeks to prevent recrudescence and, at the same time, tries to prevent the development of resistance. This is the rationale behind artemisinin-based combination therapies (ACT). Artemisinin and its derivates (artesunate, artemether, artemotil, dihydroartemisinin) are clearing both parasitaemia and symptoms rapidly (White et al. 2014; World Health Organization (WHO) 2015). There is little difference in absorption and bioavailability among the different artemisinin derivatives. Due to the extraordinary high parasite clearance rate, artemisinin and its derivates quickly reduce the total burden of parasites to very low levels (Fig. 6). Thereafter, the eradication of the remaining parasites relies on the partner drug (Fig. 6). The partner drug needs to be effective and parasiticidal concentrations have to be sustained until all parasites have been killed. A slowly eliminated drug is ideal. The artemisinin derivatives are well tolerated, although type 1 hypersensitivity reactions have been reported in a few rare cases.

ACTs with amodiaquine, atovaquone–proguanil, chloroquine, clindamycin, doxycycline, lumefantrine, mefloquine, piperaquine, pyronaridine, proguanil–dapsone, sulfadoxine–pyrimethamine, and tetracycline have been evaluated in trials. The following ACTs are currently recommended for all cases of uncomplicated



Antiprotozoal Drugs, Fig. 6 Artemisinin combination therapy (ACT): Adding a 3-days artesunate course to mefloquine clears the parasitaemia much more rapidly ($A_1 \rightarrow A$). The remaining parasites are exposed to higher mefloquine levels in ACT (B) compared to mefloquine monotherapy (B_1) (with permission White, 1997 Antimicrob Agents Chemother 41:1413–1422)

falciparum malaria: artemether–lumefantrine, artesunate-mefloquine, dihydroartemisinin-piperaquine (White et al. 2014; World Health Organization (WHO) 2015). In regions of low antifolate and amodiaquine resistance, artesunate-sulfadoxine-pyrimethamine or atresunate amodiaquine can also be used (White et al. 2014; World Health Organization (WHO) 2015). In South-East Asia particularly in western Cambodia and the Thailand-Myanmar border, artemisinin-resistant *P. falciparum* has emerged, limiting the use of ACTs. Resistant parasites show delayed parasite clearance rated of more than 3 days (White et al. 2014). Amodiaquine, sulfadoxine–pyrimethamine, and, to a lesser extent, mefloquine resistance is also present with a prevalence of approximately 20% in this region. The Worldwide Antimalarial Resistance Network (http://www.wwarn.org) provides up-to-date information regarding the prevalence and distribution of antimalarial drug resistance.

Malaria Treatment and Chemoprophylaxis in Nonimmune People

Travelers treated for malaria after returning to non-endemic regions are treated along the same lines as above regarding uncomplicated and severe forms of the disease. Considerations with respect to development of resistance can be neglected. The following drugs are recommended: artemether–lumefantrine, dihydroartemisinin-piperaquine, atovaquone–proguanil, for uncomplicated malaria and intravenous artesunate for severe malaria or, if artesunate is not available quinine plus doxycycline or clindamycin (World Health Organization (WHO) 2015). For the millions of nonimmune travelers to malaria-endemic areas, chemoprophylaxis (high-risk areas) and stand-by treatment (low-risk areas) are the corner stones of malaria prevention (World Health Organization (WHO) 2015), along with exposition prophylaxis using repellent and insecticide-impregnated bednets.

Intermittent Preventive Therapy in Pregnant Women and Infants

In endemic areas, chemoprophylaxis has been abandoned for a variety of reasons, mainly due to sustainability problems and the risk of contributing to the development of resistance. Intermittent preventive therapy (IPT), however, appears to be an alternative to protect pregnant women (IPT_p) and children during the first year of their life (IPT_i) (World Health Organization (WHO) 2015).

Antimalarial Drugs (*P. vivax, P. ovale, P. malariae*)

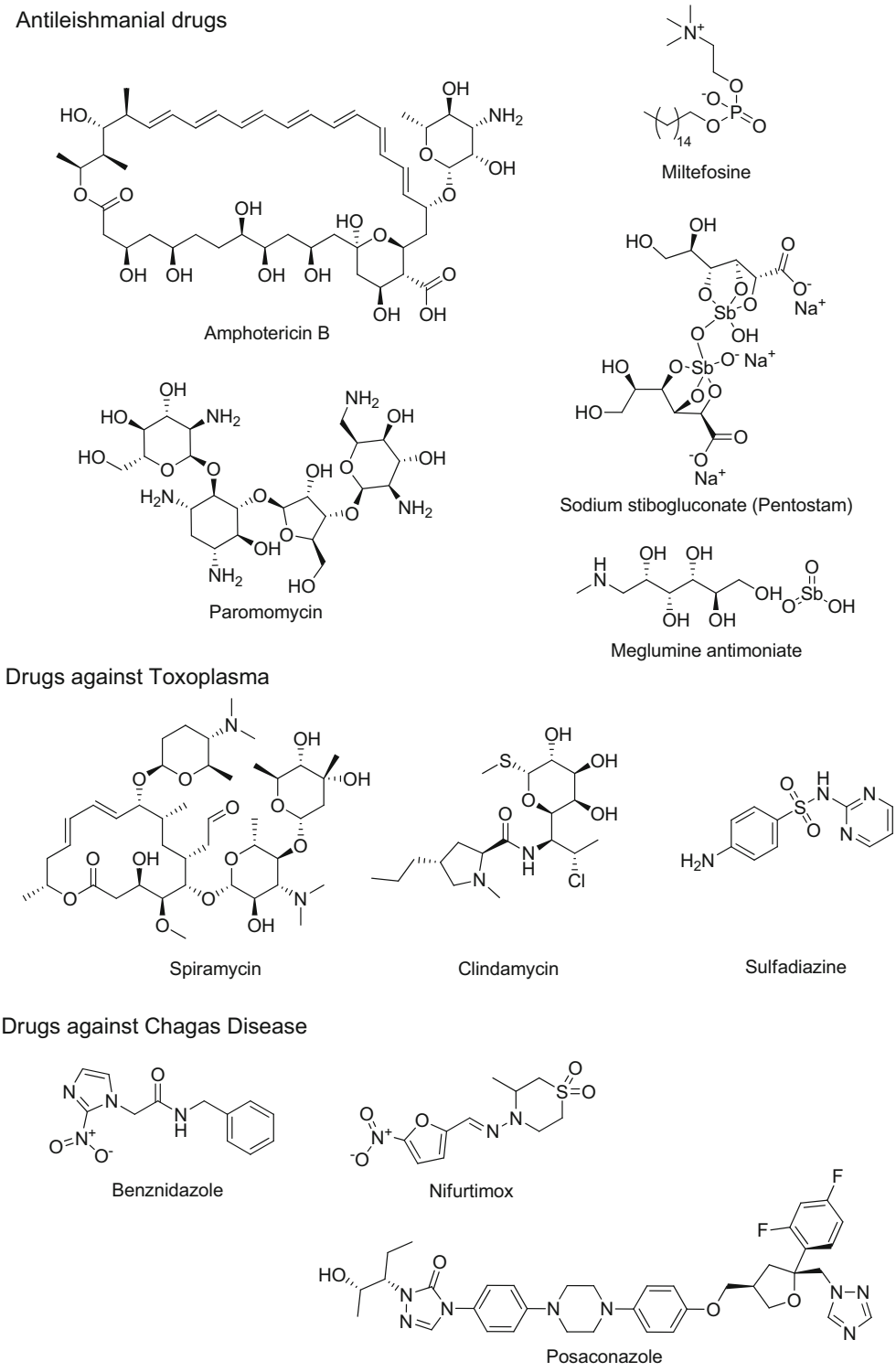
Resistance of *P. vivax* to chloroquine occurs, but it is geographically limited. Chloroquine is recommended for the treatment of chloroquine sensitive *P. vivax*, *P. ovale*, and *P. malariae* infections. Alternatively artemisinin-combination treatment can be prescribed. *P. vivax* and *P. ovale* produce hypnozoites, parasite stages in the liver that can produce multiple relapses. To achieve radical cure of *P. vivax* and *P. ovale* infection, treatment with schizonticidal drugs must be followed by treatment with primaquine to eliminate the dormant liver stages (White et al. 2014; World Health Organization (WHO) 2015).

Antileishmanial Drugs

Leishmaniasis is a disease complex caused by different species of *Leishmania*. The parasite, which is transmitted to humans by the bite of phlebotomine sandflies, multiplies within human macrophages. There are an estimated one million cases in approximately 97 countries each year and every year there are 60,000–90,000 new cases of visceral leishmaniasis.

Mechanism of Action

Amphotericin B is a polyene antibiotic (Fig. 7), used also in the therapy of systemic fungal infections. Its mode of action exploits differences in membrane composition between the pathogen and the human host. Ergosterol, the predominant sterol of fungi, plants, and some protozoan parasites, interacts with Amphotericin B, resulting in an increased ion permeability of the membrane (Ghorbani and Farhoudi 2018; Ponte-Sucre et al. 2017). Human membranes contain cholesterol, which has a low affinity for amphotericin B.



Antiprotozoal Drugs, Fig. 7 Chemical structure of relevant drugs for the treatment of Leishmaniasis, Toxoplasmosis, and Chagas disease

Miltefosine (Fig. 7), an alkylphosphocholine derivative, is a new antileishmanial drug and the first effective oral treatment of visceral leishmaniasis. However, there are concerns regarding teratogenicity, rapid emergence of resistance, and variable cure rates, possibly due to species differences in drug sensitivity. The mechanism of action of miltefosine seems to be complex, by interfering with intracellular Ca^{2+} homoeostasis, phosphatidylcholine synthesis, and mitochondrial cytochrome c oxidase (Ghorbani and Farhoudi 2018).

The pentavalent antimonial drugs, sodium stibogluconate and meglumine antimonate (Fig. 7), are prodrugs that require biological reduction to the trivalent form Sb(III) for antileishmanial activity. Sb(III) seems to inhibit the leishmanial trypanothione reductase, which, together with a depletion of thiols, results in a breakdown of the cellular thiol redox potential (Ponte-Sucre et al. 2017). *Leishmania* and other kinetoplastidae possess an unusual antioxidant termed trypanothione, which is composed of two molecules of glutathione joined by a spermidine linker. Trypanothione protects the cell from oxidative stress by reducing disulfide bonds formed within proteins. The trypanothione reductase regenerates oxidated trypanothione.

Clinical Use

Management of the disease varies with clinical presentation, host, geographic area, species, availability of drugs, and costs of treatment.

Pentavalent antimonial drugs have been the cornerstone of antileishmanial therapy for decades, in spite of their general toxicity (Burza et al. 2018; Ponte-Sucre et al. 2017). Pentavalent antimonial drugs are available in two formulations: sodium stibogluconate and meglumine antimoniate. Antimonials are cardiotoxic and painful when administered by intramuscular injection. Resistance to pentavalent antimonial drugs is widespread in India and is no longer recommended in this part of the world. They are, however, still used elsewhere, particularly in East Africa where resistance has remained low. Liposomal Amphotericin B is the drug of choice in the Indian subcontinent (Burza et al. 2018).

Liposomal Amphotericin B (LAMB) is a highly effective drug against visceral leishmaniasis with remarkably few side effects. Short treatment courses over a period of 3–5 days are recommended by the WHO in the Indian subcontinent. In some instances combination treatment is used (see below).

Amphotericin B: This second line drug has is poorly tolerated due to hypokalemia and nephrotoxicity. Prolonged treatment is necessary.

Paromomycin (PM), an oral aminoglycoside, is effective as a topical treatment for cutaneous leishmaniasis and, as a parenteral drug, against visceral leishmaniasis (Ponte-Sucre et al. 2017). Injectable paromomycin has been registered in India in 2006. It can be given in combination with LAMB or miltefosine (MF).

Miltefosine (MF): The antileishmanial activity of this anticancer drug was discovered in the mid-1980s. It has been used as an oral drug against visceral and cutaneous/mucocutaeous leishmaniasis in India in 2002. After more than a decade of use, there is evidence of reduced effectiveness. Treatment combinations with LAMB and PM exist.

Pentavalent antimonials (SbV) are the treatment of choice in combination with intramuscular PM in East Africa.

Antitoxoplasma Drugs

Infection with the obligatory intracellular parasite *T. gondii* is mainly acquired by ingestion of contaminated food or water. Approximately a third of the world's human population is infected.

Mechanism of Action

Clindamycin (Fig. 7), a lincosamide derivative, inhibits protein biosynthesis within a unique organelle of the parasite, termed apicoplast. Its mode of action is similar to that of spiramycin.

The folate antagonists, pyrimethamine and sulfadiazine, inhibit the parasite's DHFR/TS synthase enzyme complex and the DHPS, respectively (Figs. 2, 5, and 7) (see antimalarial drugs). To avoid deficiency of folic acid in patients treated with antifolate antagonists, folinic

acid supplementation is recommended to reduce bone-marrow suppression.

Spiramycin (Fig. 7) is a macrolide that inhibits protein biosynthesis by blocking transfer of the aminoacyl-tRNA along the ribosome in a unique organelle of the parasite, termed apicoplast (Dunay et al. 2018). The apicoplast is a remnant of a secondary endosymbiosis of a red algae and is only found in the phylum sporozoa.

Clinical Use

Toxoplasmosis remains a challenge to clinicians. *T. gondii* is one of the most prevalent parasites worldwide and it persists in the body for a lifetime. The infection passes unnoticed or with little signs and symptoms in immunocompetent children and adults. However, the parasite poses a major threat when acquired during pregnancy and transmitted to the fetus, and in immunocompromised patients with or without AIDS, due to a reactivation of latent disease or newly acquired infection. The major clinical conditions are congenital toxoplasmosis, ocular toxoplasmosis, and toxoplasmic encephalitis. Current treatment options are confined to the acute stage, but do not eradicate the parasite from the patient. The most commonly used treatment, and currently probably the most effective, is the combination of pyrimethamine and sulfadiazine, supplemented by folinic acid to prevent bone marrow suppression (Dunay et al. 2018; Montoya and Liesenfeld 2004). In maternal infection during pregnancy, the primary goal is to prevent transmission to the fetus, and the secondary goal, to treat the infected fetus at the earliest time possible to reduce damage. Due to the specific requirements during pregnancy, spiramycin is recommended for the first and early second trimester and pyrimethamine/sulfadiazine for the late second and third trimester (Dunay et al. 2018; Montoya and Liesenfeld 2004). In most countries, treatment is continued in the newborn for various lengths of time. The efficacy of this regimen, however, has still to await confirmation by appropriately designed studies, and different drug regimens and strategies need to be tested for different clinical settings. In the immunocompromised patients, recrudescence after successful treatment of acute toxoplasmosis is a problem. If restitution of the immune response cannot be achieved or is, for therapeutic purposes, not desired, chemoprophylaxis needs to be installed.

Drugs Against Chagas Disease

Chagas disease is a serious public health problem in Latin America, where an estimated 700,000–800,000 people are infected (Perez-Molina and Molina 2018). The causative agent is *Trypanosoma cruzi*, which is transmitted to humans by reduviid vectors (such as triatomines). During acute disease, the parasite develops intracellularly in many tissues, including nervous and muscular tissue. Focal parasite-induced degeneration of infected organs, particularly the heart and the gastrointestinal tract, characterizes the chronic form.

Mechanism of Action

Benznidazole (Fig. 7), a nitroimidazole derivative, has a mode of action that seems to involve covalent modification of macromolecules by nitroreduction intermediates.

Nifurtimox (Fig. 7), a nitrofuran, is a prodrug that is reduced to unstable nitroanion radicals, which react to produce highly toxic oxygen metabolites, such as superoxide and peroxide. Oxidative stress subsequently kills the parasite, which seems to lack effective enzymatic pathways to detoxify oxygen metabolites.

Clinical Use

Benznidazole and nifurtimox, which have been developed in the 1960s and 1970s, have remained the only two drugs available for the acute stage of the disease (Perez-Molina and Molina 2018). There is no proven effect on the chronic stage. The specific treatment of the chronic stage has gained renewed interest with recent findings where persisting parasites may play a role in the development of irreversible lesions. The side

effect of the two available compounds can be severe. The development and testing of new drugs has been slow. Recently, posaconazole, one of the most promising new drug candidates failed to cure Chagas patients (Bermudez et al. 2016).

Drugs Against African Sleeping Sickness

African sleeping sickness has reached a historical low due to sustained efforts to control the spread of this disease over the past 15 years. 2804 cases were reported by the WHO in 2015 of which 2733 were caused by *T. brucei gambiense*. The etiological agents, *T. brucei gambiense* and *T. brucei rhodesiense*, are transmitted to humans by the bite of Tsetse flies.

Mechanism of Action

Eflornithine (difluoromethylornithine, DFMO) (Fig. 8) enters the parasite via an amino acid transporter. It inhibits the ornithine decarboxylase of the polyamine pathway, in both the trypanosome and the mammalian cell, by acting as an irreversible competitor of the natural substrate ornithine. Inhibition of ornithine decarboxylase results in depletion of the polyamines, putrescine, spermidine, and spermine, which are essential for cell proliferation. Eflornithine selectively harms the parasite and not the mammalian cells, despite acting as an ornithine decarboxylase inhibitor in both cell types. This selectivity is explained by the lower rate of ornithine decarboxylase production in the parasite, as compared to mammalian cells. Due to the high turnover rate, mammalian cells are capable of quickly replenishing inhibited ornithine decarboxylase by newly synthesized enzyme. Eflornithine is only effective against *T. brucei gambiense* (Buscher et al. 2017; Hedley et al. 2016).

Melarsoprol (Fig. 8), a trivalent organic melaminophenyl arsenic compound, kills intracerebral parasites of both *T. brucei gambiense* and *T. brucei rhodesiense* via rapid cell lysis (Fairlamb and Horn 2018). Melarsoprol enters trypanosomes via an adenosine/adenine transporter and via aquaglyceroporin 2. The mode of action of melarsoprol is largely unclear. Initial evidence suggests that melarsoprol interferes with redox homoeostasis by forming a complex with the antioxidant trypanothione. The complex, termed MelT, inhibits the antioxidant enzyme trypanothione reductase. Resistance to melarsoprol is associated with mutations in aquaglyceroporin 2, leading to reduced drug uptake. Melarsoprol is highly toxic to humans.

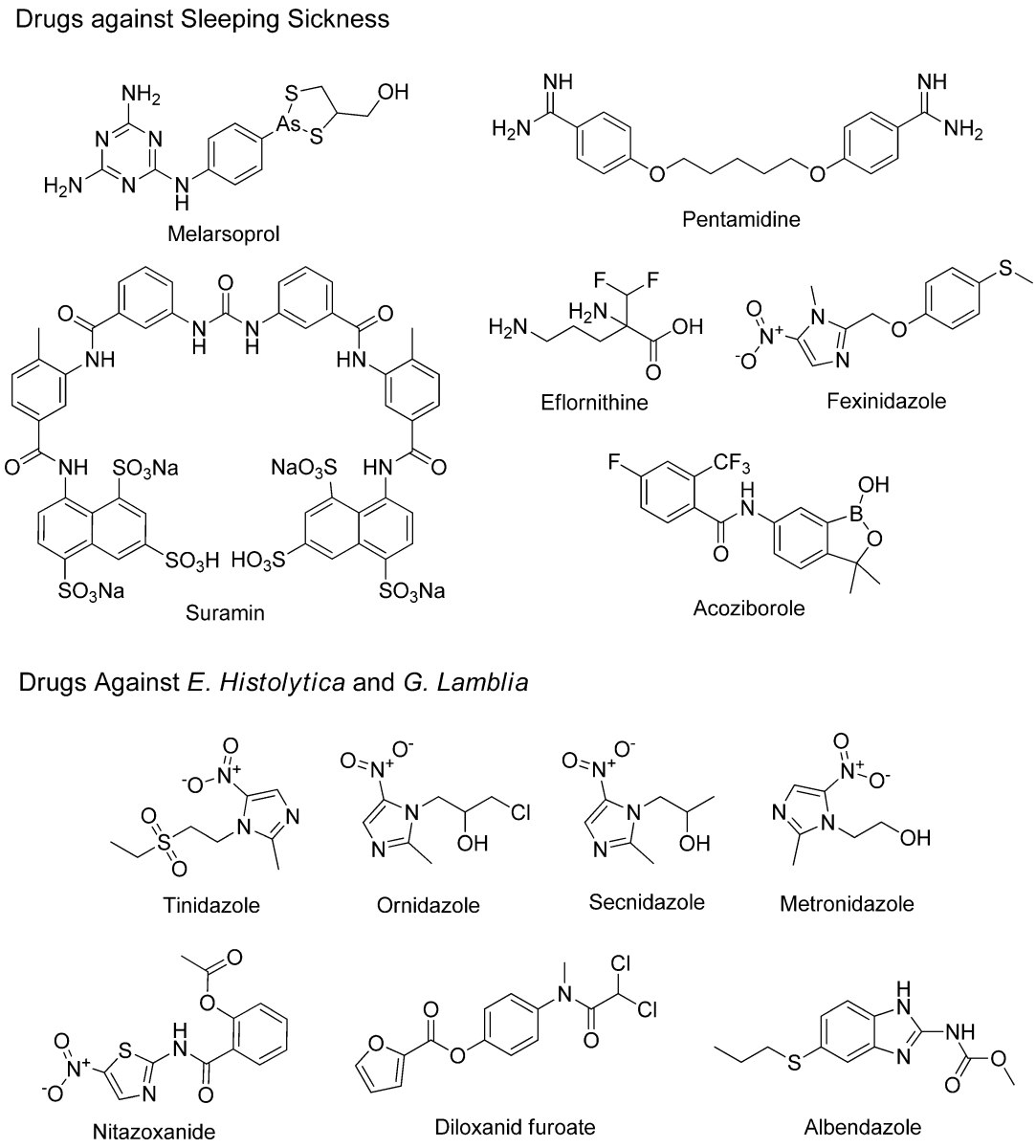
Nifurtimox and fexinidazole (Figs. 7 and 8) are nitro pro-drugs that are activated by a ubiquinone nitroreductase in the mitochondrion of the parasite. Their mode of action is unclear, but may involve disruption of mitochondrial functions.

Pentamidine is an aromatic diamidine (Fig. 8). Pentamidine uptake by the parasite is mediated by several different adenosine transporters. The mode of action is unclear. There is, however, evidence suggesting that pentamidine inhibits DNA, RNA, and protein synthesis.

Suramin (Fig. 8), a symmetrical, polysulfonated naphthylamine, is selectively taken up by the parasite via receptor-mediated endocytosis. It inhibits a number of trypanosomal enzymes, including pyruvate kinase, leading to an inhibition of energy production.

Clinical Use

There has been substantial progress in the treatment of African sleeping sickness in recent years (Buscher et al. 2017; Hedley et al. 2016). The first line treatments for the acute hemolymphatic stage are pentamidine *(T. brucei gambiense)* and suramin *(T. brucei rhodesiense)* (Buscher et al. 2017). The late central nervous system stage is treated using nifurtimox-eflornithine combination therapy in the case of *gambiense* African trypanosomiasis. The efficacy of nifurtimox-eflornithine against *T. brucei rhodesiense* has not yet been explored and melarsopol remains the treatment of first choice. Melarsoprol is highly toxic and can causes reactive encephalopathy, a lethal complication in 2–10% of treated patients.



Antiprotozoal Drugs, Fig. 8 Chemical structure of relevant drugs for the treatment of Sleeping Sickness, Amoebiasis, and Lambliasis

A cumbersome dosing schedule, used for decades, has recently been shortened to a 10-days course based on pharmacokinetic studies and controlled clinical trials. This certainly will improve patient compliance and costs. Fexinidazole has received a positive scientific review by the European Medicine's Agency in November 2018 for the treatment of *T. bucei gambiense*. WHO treatment guidelines are currently being updated to define the role of fexinidazole. Fexinidazole is registered in the Democratic Republic of Kongo (Buscher et al. 2017).

Drugs Against *E. Histolytica* and *G. Lamblia*

Entamoeba histolytica and *Giardia lamblia* are waterborne infectious diseases that cause colitis and liver abscess, and enteritis, respectively (Hemphill et al. 2019).

Mechanism of Action

5-Nitroimidazoles derivatives (Fig. 8), such as metronidazole, tinidazole, ornidazole, and secnidazole, are the drugs of choice in the treatment of anaerobic protozoa. All 5-nitroimidazoles share the same mode of action. Anaerobic microorganisms reduce 5-nitroimidazoles to their active forms. This process only occurs under strongly reducing conditions. In some anaerobic protozoa and bacteria, such conditions are achieved when ferredoxin is reduced by the fermentation enzyme pyruvate ferredoxin oxidoreductase (POR). Ferredoxin can transfer one electron to 5-nitroimidazole, resulting in the reduction of the nitro group. POR does not occur in mammalian cells. The corresponding enzyme to POR in mammalian cells is pyruvate decarboxylase, which is not able to establish a reducing potential high enough for the reduction of 5-nitroimidazoles. The reduced products of 5-nitroimidazoles disrupt the DNA structure, thereby interfering with transcription and replication.

Clinical Use

Entamoeba histolytica colonization of the large intestine is eradicated using a luminal agent such as diloxanid furoate or paromomycine (Figs. 7 and 8). Invasive amoebiasis (colitis, liver abscess) is treated with one of the 5-nitroimidazole derivatives (Leder and Weller 2019), followed by a luminal agent to prevent relapses from remaining cysts in the intestine.

Giardia lamblia is treated with 5-nitroimidazole derivatives. Albendazole, paromomycine, and nitazoxanide (Figs. 7 and 8) are second line treatments. Antimicrobial resistance is estimated at 20% and repeated treatment courses may be necessary. Patients who are repeatedly treated by monotherapy may benefit from combination treatment, that is, metronidazole plus albendazole (Bartelet 2018).

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Antiprotozoan Chemotherapeutics

► Antiprotozoal Drugs

Antiseizure Drugs

► Antiepileptic Drugs

Anti-sense Oligonucleotides

► Lipid-Lowering Drugs

Antithyroid Drugs

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Synonyms

Thionamides

Definition

Antithyroid drugs are defined as drugs that inhibit thyroid hormone synthesis. Clinically, these drugs were developed for the treatment of hyperthyroidism (Burch and Cooper 2018). The mainstay of current antithyroid drugs is thionamides, sulfur-containing derivatives of thiourea. Second-line drugs known to negatively impact thyroid production at the level of the thyroid gland include iodine, perchlorate, and cholestyramine. Drugs such as β-blockers and glucocorticoids may inhibit thyroid hormone function in peripheral tissue. Among β-blockers, propranolol has been demonstrated to impair peripheral conversion of thyroxine (T_4) to triiodothyronine (T_3). Though in its preliminary stages, a selective thyrotropin receptor (TSHR) antagonist has been shown to have antithyroid properties in an animal model (Neumann et al. 2014). This may be a promising and novel therapeutic target in the treatment of Graves' disease; however, its clinical use has yet to be determined. The focus of this entry will be on the mechanism of action, pharmacokinetics, and clinical use of thionamides.

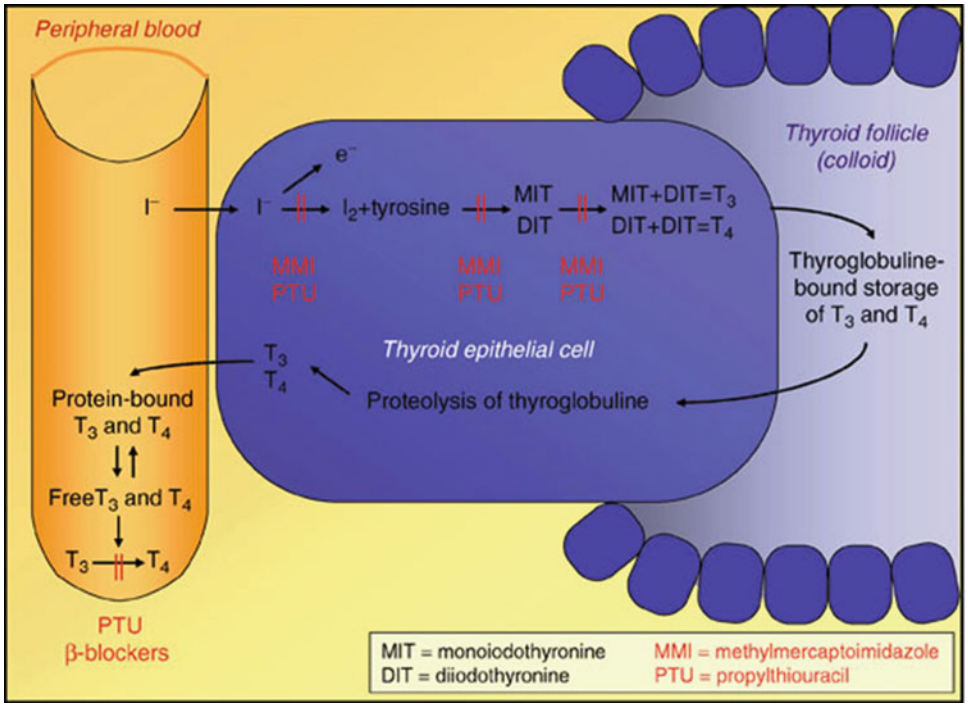
The antithyroid properties of thionamides were first noted in the early 1940s by three independent groups (Mackenzie, Richter, and Kennedy)

(Kennedy 1942; Mackenzie et al. 1941; Richter and Clisby 1942). These groups each observed goitrogenic effects of sulfaguanidine and/or thiourea in animal models. In 1942, Professor Edwin B. Astwood unified the results of these studies and proposed a novel therapy for hyperthyroidism using thiourea. Further purification of 2-thiouracil as a potent antithyroid drug led the first documentation of successful hyperthyroidism treatment (Astwood 1984). The search for more potent and less toxic agents led to the discovery of 6-*n*-propylthiouracil (PTU) in 1946, methimazole (MMI) in 1949, and carbimazole (CBM) in 1956. These antithyroid drugs remain the cornerstone of hyperthyroid treatment today, over 60 years later (Burch and Cooper 2018).

Thionamides are heterocyclic thiocarbamide compounds that are derivatives from thiourea. Thiouracil and PTU belong to the pyrimidines subgroup, whereas MMI and CBM belong to the thioglyoxalines subgroup. CBM is an MMI prodrug that was developed with the unachieved goal of less toxicity. CBM is used predominantly in the United Kingdom, whereas MMI is favored in North America, most of Europe and Asia.

Mechanism of Action

The mechanism of thionamide action is incompletely elucidated; however, our current understanding involves multiple actions within the thyroid hormone production pathway (Fig. 1). Biologically active thyroid hormones, triiodothyronine (T_3) or thyroxine (T_4), are synthesized within the thyroid in five sequential steps. First,



Antithyroid Drugs, Fig. 1 Synthesis and secretion of thyroid hormones and mechanisms of action of antithyroid drugs. Iodine is actively concentrated by the thyroid gland (sodium-iodide transporter). After oxidation it is bound to thyrosine residues, thus forming monoiodothyronine (MIT) or diiodothyronine (DIT). MIT and DIT are coupled to triiodothyronine (T_3) or thyroxine (T_4) and are stored in thyroid follicles bound to thyroglobulin. Thyroid hormones are released by proteolysis. In the thyroid and in peripheral tissue, T_4 is converted to T_3. Antithyroid drugs act by inhibiting the thyroid peroxidase-mediated formation of T_3 and T_4 and compete with iodothyronine residues for oxidized iodine. Moreover, they inhibit iodine oxidation. Propylthiouracil (PTU), but not methimazole (MMI), inhibits the monodeiodination of thyroxine to triiodothyronine

inorganic iodide is actively transported into the basement membrane of thyrocyte via the sodium-iodide symporter, a process known as trapping. Second, organification occurs at the apical-colloid interface with the oxidation of iodide to iodine (iodination) and binding of iodine to tyrosyl residues of thyroglobulin (Tg) to form monoiodothyronine (MIT) or diiodothyronine (DIT). Third, MIT and DIT are coupled (appropriately termed coupling) to form T_3 and T_4. Iodination and coupling are catalyzed by thyroid peroxidase (TPO) with hydrogen peroxide. Fourth, T_3 and T_4 are stored in the follicular lumen until the reentry of Tg in a pinocytosis process forming lysosomes. The fusion of lysosomes with colloid vesicle releases T_3 and T_4 into the circulation. Lastly, two deiodination processes occur within the thyroid cell: deiodination of MIT and DIT by intrathyroidal deiodinase releasing useable iodide and deiodination of T_4 to T_3 through Type I and Type II 5′-deiodinases (Miehle and Paschke 2003). The majority of T_4 to T_3 deiodination occurs in peripheral tissue (Fig. 1).

The predominant mechanism of thionamides as antithyroid drugs is mediated through TPO inhibition during iodine organification (Davidson et al. 1978; Taurog 1976). Specifically, the heme residues of TPO are bound to oxidized iodide prior to its subsequent iodination to thyroglobulin. In the presence of iodine, thionamides act as competitive inhibitors to TPO and become iodinated themselves thus reducing MIT and DIT levels. These inhibition rates depend mainly on the iodine to drug concentration ratio. At high iodine to drug ratio, the inhibition of iodination is reversible and TPO is only partially inactivated. Under these conditions extensive drug oxidation occurs. When the iodine to drug ratio is low, iodination is irreversibly inhibited. This is associated with rapid and complete inactivation of TPO (Miehle and Paschke 2003). Furthermore, thionamides have also been shown to a lesser degree to impair coupling and thyroglobulin synthesis and function (Engler et al. 1983; Monaco et al. 1980).

PTU has distinct inhibitory properties on Type I 5′-deiodinases, properties not shared by MMI or CBM. This results in reduction of T_4 to T_3 conversion, which theoretically results in reduced biologically active thyroid hormone action.

Lastly, thionamides have been associated with decreased thyroid-stimulating antibody titers (Fenzi et al. 1979). Antithyroid drugs have both direct and indirect immune effects that result in reduced thyrotropin receptor antibodies (TRAbs) (Hesarghatta Shyamasunder and Abraham 2017). Similar results have been noted following thyroidectomy and gradually (6–12 months) following radioactive iodine therapy. The degree of TRAb reduction associated with therapy is important in three areas: (1) prediction of Graves' disease remission (Hesarghatta Shyamasunder and Abraham 2017), (2) management of Graves' orbitopathy (Kahaly et al. 2019), and (3) risk of fetal hyperthyroidism in pregnancy (Abeillon-du Payrat et al. 2014).

Pharmacokinetics

MMI and CBM are well absorbed in the gastrointestinal tract. After oral ingestion of 15 mg MMI, peak serum concentrations increase linearly and are in the range of 300 ng/ml at 1–2 h. In vitro, CBM is an effective inhibitor of iodination without prior hydrolysis to MMI. This is contrasted in vivo, where CBM is biologically inactive prior to conversion to MMI. In human serum, CBM is almost completely converted to MMI. Ten milligram CBM is equivalent to 6.7 mg MMI (Miehle and Paschke 2003). MMI is minimally protein bound. The total volume distribution is approximately 40 L and the serum half-life is 4–6 h. MMI can be applied parenterally (Wu et al. 2013). MMI and CBM are likely metabolized by the liver as patients with hepatic disease have prolonged plasma levels, whereas the levels are unchanged in chronic kidney disease and is minimally excreted in urine (Cooper 2005).

PTU is also well absorbed in the gastrointestinal tract. After a 150 mg oral dose, peak serum concentrations are in the range of 3 μg/ml at 1 h. PTU is 80–90% protein bound. The total volume distribution is around 30 L and the serum half-life of PTU is 75 min. Drug levels are not impacted in patients with thyrotoxicosis, renal disease, or liver

disease. PTU is mostly excreted in the urine after hepatic conjugation with glucuronide (Cooper 2005). Biotransformation of PTU primarily occurs at the S group. It results in substantial loss of antiperoxidase activity. The metabolites, 6-*n*-propyluracil, *S*-methyl-PTU, PTU disulfide, and PTU glucuronide, are only weakly active or completely inactive as TPO inhibitors.

For peri- and postpartum women, the lipophilic character of MMI allows for transplacental passage and for relatively elevated levels in breast milk. While PTU also crosses the blood-placenta barrier, PTU is excreted in breast milk to a lesser degree than MMI due to its the high protein binding and ionization at a physiologic pH (Cooper 2005).

Both drugs, MMI and PTU, are actively concentrated by the thyroid gland. Intrathyroidal concentrations of MMI are in the range of 5×10^5 M. There is no difference in intrathyroidal concentrations of MMI 3–6 and 17–20 h after ingestion of 10 mg of CBM. Little is known about intrathyroidal concentrations of PTU. After a single dose of 10 mg of MMI or 100 mg of PTU, inhibition of intrathyroidal organification of iodide is about 90% and 60%, respectively, at 8 h. Thus MMI can be administered once daily, whereas PTU is dosed thrice daily.

Metabolism of the drugs by TPO is largely iodine dependent. Under conditions of reversible inhibition of iodination, the drugs are rapidly metabolized to higher oxidation products such as sulfonate and sulfate, with disulfide as an intermediate. If there is irreversible inhibition of iodination (higher drug to iodide ratio), some of the drug is oxidized only to the disulfide stage, but the TPO is simultaneously inactivated, and no iodination is observed (Miehle and Paschke 2003).

Clinical Use

Thionamide use in the treatment of hyperthyroidism is well summarized in the 2016 American Thyroid Association (ATA) Guidelines for Diagnosis and Management of Hyperthyroidism and Other Causes of Thyrotoxicosis and in the 2018 European Thyroid Association (ETA) Guidelines for the Management of Graves' Hyperthyroidism (Kahaly et al. 2018; Ross et al. 2016). Graves' disease, caused by unregulated thyroid hormone production and secretion precipitated by autoantibodies to thyrotropin receptor (TRAbs), is the most common cause of hyperthyroidism in iodine-sufficient countries. Other causes of hyperthyroidism include autonomous hormone production caused by somatic (toxic adenoma or toxic multinodular goiter) or less frequently germline activating TSH-receptor gene mutations sometimes precipitated by iodine contamination or by passive release of preformed thyroid hormone due to thyroid tissue destruction (subacute or painless thyroiditis). Less common causes include exposure to endogenous or exogenous sources of extrathyroidal thyroid hormone or rarely excessive production of thyrotropin causing unregulated thyroid hormone production and release. The consequences of untreated or undertreated hyperthyroidism include atrial fibrillation, osteoporosis, weight loss, tremor, proximal muscle weakness, mood symptoms, and at its extreme thyroid storm and cardiovascular collapse. This emphasizes the importance of effective and persistent hyperthyroid treatment.

Thionamides are used as first-line treatment for Graves' disease (Table 1). Specifically, the ETA guidelines recommend MMI or CBM for at least 12–18 months in the treatment of nonpregnant patients choosing antithyroid drugs (Kahaly et al. 2018). In North America, the trend has shifted toward increased use of antithyroid drugs over radioactive iodine therapy (Ross et al. 2016). Radioactive iodine therapy (RAI) and thyroidectomy are the other first-line therapies that should be discussed with patients. MMI or CBM is preferred over PTU in most patients except during the first trimester of pregnancy or during treatment of thyroid storm due to once-daily dosing, more potent efficacy, and less toxicity. The starting dose of MMI as per ATA task force is 5–10 mg daily if free T_4 is 1–1.5 times the upper limit of normal (ULN), 10–20 mg if free T_4 is 1.5–2 times ULN, and 30–40 mg if free T_4 is 2–3 times ULN (Ross et al. 2016). Due to its shorter duration of action, the starting dose of PTU is 50–150 mg three times daily.

Antithyroid Drugs, Table 1 Different treatment strategies depending on the cause of hyperthyroidism

	Graves' disease	Toxic adenoma and toxic multinodular goiter
Etiology	Organ-specific autoimmune disease	Constitutively activating somatic mutation in TSH receptor or in Gsα
	TSH receptor antibody production	
Remission after antithyroid drug treatment	In 30–70% of patients 1 year after treatment withdrawal	No remission
Antithyroid drug treatment	12–18 months	Until euthyroid
Treatment with radioiodine or surgery	If relapse after cessation of antithyroid drug	After euthyroidism is achieved

Remission from Graves' disease ranges from 30% to 70% following cessation of antithyroid drugs for at least 1 year (Burch and Cooper 2018). Remission occurs most frequently during the first 12 months (Paschke and Ludgate 1997). Unfortunately, Graves' disease recurs in one-third of patients, whereas one-third of patients achieve permanent remission. Antithyroid drugs are particularly favored in patients with high likelihood of remission, including women, those with mild disease, small goiters, and low-to-negative TRAb titers. Factors negatively impacting remission include tobacco smoking, age <30, increased thyroid volume and postpartum. The optimal duration of therapy is 12–18 months as there is no added benefit with respect to recurrence rate and there is an increased risk of adverse effects (Abraham et al. 2010). However, a recent study suggested that longer-term MMI use (60–120 months) in select patient populations with Graves' disease may increase remission rates (Azizi et al. 2019). Nonetheless, thyrotropin, and free T_4 (free T_3) should be monitored closely until doses are stabilized. Thionamide doses should be gradually decreased to the minimal maintenance dose as the serum thyroid hormone levels fall. The aim is to restore clinical and biochemical euthyroidism within 1–2 months.

In thyroid autonomy caused by somatic TSH receptor or Gsα mutations, a spontaneous remission (e.g., by nodule apoplexy) is very uncommon. Therefore, antithyroid drug treatment is used to render patients euthyroid before ablative RAI or surgery (Table 1). Similar to Graves' disease, MMI is favored over PTU as PTU increases the failure risk of RAI though its ability to neutralize iodinated free radicals (Cooper 2005). This effect can be overcome by increasing the radioiodine dose. Continuous MMI use up to radioiodine therapy can reduce its success rates, thus MMI should be discontinued 2–3 days prior to RAI. In patients at increased risk of complications of hyperthyroidism, MMI can be resumed 3–7 days post RAI (Ross et al. 2016).

Iodine intake impacts the responsiveness of hyperthyroid patients to thionamides. Combined iodide and antithyroid drug therapy short term has been demonstrated to reduce gland vascularity and to reduce complications of transient hypoparathyroidism and voice hoarseness following thyroidectomy (Randle et al. 2018). Response to thionamides are increased in countries with moderately low iodine intake compared with iodine-sufficient countries (Miehle and Paschke 2003).

The "block-and-replace" regiment with simultaneous administration of an antithyroid drug and L-thyroxine is used in individual cases. The initial hypothesis was that combined therapy may result in higher remission rates. This hypothesis has now been disproven with an increased propensity of adverse effects with this regiment due to the higher antithyroid drug doses used in this approach (Burch and Cooper 2018).

Use of MMI and PTU during pregnancy and postpartum is discussed in the 2017 Guidelines of the ATA for the Diagnosis and Management of Thyroid Disease During Pregnancy and the Postpartum (Alexander et al. 2017). While moderate-severe hyperthyroidism poses fetal and maternal risks, MMI and PTU both have potential teratogenic effects. Specifically, MMI exposure has been associated with aplasia cutis. Other defects associated with MMI use include choanal or

esophageal atresia and abdominal wall, eye, urinary system, and ventricular septal defects. In contrast, PTU exposure has been associated with face and neck cysts and urinary tract abnormalities in male fetuses. Due to the less severe teratogenic effects of PTU, the current guidelines recommend PTU as the first-line treatment of moderate-severe maternal hyperthyroidism until 16 weeks of gestation. After 16 weeks, MMI or PTU are both reasonable options. Small levels of PTU and MMI are detected within the breast milk; thus, the lowest effective doses should be utilized.

Side Effects

Antithyroid drugs have several notable adverse effects that should be discussed with patients prior to initiation of therapy. The most frequent side effects are allergic cutaneous reactions including skin rash, pruritus, and urticaria, which occur in up to 6% of patients (Burch and Cooper 2018). In patients with minor skin rashes, the ATA guidelines recommend consideration of antihistamine therapy without cessation of antithyroid drugs (Ross et al. 2016). The alternative is to switch to the other thionamide (i.e., MMI to PTU or vice versa), which may result in two-thirds of patients having improved minor reactions. Other common adverse effects reported for thionamides include arthralgias. In this case, switching to the alternative thionamide is also an option.

The two rare, but severe, adverse effects of thionamides are agranulocytosis and hepatotoxicity (Burch and Cooper 2018; Cooper 2005). The prevalence of agranulocytosis is reported at 0.2–0.5% of patients taking thionamides. The mechanism of thionamide-induced agranulocytosis is still unclear; however immune-medicated bone marrow suppression is likely involved. Risk of agranulocytosis is dose-dependent for MMI but not for PTU. Most reports of agranulocytosis occur within the first 3 months of treatment. It is critical that patients be advised to discontinue the offending drug immediately if sore throat, pharyngitis, or fever occur and immediately seek medical attention. Most patients recover from agranulocytosis after discontinuation of the antithyroid drug. Given that specific HLA types may be at increased susceptibilities and the severity of the effect, it is not advised to switch to alternative thionamides.

Thionamides have been associated with mild and severe hepatic dysfunction in up to 0.3% of patients. PTU, MMI, and CBM are not recommended in patients with transaminase elevation greater than five times ULN. Rates of hepatic failure were observed to be higher with PTU compared with MMI (Wang et al. 2014). Patients should be advised to discontinue the offending drug and seek immediate medical attention with new onset of jaundice, sudden abdominal pain, or dark urine. Historically, PTU was associated with hepatocellular dysfunction and MMI/CBM with cholestasis. Recent studies have challenged this notion, and caution must be applied to both classes of thionamides particularly within the first 12 weeks of therapy (Ross et al. 2016).

Lastly, antithyroid drug-induced vasculitis, associated with perinuclear anti-cytoplasmic neutrophil antibody, has been reported following years of therapy (Burch and Cooper 2018). Patient may present with cutaneous, pulmonary, and renal involvement, and the offending drug should be immediately discontinued. Persistent antithyroid drug use may require immunosuppressive therapy or even hemodialysis. Fortunately, the drug-induced vasculitis generally resolves following cessation of the offending drug.

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Anti-TNF

▶ Rheumatoid Arthritis

Antitussive Drugs

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Definitions

Cough is an essential protective reflex response to irritating stimuli in the respiratory tract. It involves the sudden, usually involuntary,

expulsion of air from the lungs. Cough can prevent foreign bodies from entering the lungs or aid the removal of mucus and irritants from the lungs. Cough is a common symptom in upper respiratory tract infections and more chronically in asthma, chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF), and lung cancer or may also indicate some other underlying disorders such as gastroesophageal reflux disease. It is also frequently caused by smoking. Conversely, in certain conditions such as Parkinson's disease, stroke, and motor neuron disease, cough may be severely affected, and the patient loses this protective function. Cough is useful if it is aiding the expulsion of foreign substances, but chronic cough is an unpleasant, difficult symptom for patients to live with and maybe debilitating.

Basic Mechanisms

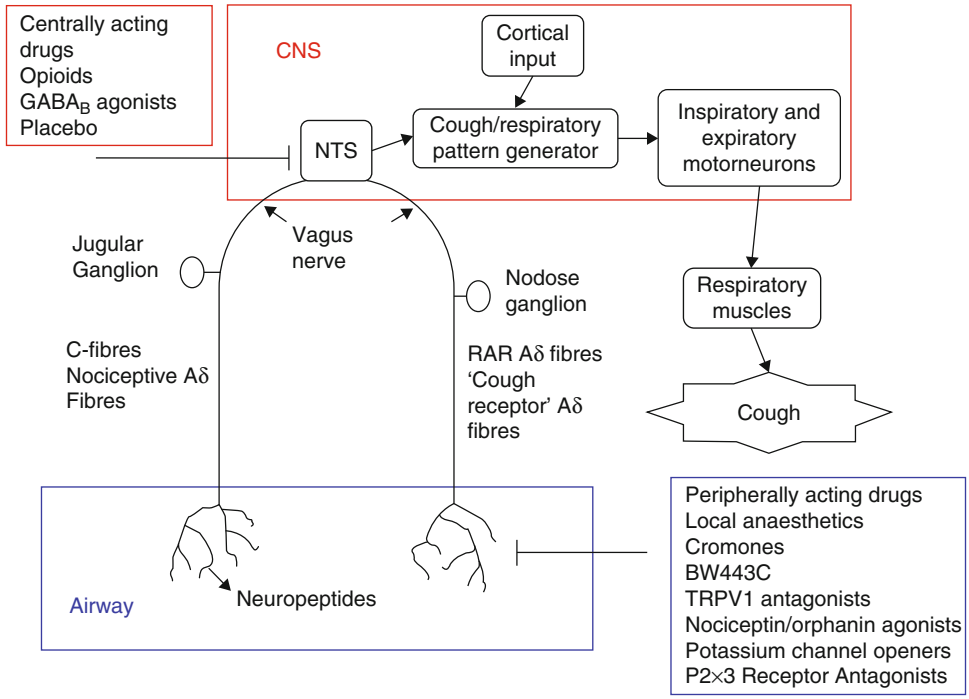
Physically, cough results from a series of events starting with the patient inhaling, often to near maximum levels. Secondly, the glottis closes, and the breathing muscles cause compression of the air in the lungs, leading to high pressure in the pleural space and alveoli. The expulsive phase occurs when the glottis reopens allowing the compressed air to escape. It is at this point that the cough sound is heard and also that foreign materials which may have been deposited in the lungs are removed. Coughing often occurs in bouts, or epochs, rather than as discrete events. *Cough* may be voluntary or involuntary – subthreshold stimuli may evoke the urge to cough, but not the reflex itself.

Airway irritation is detected by sensory nerves which are found within the walls of the airways. Various nerve endings are found in this area, but it is thought that the rapidly adapting receptors (RARs) are the predominant sensors of *cough*. RARs rapidly adapt to a maintained stimulus and have thin myelinated Aδ fibers. Slowly adapting receptors and C-fibers (C-fibers are sensory nerves with unmyelinated axons and can have a modulatory role on the cough reflex). Several different types of sensory nerves have been identified, and more than one type of fiber is involved – it is possible that the combination of fibers and the nature of the discharge regulate *cough* as much as the quantity of action potentials.

These primary afferents project from the airway into the CNS exclusively via the vagus nerve, via the nodose or jugular ganglia. These neurons enter the brainstem and terminate in the nucleus tractus solitarius. Here they synapse with other neurons which determine the various motor components of *cough*. At these central synapses, there are many neurotransmitter receptors present (e.g., tachykinins, glutamate, 5-HT, GABA, NMDA, dopamine, opioids, and nociceptin), which may be the site of action of centrally acting antitussive drugs (see below). It is also here that other sensory afferents terminate and may modulate the synaptic signalling. If there are disorders in other organs which also have sensory neurons carried in the vagus, it is here that these nerves may interact with airway neurons to cause *cough*. In contrast, in diseases where *cough* is reduced, it is generally due to problems in the efferent part of the reflex loop.

Previously it was thought that there was a central "cough center" in the CNS, but current research suggests that this is an oversimplification. The ability to coordinate a combination of a variety of motor outputs is essential for *cough*. For example, outputs go to control airway structures such as the glottis and a wide range of breathing muscles such as the diaphragm and intercostal muscles. In addition, synchronization with the respiratory rhythm is required as it is not possible to *cough* and breathe concurrently. Finally, the CNS is thought to contain a gating mechanism by which the brain determines whether the arriving stimuli are of sufficient magnitude for a cough to occur. The cortex is not necessary for cough, but it can exert descending control on the reflex (Fig. 1).

The sensory nervous system which governs cough is subject to plasticity (nerve plasticity) –



Antitussive Drugs, Fig. 1 Schematic diagram of the cough reflex and sites of action of some tussive agents. Airway sensory nerves activated in response to a tussive stimulus travel though the vagus nerve to the medulla, where they terminate in the nucleus tractus solitarius (nTS). Second-order neurons relay the message to the respiratory pattern generator, which modifies the activity of the inspiratory and expiratory motor neurons and leads to cough. Tussive agents can activate a variety of airway receptors to cause cough. Many nerve types (both peripherally and centrally) undergo phenotypic changes and have increased responses to tussive stimuli following inflammation. The sites of action of antitussive drugs are shown. Abbreviations: *RAR* rapidly adapting receptor, *CNS* central nervous system. (Figure adapted from Reynolds et al. 2004)

such that there may be an enhancement of this cough pathway, either by changes in the receptors on sensory nerve endings, the ganglia, or within the CNS which can increase the activity of these neurons leading to a hypertussive state.

Pharmacological Interventions

Cough is currently a huge unmet clinical need, as none of the currently available treatments are reliably effective (Dicpinigaitis et al. 2014). However, there are many treatments which are currently used, with variable levels of success. In addition, if it is caused by another condition, such as gastroesophageal reflux disease, then treatment of that may reduce cough.

Drugs

Centrally Acting Drugs

Opioids Opioids (opioid systems) are thought to exert their antitussive effects by acting as agonists at μ- and κ-opioid receptors in the CNS. Activation of these receptors activates various G-proteins and leads to the inhibition of the activity of most neurons, but the activity of a few is increased. Recent evidence suggests that morphine may also interact with TRPV1 receptors to explain some of its antitussive activity.

Centrally acting drugs include dextromethorphan and codeine. However the possibility that there may also be peripheral effects of these drugs has led to the development of BW443C (see below).

Opioid drugs are often more effective than other nonnarcotic treatments, but they are also associated with more side effects making them less suitable for many patients. Higher doses which are more effective are also associated with undesirable effects such as sedation.

Peripherally Acting Drugs

Local Anesthetics Local anesthetics are more consistently effective than other therapies, but they cannot be used routinely for this purpose. High concentrations are needed for therapeutic benefit, but this also increases the amount crossing the blood-brain barrier and entering the brain producing unwanted effects. Topical administration to the airways can reduce this.

Patients who do not obtain sufficient symptomatic relief from other treatments may use lidocaine, benzonatate, bupivacaine topically, or mexiletine orally.

Cromones Both disodium cromoglycate and nedocromil sodium have antitussive effects in humans, particularly against ACE inhibitor-induced cough. This suggests an effect on bradykinin-induced changes in sensory nerve function. Antitussive activity of these drugs is thought to occur by increasing the depolarization of sensory nerves, which increases the threshold for an action potential and therefore inhibits the activity of these neurons. A recent study has provided evidence of cromoglycate being effective in reducing cough in patients with IPF (Birring et al. 2017).

GABAB Receptor Agonists GABA is the predominant inhibitory neurotransmitter in the CNS. Baclofen acts centrally as an agonist at the $GABA_B$ receptor, which increases inhibition of nerves. 3-Aminopropylphosphinic acid (3-APPi) has been shown experimentally to act as an antitussive at peripheral nerves, and preclinical evidence suggests that baclofen indeed has antitussive actions clinically (Dicpinigaitis 2006).

Novel Drugs

Tachykinin Antagonists (Tachykinins) C-fiber afferents from the airways contain peptide tachykinin transmitters such as substance P (SP) and neurokinins A and B (NKA and NKB). Stimulation of these nerves can also cause local release of these mediators at their peripheral terminal, allowing them to enhance the activity of the *RARs*. SP, NKA, and NKB act at the tachykinin receptors (NK_1–NK_3), and so understandably, antagonists for NK_2 in particular are being investigated as novel treatments for cough (Dicpinigaitis et al. 2015).

BW443C BW443C is a novel opioid used for the treatment of cough, but which does not enter the brain and so exerts its effects only on peripheral nerves. It has not been tested as an antitussive in humans due to its rapid metabolism in the lungs, but the concept of a peripheral opioid is still possible.

Nociceptin/Orphanin Receptor Agonists Nociceptin and orphanin are synonyms for the peptide that acts at an opioid-like receptor. Nociceptin may act by inhibiting tachykinin release from sensory C-fibers, and a clinical trial has started to test its effects on *cough*.

TRPV1 Receptor Antagonists Capsaicin, the pungent chemical from chili peppers, induces cough. Capsaicin is an agonist at the transient receptor potential vanilloid receptor 1 (TRPV1) – a polymodal receptor which integrates several harmful stimuli such as noxious heat, low pH, and various possible endogenous mediators to mediate pain and cough. TRPV1 receptors have been demonstrated to be upregulated in patients with chronic cough (Chung 2007).

Capsaicin is used to cause cough experimentally and clinically, and the TRPV1 antagonist capsazepine can inhibit cough elicited by both capsaicin and citric acid. This suggests that other TRPV1 antagonists could be effective treatments for cough (reviewed in Dicpinigaitis et al. 2014). However, capsazepine does not block hypertonic saline-

induced cough, suggesting that other tussive receptors are also important (Birring et al. 2017) and several studies with $TRPV_1$ antagonists have failed to show clinical benefit in patients with chronic cough.

TRPA1 Receptor Antagonists TRPA1 receptors are activated by known pro-tussive agents such as cinnamaldehyde and acrolein (found in tobacco smoke) which have led to the development of novel TRPA1 antagonists as possible treatments for cough (Dicpinigaitis et al. 2015).

Potassium Channel Openers An effect of opening K^+ channels is to hyperpolarize the primary sensory neurons. Similar to local anesthetics, this makes the cell less likely to produce an action potential because more depolarizing stimuli are needed to overcome the block. NS1619 is an example of this type of drug which has initially shown antitussive activity in a variety of experimental systems.

Quaternary Ammonium Salts Quaternary ammonium salts such as carcainium chloride (RSD 931) have been shown to be antitussive while having much reduced local anesthetic activity (Dicpinigaitis et al. 2015). While the molecular mechanisms underlying this antitussive activity are not understood, RSD 931 appears to be Aδ fiber-selective and may represent a novel class of antitussive drug. More recently JMF2-1 a lidocaine derivative that blocks Na^+ channels has had beneficial effects in the airways without significant local anesthetic activity.

P2x3 Receptor Antagonists ATP is known to be an agonist of P2x3 receptor found on sensory nerves in the lung. Gefapixant is a novel P2x3 receptor antagonist that has shown promise in early clinical trials in patients with idiopathic cough and is now undergoing phase 3 clinical trials (Dicpinigaitis et al. 2014).

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Anxiolysis

▶ GABAergic System

APEL

▶ Apelin

Apelin

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Synonyms

AGTRL1 ligand; APEL; APJ endogenous ligand; APLN; XNPEP2

Definition

Apelin (APLN) is a small adipokine released primarily by adipose tissue. It is also expressed in many organs, including the brain, heart, lung, kidney, and stomach. It is a ligand for the G-protein-coupled receptor APJ. Apelin is involved in many physiological processes, such as regulation of blood pressure, body fluid homeostasis, the endocrine stress response, cardiac contractility, angiogenesis, and energy metabolism. It may also participate in pathological processes, including heart failure, obesity, diabetes, cancer, and neurodegenerative diseases.

Basic Characteristic

Apelinergic System

Apelin is an endogenous peptide first isolated from the bovine stomach in 1998, encoded by the apelin gene *APLN* located on chromosome Xq25-26.1. Its receptor APJ (APLNR) belongs to the family of seven-transmembrane proteins and is closely related to the angiotensin receptor. Apelin is secreted by adipose tissue as a 77-amino acid prepropeptide called preproapelin, which can be cleaved to produce a mature 36-amino acid apelin peptide, apelin-36, and shorter peptides, apelin-17 and apelin-13, and its modified pyroglutamyl form [Pyr1]apelin-13 (Fig. 1) (Tatemoto et al. 1998).

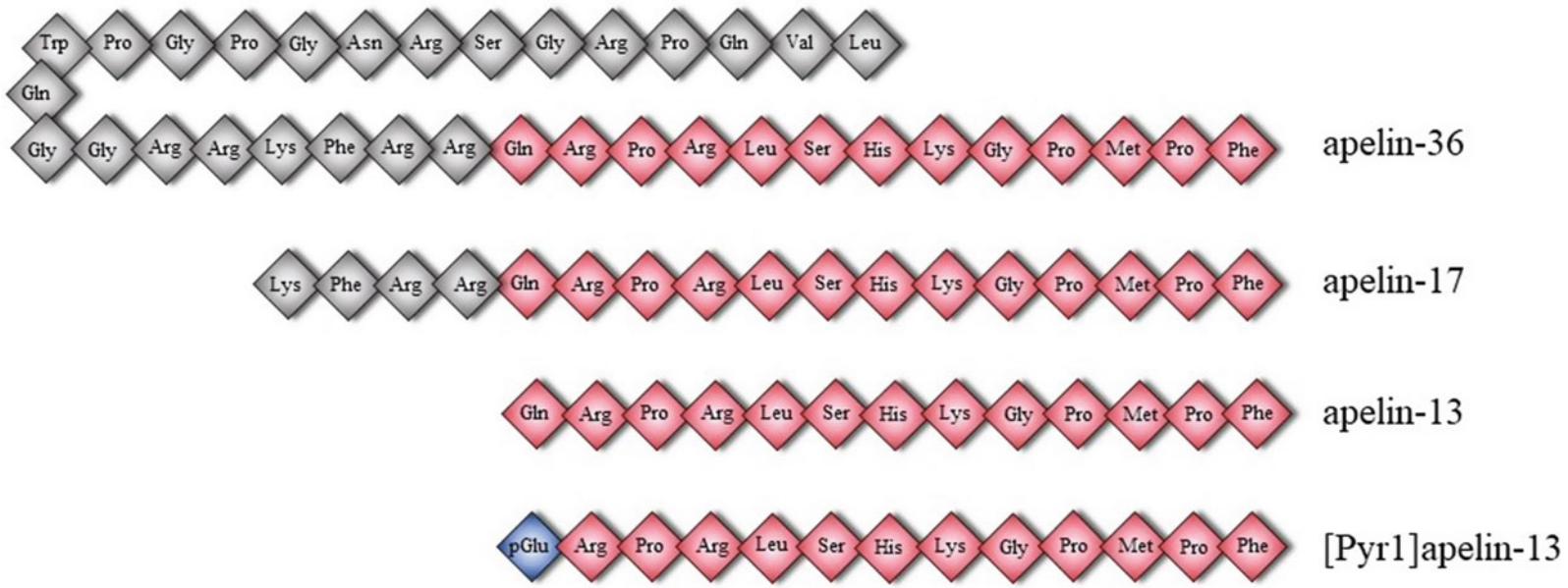
Tissue distribution of apelin prepropeptide and APJ is similar, and in humans it was found in the heart, lung, kidney, spleen, stomach, and central nervous system. This extensive expression suggests that apelin/APJ, also known as the apelinergic system, may be involved in many physiological processes, such as regulation of cardiovascular system homeostasis, angiogenesis, and carbohydrate metabolism. The role of apelin in various processes is probably mediated through several signalling pathways: the AMP-activated protein kinase/nitric oxide synthase (AMPK/eNOS), phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt), and mitogen-activated protein kinase (MAPK) pathways mediate angiogenesis and ischemia and reperfusion (I/R) protection. Processes leading to metastasis are mediated through the peroxisome proliferator-activated receptor (PPAR), PI3K/Akt/mechanistic target of rapamycin (mTOR), MAPK, and PAK1/cofilin pathways. The apelinergic system also has an influence on processes connected with energy metabolism, including glucose uptake, lipolysis, and fatty acid oxidation via the AMPK/eNOS and PI3K/Akt pathways (Fig. 2) (Wysocka et al. 2018).

Apelin in the Cardiovascular System

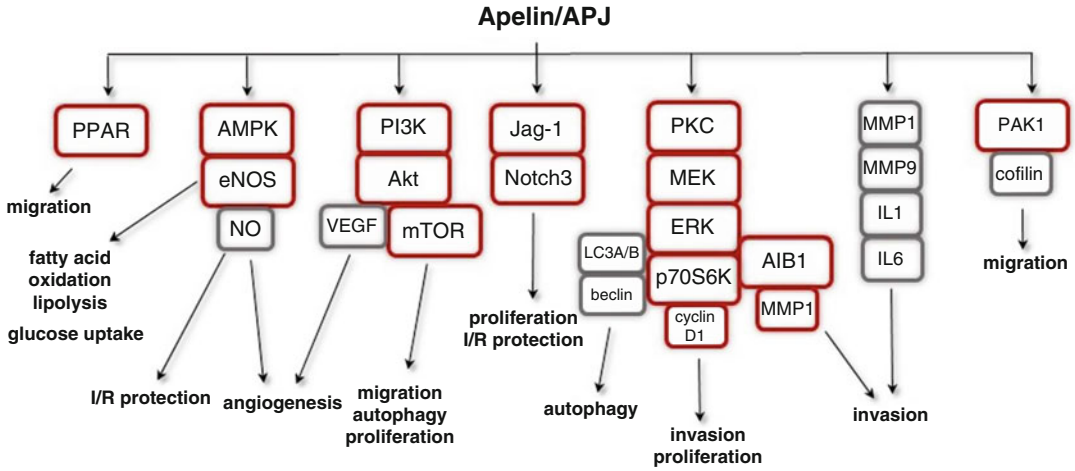
Due to the high expression level of apelin in the heart, the apelinergic system was considered to have an impact on cardiac system regulation. In humans, it can induce peripheral and coronary vasodilation, lower arterial blood pressure, and increase cardiac output. Apelin also stimulates a positive inotropic effect and upregulates the Ca^{2+} sensitivity of myofilaments in heart muscle (Kuba et al. 2019). Moreover, this peptide is also involved in physiological process of forming new blood vessels, called angiogenesis, that is crucial for supplying tissue in oxygen. Apelin upregulates hypoxia-inducible factor (HIF-1), vascular endothelial growth factor (VEGF), and vascular endothelial growth factor receptor 2 (VEGFR), as well as activates the AMPK/eNOS and PI3K/Akt/eNOS pathways.

In hypertension, a cardiovascular condition characterized by increased arterial blood pressure, apelin reduces blood pressure, which is mediated through the Akt/eNOS signalling pathway.

Under hypoxia, a pathological condition characterized by restriction of blood delivery to organs and tissues that is often caused by ischemia and reperfusion, apelin displays a protective effect. This protection is possible through activation of antioxidant enzymes reducing oxidative stress. In different cardiovascular pathologies, such as coronary artery disease and ventricular hypertrophy, the apelin level is altered, suggesting that it could be successfully used as a biomarker of cardiovascular system pathologies (Table 1) (Wysocka et al. 2018).



Apelin, Fig. 1 Amino acid sequences of apelin peptides



Apelin, Fig. 2 An overview of the apelin-induced signalling pathways

Apelin in Obesity and Diabetes

Apelin belongs to the family of adipokines, biologically active molecules secreted by adipose tissue. Therefore, this peptide was suggested to be involved in some obesity-related disorders, but its role remains unclear. In several studies, apelin plasma level was increased in obese individuals in comparison to nonobese patients (Table 1). It can also mediate angiogenesis regulation correlated with adipose tissue growth (Wysocka et al. 2018). Moreover, apelin plays a beneficial role in carbohydrate metabolism by increasing glucose uptake and insulin sensitivity through translocation of glucose transporter type 4 (GLUT-4) in a PI3K/Akt-dependent manner. Several investigations have demonstrated involvement of this peptide in the regulation of diabetic complications, including cardiomyopathy, retinopathy, and nephropathy, where apelin acts as a protector against oxidative stress and apoptosis through the mTOR pathway (Cheng et al. 2019; Wysocka et al. 2018).

Apelin in Cancer

In many cancer types, the level of apelin is upregulated (Table 1), suggesting a beneficial role of apelinergic system in cancer progression. It has a potential to stimulate tumor growth, cancer cell migration, and metastasis through several mechanisms, including AMPK, PI3K/Akt, PPAR, MAPK, and p-21-activated kinase (PAK1)/cofilin pathways. Under tumor hypoxia, the angiogenic balance is shifted to pro-angiogenic conditions, connected with upregulation of apelin and its receptor. During cancer development, this peptide can also suppress apoptosis, programmed cell death, by decreasing the level of caspase-3 activity, which is a crucial apoptotic executor. Interestingly, apelin is especially involved in progression of obesity-related cancer. Numerous studies have revealed that increased body mass index (BMI) is correlated with the risk of developing tumors, such as prostate cancer, breast cancer, and esophageal adenocarcinoma. Several investigations have demonstrated that in obese individuals with cancer, the apelin peptide level was elevated compared to nonobese patients and positively correlated with BMI, fasting insulin level, hyperinflammation, metabolic changes in adipose tissue, and neovascularization (Table 1) (Wysocka et al. 2018).

Apelin in Neurodegenerative Diseases

Apelin is widely expressed in the central nervous system, and hence it was suggested to be involved in development and progression of neurodegenerative disorders. It can inhibit Alzheimer's disease (AD) by regulating neuron apoptosis, inflammatory mediators, Tau activation, as well as amyloid-β accumulation. Tau and amyloid-β are molecules

Apelin, Table 1 Expression of apelin/APJ in different types of pathologies

Disease	Patient/tissue/cell line	mRNA	Protein	References[a]
Failing heart	Human idiopathic dilated cardiomyopathy tissue	*APLN* ↑	–	Földes et al. (2003)
Hypertension	Human plasma Rat plasma Rat left ventricular myocytes	– *APLN* ↓ *APLNR* ↓	Apelin-12 ↓ – APJ ↓	Sonmez et al. (2010) Akcılar et al. (2013) Pang et al. (2014)
Coronary artery disease	Human plasma	–	Apelin-12 ↓	Kadoglou et al. (2010)
Left ventricular hypertrophy	Human left ventricular myocytes Human plasma Rat left ventricular myocytes Rat plasma	– – – –	Apelin-12 ↓ Apelin-12 ↓ Apelin-36 ↓ Apelin-36 ↑	Chandrasekaran et al. (2010) Ye et al. (2015) Falcao-Pires et al. (2010)
Retinal ischemia	Rat retinal Müller cells	*APLN* ↑ *APLNR* ↑	Apelin ↑ (unspecified) APJ ↑	Wang et al. (2012)
Heart ischemia	Rat hearts	–	APJ ↑	Rastaldo et al. (2011)
Brain ischemia	Rat and mouse hippocampus	–	APJ ↓	Fan et al. (2017)
Pulmonary hypertension	Human plasma Mouse lung	– *APLN* ↑ *APLNR* ↑	Apelin-12 ↓ –	Chandra et al. (2011)
Ischemic retinopathy	Mouse retinas	*APLN* ↑ *APLNR* ↑	APJ ↑	Kasai et al. (2010)
Portal hypertension	Rat mesentery, intestine, portal vein and mesenteric artery	*APLN* ↑ *APLNR* ↑	–	Tiani et al. (2009)
Obesity	Human plasma	– –	Apelin-12 ↓ Apelin-12 ↑	Tapam et al. (2010) Krist et al. (2013) Kotanidou et al. (2015) Ba et al. (2014)
Diabetes mellitus	Human plasma Rat heart	– –	Apelin-12 ↑ Apelin ↑ (unspecified)	Habchi et al. (2014) Ma et al. (2014) Akcılar et al. (2015)
Diabetic retinopathy	Human vitreous body	–	Apelin-13 ↑	Tao et al. (2010)
Diabetic nephropathy	Mouse kidney cortex	–	Apelin-13 ↓	Day et al. (2017)
Diabetic cardiomyopathy	Mouse heart	–	Apelin ↑ (unspecified)	Zeng et al. (2013)
Lung cancer	Non-small cell lung carcinoma Adenocarcinoma	*APLN* ↑ –	– APJ ↑	Berta et al. (2010) Yang et al. (2014)
Cholangiocarcinoma	Cholangiocarcinoma Cholangiocarcinoma cell lines	*APLN* ↑ *APLNR* ↑ –	– Apelin-36 ↑ APJ ↑	Hall et al. (2017)
Liver cancer	Hepatocellular carcinoma	*APLN* ↑	–	Muto et al. (2014)
Gastric cancer	Gastroesophageal cell carcinoma Plasma Gastric cancer Adenomas and adenocarcinomas Colon cancer cell lines	– – – – –	Apelin ↑ (unspecified) Apelin ↑ (unspecified) APJ ↑	Diakowska et al. (2014) Feng et al. (2016) Diakowska et al. (2014)

(continued)

Apelin, Table 1 (continued)

Disease	Patient/tissue/cell line	mRNA	Protein	References[a]
			preproapelin ↑ APJ ↑ preproapelin ↑ APJ ↑	Hao et al. (2017) Picault et al. (2014)
Prostate cancer	Prostate cancer	*APLN* ↑	–	Wan et al. (2015)
Ovarian cancer	Ovarian cancer	*APLN* ↑ *APLNR* ↑	–	Hoffmann et al. (2017)
Breast cancer	Plasma	–	Apelin-36 ↑	Salman et al. (2016)
Multiple myeloma	Plasma	–	Apelin ↑ (unspecified)	Maden et al. (2017)
Obesity-related colon cancer	Human plasma	–	Apelin-12 ↑	Al-harithy et al. (2015)
Obesity-related endometrial cancer	Human plasma	–	Apelin-36 ↑	Altinkaya et al. (2015) Salman et al. (2016)

[a]Details of bibliographic data are available in review "The role of apelin in cardiovascular diseases, obesity and cancer" by Wysocka et al. (2018)

crucially involved in Alzheimer's disease as the microtubule stabilizer and main component of amyloid plaques found in AD patients' brains, respectively. Apelin is able to suppress Tau activation via PI3K/AKT/glycogen synthase kinase-3β (GSK-3β) signalling pathway regulation. Furthermore, in Parkinson's disease (PD) apelin plays a protective role, although the mechanism is still unknown. Activation of the apelin receptor has a neuroprotective and antiapoptotic effect in PD. Additionally, apelin is connected with progression of Huntington disease (HD). It can delay HD progression through inhibition of the tumor necrosis factor/nuclear factor kappa B (TNF/NF-κB) pathway connected with increased neurodegeneration induced by mutant huntingtin protein (Luo et al. 2019).

Drugs

Apelin as a Therapeutic Target

Despite the fact that no clinical trials focusing on apelin have been conducted, many scientists still focus on the possible application of this peptide in pathological disorders. Apelin has an inhibitory effect against atherosclerosis, which is a chronic inflammatory disease. It can reduce lipid accumulation of foam cells through autophagy activation via PI3K/Beclin-1 pathways. Moreover, apelin signalling blocks angiotensin II-induced atherosclerosis, which is probably caused by eNOS pathway activation.

Under hypertension, exogenous peptide injected into rats decreased systolic and diastolic blood pressure immediately. Apelin also reduced the mean arterial pressure in rats, and this mechanism is probably connected with nitric oxide production. Moreover, in patients with hypertension, the level of serum apelin was lower than in healthy controls. The same results were obtained in patients with left ventricular hypertrophy or hemodialysis patients with pulmonary arterial hypertension.

The main feature of myocardial infarction is left ventricular enlargement and reduced capillary density. Several studies have indicated that the concentration of serum apelin was lower in patients with acute myocardial infarction in comparison to healthy individuals. Furthermore, exogenous administration of apelin reduced infarct size and improved heart muscle functions after ischemia through phosphorylation of PI3K/Akt and extracellular signal-regulated kinase (ERK) pathways. Additionally, apelin treatment

led to an increase in myocardial capillary density and formation of new blood vessels.

In diabetes mellitus, more evidence suggests that apelin can lower blood glucose level and improve insulin sensitivity. It stimulates glucose uptake via the PI3K/Akt pathway. This peptide also plays a protective role in the regulation of diabetic complications. It inhibited the progression of diabetic nephropathy in the model of diabetic mice. Moreover, apelin suppressed hypoxia-induced apoptosis of pericytes, cells involved in normal vascular structures, by downregulating the expression of caspase-3 in diabetic retinopathy (Cheng et al. 2019).

Drugs Targeting Apelin/APJ

At present, there are several apelin receptor antagonists that have been described and used to examine therapeutic effects in cell cultures, animal models, and patients. F13A is one of the natural antagonist isoforms of APJ that is highly selective and exhibits similar binding affinity but opposite functions to those of natural apelin-13. It can inhibit tumor growth and dilation of arteries in a hepatocellular carcinoma model. It also promotes hepatocyte proliferation and liver regeneration after hepatectomy. The first synthetic peptidic compound described as an APJ antagonist is ALX40-4C. It was first used in phase I/II clinical trials to investigate its therapeutic effects against HIV-1 virus. Several studies suggested that it could also block ligand-induced APJ internalization and intracellular calcium mobilization. There are also several cyclic peptides synthesized as APJ antagonists that are more protected from proteolytic degradation and have a long half-life. Several nonpeptidic small molecule compounds also target the APJ receptor. Well-known ML221 is described as the first reported APJ functional antagonist in cell-based assays. It could inhibit migration and invasion, as well as proteolytic activities of colon cancer cells. It also inhibits pathological angiogenesis and at the same time accelerates the recovery of normal vessels in the ischemic regions in the retina of oxygen-induced retinopathy model mice. To conclude, every drug targeting the apelin receptor has therapeutic potential, but further research is necessary (Huang et al. 2018).

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APJ Endogenous Ligand

▶ Apelin

APLN

▶ Apelin

ApoE Receptors

▶ Low-Density Lipoprotein Receptor Gene Family

Apolipoprotein E Receptor Family

▶ Low-Density Lipoprotein Receptor Gene Family

Appetite Control

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Synonyms

Control of food intake; Regulation of ingestive behavior

Definition

Appetite control is a complex function of the brain that regulates feeding behavior. This function integrates cognitive and emotional factors with a complex array of peripheral signals originated from the gastrointestinal tract, adipose tissue.

Basic Mechanisms

Feeding behavior is subjected to both short-term regulation during a single meal and long-term regulation related to the maintenance of body weight and fat content. As a complex function of the brain, ingestive behavior is controlled by psychological and cognitive factors such as sociocultural context (e.g., eating habits), experience (sensory preferences), or emotional status (mood). Appetite control is regulated by the nutritional status, which can be sensed in the brain by glucose-sensing neuronal and non-neuronal cells to elicit an adaptive response. Appetite control also integrates information about the status of peripheral organs, particularly the gastrointestinal tract and adipose tissue (Morton et al. 2006). Two main groups of signals can be distinguished: (i) satiety signals (short term) secreted from gastrointestinal organs and (ii) adiposity-related signals (long term) that are proportionate to body fat stores (Morton et al. 2014). A key factor of this control system is that energy intake is primarily controlled by adjustment of the meal size rather than the meal onset, allowing the organism to initiate meals at times that are convenient and to adapt eating patterns to individual constraints (food availability) and activities (circadian rhythm).

Satiety Signals

Satiety-inducing signals are conveyed to the brain by afferent nerve fibers that are sensitive to mechanical or chemical stimulation of the stomach and small intestine during food ingestion. In addition, humoral signals such as cholecystokinin (CCK) are released upon nutrient stimulation of neuroendocrine cells located in the gastrointestinal wall. These satiety signals converge in the nucleus tractus solitarii in the brainstem and induce meal termination in the absence of hypothalamic control, as demonstrated in decerebrated rats. CCK is the paradigmatic humoral satiety signal, and its action has been studied extensively in multiple species including humans. Exogenous administration of CCK dose dependently reduces meal size. This effect is synergistically enhanced by other factors that limit meal size, such as gastric distension. Specific CCK-A receptor antagonists stimulate food intake in rats, indicating that endogenous CCK contributes to the termination of meals. However, repeated administration of CCK before each meal does not reduce caloric intake of free-feeding mice or rats, because the animals compensate the reduced meal size by increasing the number of meals. In obesity there is CCK resistance, a phenomenon where the body does not react to physiological concentrations of CCK.

Adiposity Signals

Insulin as a Satiety/Adiposity Signal

The first hormonal signal found to comply with the characteristics of both a satiety and an

adiposity signal was insulin (Morton et al. 2006). Insulin levels reflect substrate (carbohydrate) intake and stores, as they rise with blood glucose levels and fall with starvation. Increased insulin secretion in obesity can be explained by reduced insulin sensitivity of the liver, muscle, adipose tissue, and brain. Insulin is known to enter the brain via a receptor-mediated transport, and direct administration of insulin to the brain reduces food intake. The adipostatic role of insulin is supported by the observation that mutant mice lacking the neuronal insulin receptor (NIRKO mice) develop obesity. Further, sole brain insulin signaling activation in men by administering intranasal insulin reduces food intake confirming insulin's suppressing effect on appetite. Insulin resistance occurs in obese conditions attenuating the effect of insulin on food intake and causing hyperglycemia and impaired lipid storage.

Leptin as an Adiposity Signal

Leptin is a cytokine produced and secreted by adipose tissue in proportion to the body fat content (Friedman and Halaas 1998). Overall leptin synthesis is higher in subcutaneous than in visceral adipocytes. Mice lacking leptin receptor in the brain develop a severe hyperphagia and a dramatic degree of obesity which is considerably more pronounced than that of the NIRKO mouse. Thus, leptin is the key adiposity signal in rodents and humans. Leptin secretion reflects the metabolic status of the adipocyte rather than the sheer size of triglyceride deposits, and leptin levels may transiently be dissociated from total body fat. Nonetheless, over the course of a day with unrestricted food supply, plasma leptin levels reliably reflect the amount of total body fat. Local administration of leptin into the brain results in reduced food intake. The vast majority of patients with obesity have elevated serum levels of leptin. Thus, it is believed that the polygenic obesity is due to leptin resistance. Data are emerging that reducing leptin secretion in obesity improves leptin resistance and insulin sensitivity, suggesting that primary hyperleptinemia might be causal for altered leptin signaling in the long term (Zhao et al. 2019).

Appetite-Regulating Pathways in the Arcuate Nucleus of the Hypothalamus

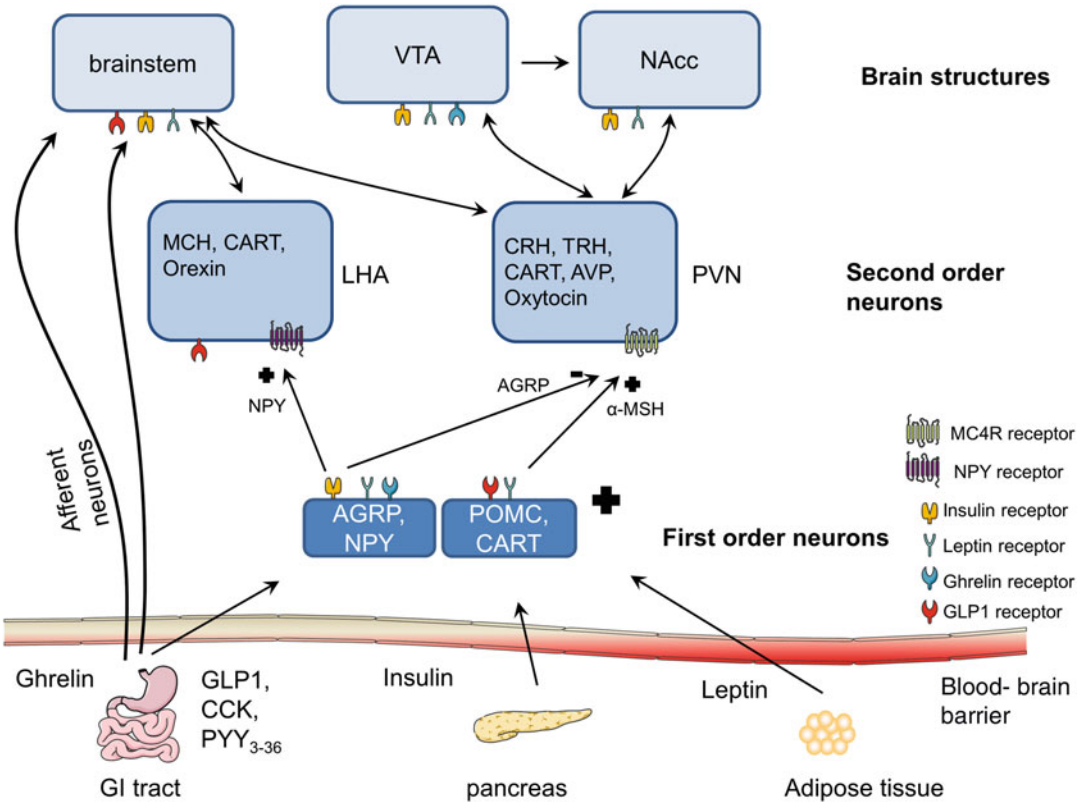
Two distinct populations of neurons in the arcuate nucleus have been identified as the most relevant target cells of leptin (Fig. 1) (Timper and Bruning 2017). Leptin inhibits expression of the orexigenic peptides NPY (neuropeptide Y) and AgRP (agouti-related protein) in one subset of neurons and stimulates production of the anorexigenic peptides α-MSH (α-melanocyte-stimulating hormone) and CART (cocaine- and amphetamine-regulated transcript) in the other. Insulin receptors are also highly concentrated in the arcuate nucleus, and insulin appears to elicit similar changes in these neuropeptides as leptin. Further insulin and leptin signaling can cross activate each other in the hypothalamus, suggesting a high degree of functional overlap. Nevertheless, recent studies reveal heterogeneity in these hypothalamic neuronal populations highlighting the importance of further detailed investigation to gain better insights into the complex interplay of hormonal control of appetite control.

The Melanocortin Signaling System

Considerable evidence indicates that the molecules of the melanocortin system are key mediators of the response to leptin. AgRP and α-MSH are antagonistic ligands for a common receptor, the melanocortin-4 receptor (MC4R). α-MSH is an anorexigenic neuropeptide that activates MC4R and thereby reduces appetite, whereas AgRP is an orexigen that acts as an endogenous antagonist of the receptor and suppresses its activation by α-MSH. The critical role of the melanocortin system in appetite regulation is supported by the effects of spontaneous and experimental mutations of AgRP, α-MSH, and MC4R in mice. Moreover, patients with complete loss of proopiomelanocortin (POMC), the precursor molecule of α-MSH, develop severe hyperphagia and overweight, and 4–5% of all cases of severe human obesity appear to be due to mutations in the *MC4R* gene.

Role of NPY/AGRP Neurons

NPY has long been known to be a potent orexigen when directly injected into the hypothalamus.



Appetite Control, Fig. 1 Diagram of pathways integrating appetite control. Satiety is the net output from brainstem centers that leads to the termination of an individual meal. Satiety is primarily determined by neural and humoral inputs from the gastrointestinal tract (short-term satiety signals). Response to satiety signals is modulated by descending anabolic or catabolic pathways originating in the hypothalamus. Appetite-regulating hormones are released in proportion to total body fat (leptin), glucose levels (insulin), or changes in food intake (ghrelin). Leptin and insulin stimulate secretion of anorexigenic peptides (α-MSH, CART) and inhibit expression of orexigenic ones (NPY, AgRP) in the arcuate nucleus (ARC), whereas ghrelin exerts opposite effects. Secondary target neurons in the paraventricular nucleus (PVN) and the lateral hypothalamic area (LHA) integrate signals from neurons in the ARC and connect them with satiety centers in the brainstem. In addition, recent evidence shows that afferent signals such as leptin and ghrelin also directly target brain centers known to play a key role in reward and addiction. Areas where such neurocircuitry is located include the ventral tegmental area (VTA) or the nucleus accumbens and appear to control mostly the hedonic components of feeding behavior

Hyperphagia of the leptin-deficient ob/ob mice is attenuated by knockout of NPY, supporting the role of NPY as a downstream effector of leptin. The effects of NPY on appetite regulation are mediated by different receptor subtypes (NPY1R and NPY5R). However, the neuropeptide is not an indispensable transmitter of adiposity signals, since lean mice which lack NPY show a normal feeding behavior. On the other hand, it has been shown that the NPY/AGRP neurons play an essential role for basal orexigenic drive. Ablation of these neurons from the arcuate nucleus in adult mice reduces drastically food intake with a substantial loss of body fat. In conclusion, NPY/AGRP neurons likely play an essential role in the control of feeding behavior, but their function cannot be easily explained solely based on the expression of the neuropeptides NPY and AGRP.

Second-Order Hypothalamic Targets in Adiposity Signaling

Lesions of the lateral hypothalamic area (LHA) cause anorexia, whereas ablation of the paraventricular nucleus (PVN) causes a hyperphagic

obesity syndrome. Consistent with these results, LHA neurons express the orexigenic neuropeptides MCH and orexin. PVN neurons produce several neuropeptides that are anorexigenic when administered directly into the brain (CRH, TRH, oxytocin), in addition to their better known roles as endocrine regulators. LHA and PVN receive rich inputs from axons of NPY/AgRP and α-MSH/CART-producing neurons in the arcuate nucleus.

Other Hormones, Peptides, and Neurotransmitters Involved in Appetite Control

Many other peptides including galanin, ghrelin, and glucagon-like peptide-1 and glucagon-like peptide-2 (GLP-1 and GLP-2) have been described to participate in appetite control (Table 1). In addition, the neurotransmitters norepinephrine, dopamine, and serotonin are known to be involved in appetite regulation. The role of the monoamines in energy homeostasis is illustrated by effects of drugs (see below). Agonists of α_1 adrenoceptors, 5-HT_{2C} serotonin receptors, and dopamine receptors (D1 and/or D2) suppress appetite. The relevant neural circuits that use these transmitters are complex but include projections to the arcuate nucleus. A control system mediating appetite-stimulating effects is the cannabinoid signaling. Endocannabinoids have been added to the list of signals that act downstream of leptin. Leptin reduces levels of the endocannabinoid anandamide in the hypothalamus of normal rats, and mice that lack the cannabinoid receptor 1 (CB1) showed reduced food intake under conditions of low leptin levels (after fasting).

Pharmacological Intervention

Appetite-Suppressing Drugs

The increasing prevalence of obesity and its consequences has stimulated the search for appetite-suppressing drugs as anti-obesity agents (Timper and Bruning 2017). Therapy based on nutritional and behavioral counseling produces almost always only a temporary weight loss. Past anti-obesity drugs targeted predominantly adrenergic and serotonergic pathways (e.g., fenfluramine, sibutramine), and many had been withdrawn from the market because of side effects (e.g., rimonabant, sibutramine). The recent insights in appetite control as outlined above have provided new candidate targets for the search of appetite-suppressing drugs. New existing drugs target various centrally acting pathways (e.g., serotonergic, dopaminergic, GLP-1 signaling) but have common negative side effects such as nausea or dry mouth. Due to novel insights into neuronal control of food intake, combination therapies are now present such as bupropion/naltrexone, which improve metabolic outcome and can reduce adverse effects. Since obesity is usually a chronic disorder which requires lifelong therapy, anti-obesity drugs need to meet high safety standards.

Incretin Mimetics

Liraglutide, a GLP-1 receptor agonist, has been initially prescribed as treatment option for type 2 diabetes but has now been also approved for the treatment of obesity. At a dose of 3 mg/day and a half-life of 13 h, liraglutide – in addition to peripheral actions – reduces food intake by modulating the reward and the homeostatic system in the brain. It activates the reward system in the ventral tegmental area in addition to POMC stimulation in the hypothalamus resulting in reduced appetite.

Exenatide, a 39-amino-acid peptide from the Gila monster (*Heloderma suspectum*), is an incretin mimetic, a functional analog of human glucagon-like peptide-1 (GLP-1). Because of its resistance to degradation, *in vivo* potency of exenatide is much greater than that of GLP-1. Exenatide improves glycemic control through glucose-dependent secretion of insulin and suppression of high glucagon levels in patients with type 2 diabetes, delays gastric emptying, and reduces of food intake. Exenatide is administered at doses of 5 and 10 μg twice daily; its most frequent adverse effects were nausea and hypoglycemia.

Phentermine

Phentermine is a sympathomimetic and enhances neurotransmitter release, primarily the release of norepinephrine with minor dopamine release. It is

Appetite Control, Table 1 Hormones, peptides, and neurotransmitters implicated in appetite control

Hormones, peptides, and neurotransmitters	Effect of ICV injection on food intake	Effect of gene deletion on food intake	Response to adiposity signals	Receptor	Effect of receptor defect on food intake
Satiety signals					
Cholecystokinin (CCK)	↓		–	CCK-A	↑(↔[a])
Adiposity signals					
Leptin	↓	↑↑	–	LEPRb	↑↑
Insulin	↓		–	IR	↑[b]
Orexigenic					
Neuropeptide Y (NPY)	↑	↔(↓[c,d])	↓	NPY1R	↓
				NPY2 and 5R	↑
Agouti-related peptide (AgRP)	↑	↔(↓[d])	↓	MC3R, MC4R[e]	↑
Melanin-concentrating hormone (MCH)	↑	↓	↓	MCHR1	↑
				MCHR2	
Orexin A and B (hypocretins)	↑	↔[f]	↓	HCRTR1	
				HCRTR2	
Galanin	↑	↔		GALR1–GALR3	
Galanin-like peptide	↑(↓)[g]		↑	GALR1–GALR3	
Ghrelin	↑	↔	↓	GHSR	↓[h]
Endocannabinoids	↑			CB1	↔(↓[i])
Anorexigenic					
α-Melanocyte-stimulating hormone (α-MSH)	↓	↑[j]	↑	MC4R	↑
Cocaine- and amphetamine-regulated transcript (CART)	↓	↔	↑	Inhibitory G-protein-coupling receptor?	
Corticotropin-releasing hormone (CRH)	↓	↔versus↓	↑	CRHR1	↔(↓[k])
				CRHR2	↔
Urocortin	↓		↑	CRHR1	↔(↓[k])
				CRHR2	↔(↓[l])
Thyrotropin-releasing hormone (TRH)	↓		↑	TRHR	↔
Glucagon-like peptide (GLP-1 and GLP-2)	↓		↑	GLP1R	↔
				GLP2R	↑[m]
Serotonin	↓		↑	5-HT_{1B}	↑
				5-HT_{2C}	
				5-HT_4	
Noradrenaline	↓(↑)			α_1,(α_2)	

[a]Normal basal food intake in knockout mice, but stimulatory response to CCK is abolished
[b]Neuron-and NPY-specific insulin receptor knockout
[c]Reduction of hyperphagia was observed in leptin-deficient mice that also lack NPY
[d]NPY/AgRP neuronal ablation
[e]AgRP acts antagonistic on MC4 receptors
[f]Knockout mice exhibit narcolepsy
[g]Acute food intake increases, but 24-h food intake decreases after GALP injection
[h]Mice under chronic high-fat diet
[i]Reduced feeding response to fasting in CB1 knockout mice
[j]α-MSH deficiency in patients with mutations in the precursor, proopiomelanocortin (POMC)
[k]Normal basal food intake in knockout mice, but inhibitory response to urocortin is attenuated
[l]Attenuation of urocortin-induced food intake by CRHR2 antagonism
[m]Knockout only in POMC neurons

an appetite suppressant by reducing the perception of hunger. Weight loss is about 5% of initial body weight. Adverse effects include elevated heartbeat, high blood pressure and restlessness but may also include pulmonary hypertension and valvular heart disease in severe cases. Due to severe adverse effects, phentermine has been withdrawn from the market in Germany.

Weight was regained when the drug was stopped, indicating that a continuous therapy would be necessary to achieve the useful but limited therapeutic effect. This general limitation is likely to apply for any novel drug that targets central pathways. Phentermine is currently available as a combinational therapy with topiramate, a sulfamate derivative of fructose, which decreases neuronal activation and may enhance thermogenesis. This combination exhibits beneficial effects on body weight reduction, in addition to improved blood pressure, HbA1c levels, and quality of life. However, topiramate causes a marked impairment of cognitive functions that outweighs its therapeutic benefit.

Bupropion/Naltrexone

Bupropion is a monoamine reuptake inhibitor and increases dopamine and norepinephrine action. Further bupropion activates POMC neurons resulting in reduced appetite and energy expenditure. Naltrexone represents an opioid antagonist and blocks the orexigenic effects of β-endorphin, which is co-spliced with α-MSH upon POMC activation. Thus this combination causes a more profound activation of the appetite-suppressing effects due to POMC activation. A weight loss about 5% is achieved after a year of treatment. A common side effect is nausea.

Lorcaserin

Lorcaserin is a selective 5-Ht_{2c} receptor agonist and causes weight loss by improving satiety, reducing food craving and impulsivity, and reducing overall the activity in the reward system. A common dose is 10 mg/day and results in average in a 4% body weight loss compared to control subjects. Adverse effects are headache, back pain, and nausea.

Tesofensine

Tesofensine is a serotonin-noradrenaline-dopamine reuptake inhibitor and was initially developed as a potential medication for the treatment of Alzheimer's and Parkinson's disease. The efficacy criteria were not met, but an unexpected side effect of profound weight loss in these patients was identified. Tesofensine is currently in phase III clinical trials for obesity and at an oral dose of 0.5 mg/day reduces body weight by up to 10% in obese patients. Rodent data suggest that tesofensine reduces food intake by activating α1 adrenoreceptors and also D1 dopamine receptors. Common side effects in obese patients are dry mouth, headache, and nausea. Elevations in blood pressure and pulse rate have also been reported.

Leptin and Leptin Sensitizers

Leptin has been shown to markedly reduce appetite and weight in extremely rare individuals who lack leptin. In contrast, in the first clinical study of patients with polygenic obesity and elevated leptin levels, weight loss was variable and relatively small. This disappointing result may be explained by the leptin resistance consistently observed in obese humans and rodents and novel data showing that hyperleptinemia per se might be a cause of leptin resistance (Zhao et al. 2019). Leptin is used for the treatment of generalized lipodystrophy. Celastrol, a pentacyclic triterpene and major component of *Tripterygium wilfordii*, reduces body weight up to 45% due to suppression of food intake in leptin-resistant animal models. In particular, celastrol enhances leptin sensitivity independent of the melanocortin system but via activation of IL-1 receptor, suggesting that activation of inflammatory processes can be beneficial (Feng et al. 2019). Clinical trials are currently in early phase I.

Drugs with Appetite-Stimulating Effects

Psychotropic Drugs

Stimulation of appetite and weight gain has frequently been observed as a side effect of long-term therapy with various psychoactive drugs. Prominent examples not only are the tricyclic (e.g.,

imipramine) and heterocyclic (e.g., mirtazapine) antidepressants but also selective serotonin reuptake inhibitors (e.g., paroxetine), neuroleptic drugs altering dopamine or histamine signaling (e.g., olanzapine), and lithium. These drugs modulate central monoamine signaling by, e.g., altering serotonin, dopamine, or histamine receptor activation, which is believed to explain their appetite-stimulating effect. Stimulation of appetite by cyproheptadine, a nonselective serotonin receptor antagonist and histamine receptor blocker, is presumably caused by mainly inhibiting serotonin receptor activation.

Treatment of Cachexia and Anorexia

In the palliative treatment of cachexia and anorexia in advanced cancer and AIDS patients, modest relief can be achieved with appetite-stimulating drugs. Various pharmacologic strategies have been tested, including corticosteroids, anabolic steroids, megestrol acetate, cyproheptadine, melatonin, and dronabinol (delta-9-tetrahydrocannabinol). The cannabinoid receptor agonist dronabinol is approved in the USA for stimulation of appetite in AIDS patients. Thalidomide also improves appetite and progressive weight gain in AIDS patients. Cyproheptadine improves appetite in patients with cystic fibrosis. Megestrol has been successfully used to increase appetite and weight gain in patients with cancer.

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Aptamers

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Definition

An aptamer is a short 20–100 nucleotide-long polymer of single-stranded DNA or RNA that binds specifically to a molecular or cellular target, such as a protein, small molecule, or even a whole cell (e.g., lysozyme, thrombin, vascular endothelial growth factor, ATP, IgE, nucleolin, ochratoxin A, among others). Although aptamers tend to be synthetically derived, there are examples of naturally existing riboswitches, which contain an aptamer-like region and, upon binding a small molecule, induce a structural change. Analogous to antibodies, aptamers act as molecular recognition elements, binding tightly to their intended target (Nimjee et al. 2017).

In the consideration of aptamer utility, aptamer structure and nucleic acid composition play a critical role. Watson-Crick base pairing creates the secondary structure (e.g., stems, hairpins, bulges, etc.) with additional non-covalent interactions forming the tertiary structures (e.g., pseudoknots, G-quadruplexes, etc.). Secondary

structures may be computationally predicted (e.g., MFold program), which provide aptamers with a unique versatility that permits the design and creation of aptamer constructs by simple adjustments of the oligonucleotide sequence. For example, the stability of the hairpin structure can be increased through the addition of complementary nucleotides on the 5′ and 3′ ends of the oligonucleotide or an oligonucleotide sequence may be inserted to link together aptamer sequences. Further, aptamers may be predictably denatured, rendering them inactive, and refolded into their native, functional structures. The versatility of aptamers allows for optimization in diagnostic assays, molecular sensing, therapeutics, and drug delivery, allowing for a single aptamer to serve multiple purposes.

Aptamers are typically isolated through a process known as SELEX (*Systematic Evolution of Ligands by EXponential enrichment)*. In general, oligonucleotides that bind the target are isolated and amplified, thus enriched in an iterative process that is repeated until oligonucleotide species that bind with high affinity are enriched and identified using sequencing and binding assay analyses.

Basic Mechanisms

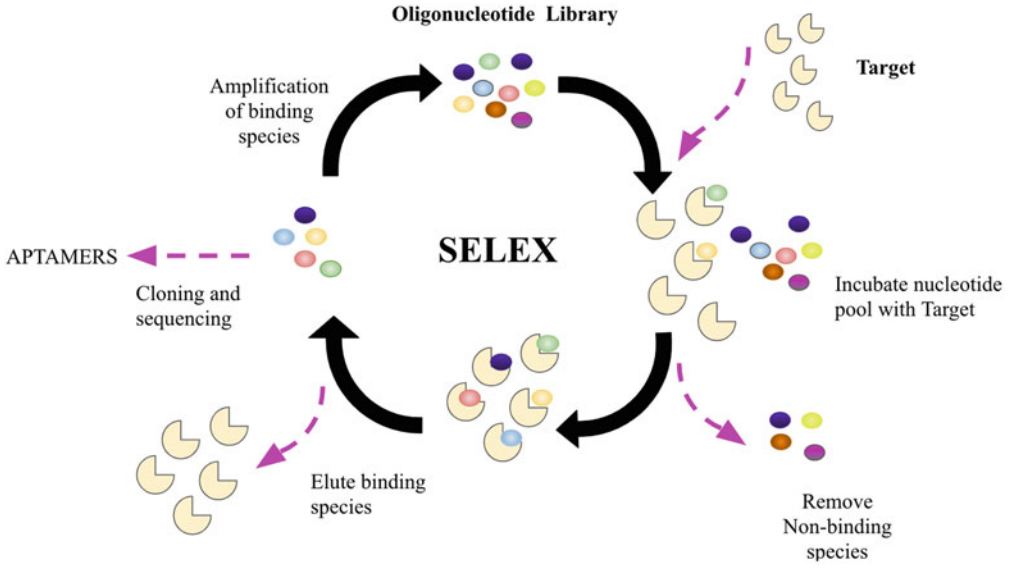
Aptamer Selection

Aptamers are typically isolated in a process known as the *Systematic Evolution of Ligands by EXponential enrichment* (SELEX) or in vitro aptamer selection. In SELEX, a nucleic acid library, containing 10^{14}–10^{15} random species, is enriched for species that bind to the target. In practice, the library is designed with each oligonucleotide species containing a variable or random region flanked by static regions that include PCR primer binding sites. The design of the nucleic acid library includes consideration of the length of the randomized region, nucleic acid type (i.e., canonical A, C, G, T/U, modified, or a combination thereof), flanking static regions (e.g., PCR primer design, inclusion of an RNA polymerase start site/promoter, etc.), and potentially the target (e.g., hydrophobic/hydrophilic, positively/negatively charged, etc.) (Stovall et al. 2014).

The enrichment of target-binding species is accomplished via iterative cycles of the following: target and oligonucleotide pool incubation, selection, and amplification of the target-bound oligonucleotide species (see protocol in Stovall et al. 2014). First, random nucleic acid libraries are incubated with targets. The selection step partitions target-bound oligonucleotide species from unbound species, using a physical partitioning methodology (e.g., nitrocellulose filter-based, magnetic bead-based, affinity chromatography, gel-shift selections, etc.). In the final step, isolated target-binding species are amplified, typically by polymerase chain reaction (PCR) (Fig. 1).

After target-pool incubation and application of selection pressure in an RNA aptamer selection, amplification of target-bound RNA occurs through conversion to cDNA via reverse transcription and subsequent PCR amplification. During PCR, a primer with an RNA polymerase promoter sequence is reappended to enable subsequent regeneration of RNA. Upon completion of PCR, a transcription reaction generates the selected RNA pool for additional rounds of in vitro aptamer selection. Typically, 6–20 rounds are performed with increasing stringency of selection conditions (e.g., pH, temperature, pool/target ratio, monovalent/divalent salt concentrations) to enrich the nucleotide pool, reducing the complexity of the original library (Stovall et al. 2014).

The efficacy of the selection can be assessed by sequencing clones in the selected pool (Stovall et al. 2014). The increased abundance of short sequence motifs (e.g., 4–10 nucleotide-long recurring sequences) or even full-length clones across rounds is evidence that a selection occurred, although a binding assay is necessary to confirm an aptamer selection. Binding assays, such as radioactive binding assays, microscale thermophoresis, surface plasmon resonance (SPR), etc., determine the affinity and specificity of the selected pool and aptamer candidates to bind their target (Plach and Schubert 2019). Further, identified aptamers can be truncated and assayed to determine the minimized variant or



Aptamers, Fig. 1 Schematic illustration of the SELEX process for the generation of aptamers. The procedure consists of multiple rounds of three major steps: (I) binding/incubation of the aptamer target with the nucleotide library, (II) elution of binding oligonucleotide species, and (III) amplification of collected oligonucleotides (i.e., selected pool)

minimum sequence/region required to preserve binding and specificity (Stovall et al. 2014).

The SELEX procedure has been modified in a number of ways to both improve efficacy and to cater to a diverse set of applications. Some commonly utilized examples include negative SELEX, toggle SELEX, and in vivo SELEX (for more examples of SELEX methodologies, see table in Darmostuk et al. 2014).

Interactions Between the Target and Aptamer

The aptamer's folded structure and the subsequent binding interactions with the target play a key role in formulating the intended application. Single-stranded aptamers, primarily via Watson-Crick base pairing and other non-covalent interactions, form structural motifs such as hairpins, pseudoknots, stems, bulges, and G-quadruplexes. The stability and secondary structures of the aptamers can be computationally predicted using programs such as MFold. Binding interactions between the target and aptamer are driven by hydrogen bonding, electrostatic interactions, hydrophobic interactions, π-π stacking, and van der Waals forces (Sakamoto et al. 2018). The specific structural characterization of these interactions may be ascertained through nuclear magnetic resonance spectroscopy (NMR), X-ray crystallography, and electron microscopy.

Quantifying the affinity of the aptamer to the target can be found through a number of techniques, generally referred to as binding assays. Common binding assay techniques include surface plasmon resonance (SPR), biolayer interferometry (BLI), microscale thermophoresis (MST), fluorescence polarization (FP), flow cytometry, filter binding assay, and electromobility shift assay (EMSA) (Plach and Schubert 2019).

Aptamer to target binding affinity is typically expressed by the dissociation constant (K_d), the rate of dissociation between the aptamer and the target. A smaller K_d reflects a higher binding affinity between the aptamer and the target. The K_d is related to Gibbs free energy (ΔG) by the following equation: $\Delta G = -RT\ln(1/K_d)$.

Observing the thermodynamic properties of the aptamer-target interaction is another approach of assessing the binding affinity. For example, isothermal titration calorimetry (ITC) is a label-free method frequently used to quantify

aptamer-target thermodynamic parameters (ΔG, ΔH, and ΔS) and K_d by measuring the enthalpic change between the bound and unbound aptamer-target species. An ITC binding assay is typically performed by maintaining one interacting partner at constant concentration and volume and incrementally titrating the other interacting partner into the system (Plach and Schubert 2019).

Pharmacological Relevance

Aptamers as Diagnostic Agents

Due to their molecular recognition properties as well as their structural versatility (including computationally predicted structures, ability to refold, etc.), aptamers are well suited as diagnostic and sensing reagents. The hairpin structure of aptamers allows for conjugation of molecules to the 5′ and/or 3′ terminal site or intercalation within the stem without impacting the structure or binding. From binding the target, a conformational change can result in observable changes in fluorescence, electrochemistry, or colorimetry. Widely used methodologies that are coupled with aptamers to suit these observable changes include optical sensing, electrochemistry techniques to create "aptasensors", and nanotechnology.

Aptamer-Fluorophore Conjugates and Optical Sensing

Aptamers are advantageous in optical imaging techniques due to the ease of fluorescent labeling and the availability of a variety of fluorophores and quenchers. There are several methods for optical detection using aptamers, two of which will be highlighted here. Both approaches are based on the flexible structure of the unbound aptamer and the more rigid structure of the target-bound aptamer. In one approach, fluorophore and quencher are conjugated to opposing termini (5′ and 3′) of a destabilized hairpin structure. Upon binding of the analyte, the conformation of the aptamer is altered, thereby altering the distance of the fluorophore and quencher and resulting in a change in fluorescence. The other approach involves strand displacement. In this example, the system contains an optically labeled aptamer recognition element (i.e., something to bind the target) and an oligonucleotide that is complementary to that aptamer (which is it attached at a distance from the aptamer or a different strand altogether). The complementary strand is labeled with a quencher and bound to the linearized aptamer. Upon the aptamer binding the target, the complementary quencher strand is displaced through a competitive binding interaction, resulting in a colorimetric response (Ilgu and Nilsen-Hamilton 2016).

Electrochemical Sensing

Electrochemical sensing with aptamers involves the use of an electrode to measure changes in impedance, current, or resistance and detect the presence of the analyte. The mechanisms underlying electrochemical sensing are diverse. One method relies on changes in the aptamer-target interaction that can alter the catalytic activity of an oxidative or reductive enzyme resulting in signal detection. Another method, dubbed "strand displacement," involves the displacement of a complementary strand with a redox reporter or the sole displacement of a redox reporter. For example, in a complementary strand and redox reporter displacement system, when the target binds to the aptamer, the release of the complementary strand results in the displacement of the redox reporter to generate the electrochemical signal (Ilgu and Nilsen-Hamilton 2016).

Nanomaterials, Aptamers, and Sensing

The stability, controllable chemical and physical properties, and large surface areas of nanomaterials can be coupled with the high selectivity of aptamers enabling for the design of highly selective and sensitive sensors. The common aptasensors utilizing nanomaterials include colorimetric detection using gold nanoparticles (AuNPs). The AuNPs adsorb the aptamers onto the surface. Through competitive displacement, target binds the aptamer. When the aptamer is displaced, the AuNPs aggregate resulting in a change in color from red (disaggregated) to purple (aggregated). The change in color is attributed to the change in the interparticle distance between the AuNPs (Jo and Ban 2016). Other examples of

nanomaterials employed in aptasensors include quantum dots, magnetic nanoparticles, gold nanorods, and graphenes.

Aptamers as Therapeutic Agents

Aptamers can be used directly as therapeutic agents. Acting primarily as antagonists, aptamers can competitively inhibit their molecular targets in vivo (e.g., enzymes, receptors, etc.). A comprehensive list of aptamer therapeutics that have undergone clinical testing can be found in the Haßel and Mayer (2019) review.

The only currently FDA-approved aptamer therapeutic Macugen® (pegaptanib) is a treatment for wet, age-related macular degeneration. Approved in 2004, the Macugen aptamer binds a 165 amino acid-long isoform of the vascular endothelial growth factor (VEGF) protein and inhibits its function, thereby suppressing angiogenesis and decreasing swelling and bleeding. The aptamer is constructed from a 2′-fluoro modified pyrimidine and 2′-methoxy modified purine library and contains a PEG addition and 3′ inverted dideoxythymidine cap for enhanced in vivo stability (Haßel and Mayer 2019).

Despite their limited translation to clinical use, aptamers are well suited to the therapeutic space, as they exhibit high tissue and cell penetration, allowing for efficient and differential take-up by the target cells (Hori et al. 2018). The use of covalent and non-covalent conjugation strategies allows for different aptamers to serve as easily exchangeable building blocks for functionalization of other therapeutic agents, such as liposomes and gold nanoparticles (Hori et al. 2018). Several optimizations can be made to aptamer therapeutics, which include functional regulation via aptamer antidotes and chemical modifications to improve longevity and specificity in vivo.

Functional Therapeutic Designs Using Antidotes

Because of the nature of oligonucleotides, Watson-Crick base pairing, and solved secondary structures, functional aptamer therapeutic designs are common, as in the development of "antidotes." In instances in which an aptamer's activity needs to be neutralized quickly, an oligonucleotide antidote, with a sequence complementary to the aptamer, may be employed. In vivo, the antidote oligonucleotide binds the aptamer and prevents secondary structure formation, thus quenching the functional aptamer. This methodology has been successfully employed in the delivery of an anti-coagulation factor IXa aptamer and its antidote, thus dosing the clotting response (Nimjee et al. 2017). Aside for this point, the aptamer REG1 developed for this coagulation factor IXa target failed Phase III clinical trials in 2014, because of adverse anaphylactic effects which were likely the result of the PEG addition and not the aptamer itself (Haßel and Mayer 2019).

Modifications in the Therapeutic Space

Due to their small size and hydrophilicity, aptamers are susceptible to clearance from the bloodstream via renal filtration and degradation by nucleases. There are several strategies to mitigate these effects. Conjugation of moieties such as PEG, zwitterionic poly(carboxybetaine) (PCB), or poly(2-methacryloyloxyethy phosphorylcholine) (PMPC) slows renal or hepatic clearance. Recent studies have found, however, that PEGylation elicits an immune response that inhibits binding and anaphylactic effects while PCB and PMPC exhibit low to non-immunogenicity. Further, the use of chemical modifications of the phosphor-sugar backbone of RNA aptamers (such as replacing 2′OH with 2′F or 2′-*O*-methyl, locked nucleic acids, xeno nucleic acids, or phosphorothioates) introduces resistance to nuclease degradation improving half-life and stability in vivo. Similarly, spiegelmers, which are a class of aptamers whose sugars are the enantiomer of those present in living systems, confer resistance to nuclease degradation due to the stereoselective nature of enzymatic processes. Further, three of such spiegelmer aptamers are currently in or completed Phase II clinical trials (Haßel and Mayer 2019).

Introduced by the company SomaLogic, another class of molecules known as SOMAmers (slow off-rate modified aptamers) optimize binding affinities and lower dissociation rates by using oligonucleotide libraries containing deoxyuridine bases with five-position modifications that

contain functional groups akin to amino acids. The addition of the modified uridines potentially increased the diversity nucleic acid libraries and, subsequently, improved selection hit rates to identify aptamers (Nimjee et al. 2017).

Aptamers as Drug Delivery Agents

In addition to being used as direct therapies, aptamers can be adapted for targeted drug delivery. They can bind to cell surface targets, delivering therapeutic payloads to a particular cell or tissue type. For example, aptamers may deliver small compound drugs, oligonucleotide-based drugs, or nanoparticles.

Aptamers Conjugated to Small Compounds

The conjugation of aptamers to small compounds, such as drugs, toxins, etc., allow for targeted delivery and thus a reduction of systemic exposure and toxicity. This aptamer-targeted drug delivery is particularly promising in the development and refinement of anticancer drugs such as doxorubicin, which induces cancer cell death by intercalating into DNA. The drug cargo (i.e., anthracycline drugs, doxorubicin, daunorubicin, etc.) can be linked to the aptamer by intercalation in GC-rich regions of the aptamer, conjugation of a CG-cargo structure containing the intercalated payload, or creation of a dimeric aptamer complex with CpG sequences containing the intercalated payload (Hori et al. 2018). GC regions are typically used in designs implementing doxorubicin because it is known to bind preferentially to 5′-CG-3′ and 5′-GC-3′ regions. Additionally, the high GC content provides added stability (Hori et al. 2018). Other conjugation strategies include direct conjugation via amine coupling utilizing N-hydroxysuccinimide and crosslinking the payload and aptamer with formaldehyde.

Aptamer-Oligonucleotide-Based Drug Conjugates

In addition to conjugation to drugs and toxins, aptamers can deliver oligonucleotide therapeutics (i.e., siRNAs, anti-miRNA, or miRNA), targeting undruggable oncogenes at the level of protein expression. Importantly, because oligonucleotide-based therapeutics function inside the cell, the therapeutic requires targeted delivery. Delivery is achieved through the covalent conjugation of the aptamer to the oligonucleotide therapeutic. Aptamer-siRNA chimeras using anti-epithelial cell adhesion molecule (EpCAM) aptamers are widely used for epithelial cancers and cancer stem cells (Hori et al. 2018). For example, Judy Lieberman received a patent in 2019 for a novel aptamer-siRNA molecule (AsiCs) targeting breast cancer using EpCAM (patent number, 10385343). An overview of aptamer-oligonucleotide therapeutic approaches can be found in the Hori et al. (2018) review.

Aptamer-Nanoparticle Conjugates

Aptamers can be used to deliver or functionalize nanoparticles including liposomes, gold nanoparticles (AuNPs), polymers, and dendrimers by covalent or non-covalent conjugation to these constructs, decreasing systemic toxicity. Liposomes show promise as carriers for anticancer drugs due to their ability to target tumor tissue. Recent work has shown reduced systemic toxicity and increased cellular uptake and cytotoxicity of Doxil, a PEGylated liposomal doxorubicin, by conjugating AS1411, a nucleolin-targeting aptamer. AuNPs also show promise as constructs for targeted drug delivery due to their biocompatibility, low toxicity, and large surface area. Aptamers conjugated to AuNPs carrying drug payloads such as doxorubicin increased cellular intake and cytotoxicity in several cancer cell lines (Hori et al. 2018). A full review of approach to functionalizing nanoparticles for increased efficacy can be found in the Hori et al. (2018) review.

Future of Aptamers

Challenges to Widespread Use in Clinical Diagnostics

Despite their promise in clinical diagnostics, there are few clinical aptamer-based diagnostics currently in use due to what has been coined the "thrombin problem." The "thrombin problem" refers to the predominant focus on creating novel

clinical diagnostic techniques using the most well-characterized aptamers, such as the anti-thrombin aptamer, rather than discovering new clinically relevant aptamers against new biomarkers (Famulok and Mayer 2011). Thus, the generation of aptamers for clinically relevant biomarkers for which alternatives are not available could increase use in a clinical setting.

In addition to the limited number of clinically relevant aptamers, aptamer-based sensors (i.e., aptasensors) often fail to detect the analyte in "real" patient samples. Preanalytical handling and processing of patient samples, which can potentially denature proteins and hence alter the ability of the aptamer sensor to detect the analyte with a high degree of reliability and sensitivity, hinder translation to the clinic. Recent work, however, has successfully employed protease inhibitors and bivalent aptamers (i.e., a construct of two or more aptamers) to allow for the detection of thrombin in patient samples (Famulok and Mayer 2011).

Challenges to Widespread Use in Therapeutics

A limited number of aptamers have reached late-stage clinical trials, with only one aptamer-based drug having received FDA approval, Macugen®, an anti-VEGF aptamer used to treat age-related wet macular degeneration. There are several impediments to in vivo application of aptamers including their short half-life due to nuclease-mediated degradation, rapid renal filtration, unknown and variable immunogenicity and toxicity with systemic administration, and entrapment in cellular organelles (Haßel and Mayer 2019). In addition to recent advancements in the stability and longevity of aptamers in vivo, the use of deep sequencing, live-animal-based SELEX, and high-throughput SELEX has increased efficiency while increasing the likelihood of identifying aptamers (Hori et al. 2018).

Though aptamers show great promise as therapeutics, the lack of commercial infrastructure, the high cost of modified oligonucleotides, and the previous failures of aptamer therapeutics to attain FDA approval hinder their widespread use in a clinical setting. Anti-nucleolin aptamer AS1411, for example, showed antitumor activity in patients with metastatic renal cell carcinoma and acute myeloid leukemia without exhibiting systemic toxicity but failed in Phase I (2009) and Phase II (2011) clinical trials respectively. The failure of these clinical trials was largely due to the lack of characterization of the fate of the AS1411 aptamer within the cell. Undoubtedly, expanding the use of aptamers in a biochemical and clinical setting will require thorough characterization of the fate of aptamers within their target cells and in the body (e.g., pharmacokinetic-pharmacodynamic (PK/PD) profiles), optimal pharmacological formulation, immunogenicity and toxicity profiles, as well as increased funding by biopharmaceutical companies for pre-clinical and clinical trials (Haßel and Mayer 2019).

Despite these hurdles to effective implementation of aptamer-based therapeutics, the funding of other RNA-based therapeutics by biopharmaceutical companies has increased, resulting in the approval of six oligonucleotide-based (non-aptamer) therapies. This financial support indicates a push in recent years toward the use of RNA-based therapeutic intervention and perhaps positively foreshadowing the emergence of additional aptamer thereapeutics in the future (Haßel and Mayer 2019).

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AQP

► Aquaporins

Aquaporins

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Synonyms

AQP; Water channels

Definition

Aquaporins, AQP, are cellular channel proteins that are permeated by water and small, uncharged solutes. The AQP protein family is functionally divided into water-specific channels, often referred to as "orthodox aquaporins," and solute channels that mainly conduct glycerol, termed "aquaglyceroporins." Besides glycerol, other similar or smaller sized solutes, such as urea, hydrogen peroxide, and gases including ammonia and carbon dioxide pass certain AQP isoforms. As passive channel elements of cellular membranes, water and solute passage via AQPs is driven solely by osmotic or chemical concentration gradients, respectively.

Basic Mechanisms

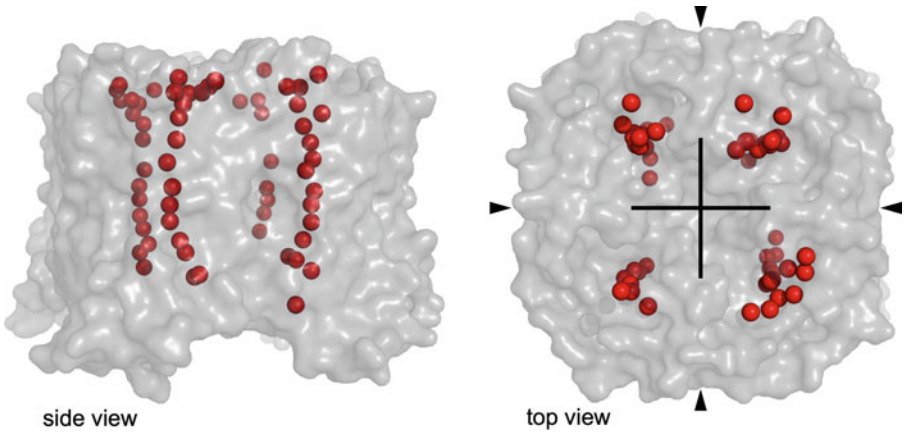
Aquaporin Structure-Function Relationships

The human AQP protein family comprises 13 isoforms, see Table 1 (Day et al. 2014). All share the same general fold and quaternary protein structure consisting of homotetramers with one individual water/solute pore in each protomer (Fig. 1). About four fifths of the volume of the AQP tetramer are buried in the lipid bilayer with the extra- and intracellular protein surfaces exposed and accessible for water and solute substrate molecules. The transduction channels are narrow allowing water or solute molecules to pass only in single files. Substrate selection occurs by two principles: (1) size and (2) exclusion of charged compounds and ions (Beitz et al. 2006). Consequently, water-specific, orthodox AQPs exhibit pore entry sites with a small diameter of 2.4 Å matching the diameter of a water molecule, whereas the filter region of aquaglyceroporins is wider at around 3.4 Å accommodating passage of glycerol in a longitudinal orientation. The channel interior provides suitably spaced hydrogen bond interaction sites for passing water or solute molecules, and an electrostatic environment that prohibits entry of charged entities due to a high energetic barrier. With respect to the handling of ions, AQP6 is rather special because it functions as a chloride (and nitrite, nitrate) channel in intracellular vesicles of intercalated cells of the kidney collecting duct. It accompanies the vacuolar proton ATPase permitting accumulation of hydrochloric acid in the vesicles, which release upon signaling their acid load into the primary urine for body pH regulation.

AQP water permeability is characterized by three hallmarks, which even before the discovery of the AQPs in 1992 (Preston et al. 1992) led to the assumption that proteinaceous water channels must exist in the cell membrane. First, water

Aquaporins, Table 1 Aquaporin isoform tissue localization and function

Isoform	Tissue (selection)	Functions (selection)
AQP0	Eye lens	Cell–cell contact, transparency
AQP1	Erythrocytes, kidney proximal tubule, lung, brain choroid plexus, eye	Colton blood group, primary urine concentration, lung moistening, cerebrospinal fluid regulation, intraocular pressure regulation
AQP2	Kidney collecting duct principal cells	Vasopressin-dependent body water homeostasis
AQP3	Erythrocytes, kidney, adipocytes, skin	Gill blood group, body water homeostasis, lipolysis, skin moistening
AQP4	Kidney, brain, neuronal tissue	Body water homeostasis, cerebrospinal fluid regulation, target of autoantibodies causing NMO
AQP5	Sweat and salivary glands	Sweat and saliva production
AQP6	Kidney collecting duct intercalated cells	Vesicular chloride channel, body pH regulation
AQP7	Adipocytes, intestine	Lipolysis, intestinal water facilitation
AQP8	Mitochondrial	Highly ammonia permeable
AQP9	Liver	Lipogenesis, gluconeogenesis
AQP10	Adipocytes, intestine	Lipolysis, intestinal water facilitation
AQP11	Kidney	Deletion causes polycystic kidney disease
AQP12	Pancreas	Unknown



Aquaporins, Fig. 1 Aquaporin structure and single-file water passage

permeation via AQP channels is at least one order of magnitude faster than simple transmembrane diffusion; in fact, it can be as fast as the unhindered mobility of water molecules in free aqueous solution. Second, at < 5 kcal mol^{-1}, the Arrhenius activation energy of AQP water permeability is lower than that of transmembrane diffusion (> 10 kcal mol^{-1}); as a consequence, the AQP water channel function is largely temperature independent. Third, AQP water permeability is inhibitable, e.g., by organic mercurials (isoform-dependent), whereas transmembrane water diffusion is not (Agre 2004).

The permeability properties of AQPs for solutes largely adhere to the above-described characteristics. Yet, the discrimination between solute passage via an aquaglyceroporin and transmembrane diffusion is sometimes less clear in particular for small, lipophilic gas molecules, e.g., ammonia, NH_3, or carbon dioxide,

CO_2, which directly penetrate the lipid bilayer quite easily.

The driving force for diffusion in general, irrespective of passing an AQP channel or a cell membrane, is entropy. Particles will move along an existing gradient towards establishment of an equilibrium. For orthodox AQPs, this means specifically, that a net water movement will occur only when a transmembrane osmotic gradient exists. In this case, water molecules will pass the AQP in the direction towards the compartment holding the higher particle concentration (Fig. 2). This occurs prominently in the salt-laden tissue surrounding the nephron (up to 1200 mM salt compared to 300 mM in the remaining body) driving the concentration process of the primary urine by a transcellular water movement of more than 150 l per day. Yet, also local intracellular changes in particle concentrations will initiate AQP-carried water fluxes across the membrane. Ion channels in neuronal cells and the ubiquitous sodium-potassium ATPase, Na^+/K^+-ATPase, transiently disturb the equimolar transmembrane ion distribution and trigger an equilibrating water movement into or out of a single cell. Likewise, a local breakdown of polymerized filamentous actin into individual soluble actin molecules will locally increase the osmolarity and lead to water influx. This mechanism promotes local cell-swelling and protrusion of lamellipodia ("cell-feet") that are central to cell motility, e.g., of cells of the immune system but also of migrating tumor cells. Aquaglyceroporin solute channels will permit a net flux of a specific solute when its concentration is uneven at both sides of a membrane leading, for example, to an efflux of glycerol from adipocytes during lipolysis.

Aquaporin Regulation

Almost all human AQPs appear not to be gated, i.e., contrary to certain plant AQPs, there are no molecular closing mechanisms contained in the AQP protein itself sensing, for instance, pH conditions or ion concentrations, so that the channels remain open at all times. AQP10 of the intestine and adipose cells poses an exception in that it is activated by acidic pH conditions (Gotfryd et al. 2018). It is further rather typical that AQPs are constitutively present at the cell plasma membrane as permanent water and solute pore elements (Fig. 3). Regulation occurs, however, on the transcriptional level, e.g., in the lung, hypoxia induces the expression of the AQP1 gene (Agre 2004), and glucocorticoids via nuclear receptors that of AQP4. By this long-term regulation mechanism, more AQP channels will be inserted into the plasma membrane increasing water permeability. In the case of the mentioned hypoxia-induced orthodox AQP1, this facilitates lung moistening at conditions of low air pressure, e.g., at high altitudes. A second, short-term regulation mechanism exists for specific human AQP isoforms. Here, AQP protein is stored in intracellular vesicles that will traffic to and fuse with the plasma membrane upon a signal. A prominent example for this mechanism is the vasopressin-dependent body water homeostasis (Tamma et al. 2005): vasopressin secreted from the posterior pituitary activates the G-protein-coupled receptor type 2 for vasopressin of principal cells in the kidney collecting duct, adenylyl cyclase/cAMP-signaling will lead to phosphorylation of water-specific AQP2 residing in perinuclear vesicles and initiate transport and insertion into the apical plasma membrane. As a result, the cells are rendered more water permeable increasing concentration of the primary urine by water reabsorption. When the vasopressin signal ceases, AQP2 will be dephosphorylated by protein phosphatases initiating internalization back into the pool of intracellular vesicles. Vasopressin has an additional long-term effect on AQP2-facilitated water permeability via gene induction by the cAMP-dependent transcription factor cAMP response element-binding protein, CREB.

Aquaporins as Surface Antigens and Cell–Cell Contact Proteins

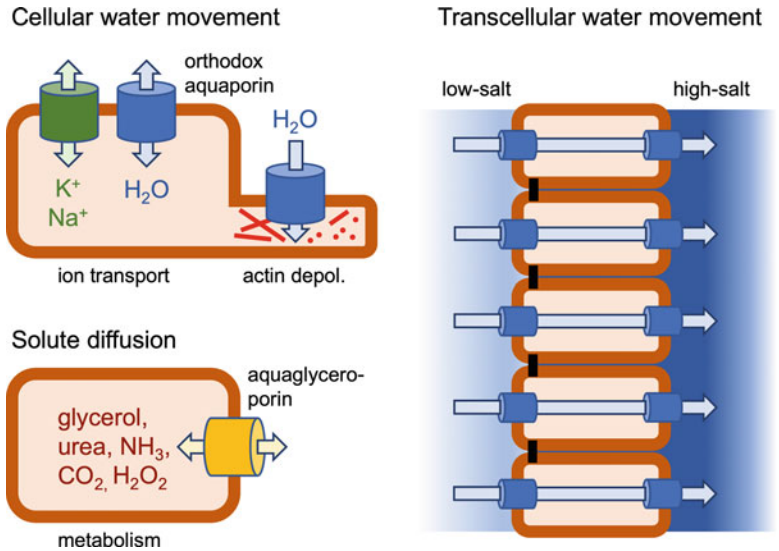
Independent from their channel function, AQP surface structures act as antigens (Agre 2004). Human erythrocytes carry AQP1 and the aquaglyceroporin AQP3 in their plasma membranes, and both AQPs represent antigens in the blood group system. The Colton blood group is due to an alanine versus valine variation of AQP1, and the Gill blood group to AQP3. The orthodox

AQP4 is strongly expressed in neuronal tissue including the optic nerves. In rare cases, individuals produce autoantibodies against AQP4 resulting in neuromyelitis optica, NMO, characterized by demyelination and inflammation that damage neuronal tissue and functionality. AQP4 autoantibodies are used as diagnostic marker for NMO. The extracellular surfaces of AQP0, which is exclusively expressed in the eye lens, interact with each other facilitating cell–cell contacts of lens fiber cells. Their proper alignment is responsible for the transparency of the lens. Certain polymorphisms of the AQP0 gene that result in alterations of the cell–cell contact sites on the AQP0 protein cause early-onset cataract.

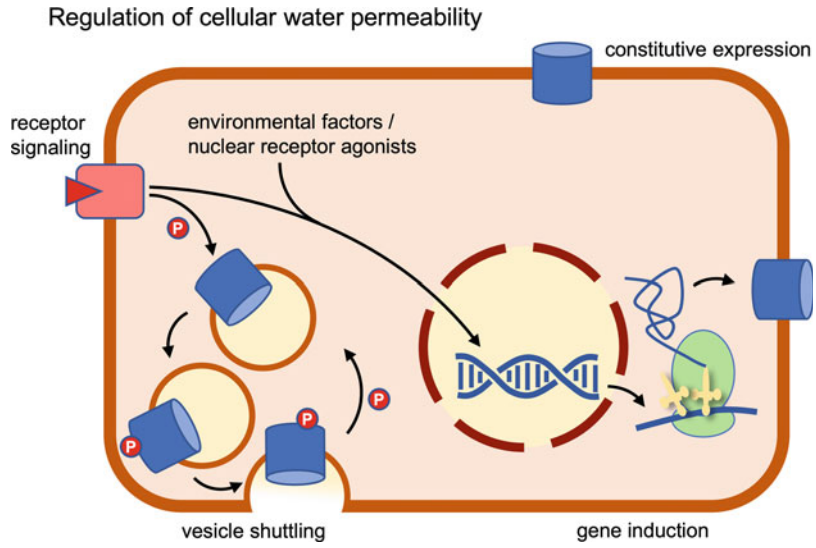
Physiological Functions of Aquaporin Water Permeability

Transmembrane water facilitation is a main task of the AQPs (Day et al. 2014). Since water makes up two-thirds of the body weight and all organs and tissues are exposed to and regulate aqueous interstitial, serum, or surface fluids, AQP water permeability is ubiquitously relevant. Their functionality is intricately connected to the transport of ions or osmotically active compounds (Fig. 2) that drive transmembrane water movement. Such mechanisms are specific to individual tissues and cells. The kidney is the central organ maintaining the body water homeostasis. Several AQP isoforms are expressed in the various kidney cell types. Eighty percentage of the filtrated primary urine volume are reabsorbed in the proximal tubule and Henle loop of the nephron. This transcellular water flow (Fig. 2) is almost exclusively carried by AQP1. The fine-tuned urine volume regulation takes place in the collecting duct. It involves the vasopressin-regulated, apical AQP2 of the principal cells via vesicle shuttling, and long-term AQP2 gene induction via CREB (Fig. 3), as well as the constitutively expressed AQP3 and AQP4 at the basolateral membranes. This way the blood osmolarity is kept even within $<1\%$ accuracy. Accordingly, erythrocytes usually experience a highly constant osmotic environment in the blood stream; however, 20–30 times per day, they will pass the high-salt tissue of the kidney and are subjected to a volume decrease by more than 20%. AQP1 and AQP3 of the erythrocyte membrane are thought to preserve membrane integrity by letting water pass swiftly and in an ordered way.

Water regulation in the brain is particularly critical due to the inflexible bone-enclosed compartment. Any overpressure, edema, will immediately inflict cell stress and ultimately cell damage. AQP4 and AQP1 are relevant isoforms in the production and release, respectively, of the cerebrospinal fluid. Similar situations, although at a smaller volume scale, are present and regulated by



Aquaporins, Fig. 2 Driving forces of cellular and transcellular water and solute movement



Aquaporins, Fig. 3 Regulation of cellular water permeability via aquaporins

AQPs in the enclosed eye and inner ear compartments. Here as well, diseases are linked to dysregulated fluids, i.e., glaucoma and Menière's disease, respectively.

AQP water permeability is further required in cells that are exposed to external body surfaces present in the lung (AQP1, AQP4), mucosal tissues (various AQPs), and secretory glands, e.g., for sweat and saliva (AQP5). In this encyclopedia entry, a listing of physiologically relevant situations of AQP-driven water permeability cannot be conclusive; it is referred to the cited literature and reference therein for further details.

Role of Aquaporins in Glycerol Metabolism, Gas Transport, and Redox Signaling

Solute-permeable aquaglyceroporins assume key functions in the adipocyte and liver glycerolipid metabolism and glucose neogenesis pathways (Gotfryd et al. 2018). During lipolysis in adipocytes, lipase enzymes catalyze the chemical hydrolysis of the ester bonds between fatty acids or cholesterol and the glycerol hydroxyl moieties. The freed fatty acid portion will be used mainly in the same cell for the energy metabolism process of β-oxidation, whereas glycerol will be released from the cell via aquaglyceroporins, predominantly AQP7 and AQP10. Accumulation of glycerol in the adipocytes due to malfunction or absence of AQP7 causes adipocyte swelling by increased glycerolipid storage resulting from product-inhibition of the catabolic lipase reaction. Glycerol will travel with the blood to the liver where AQP9 facilitates uptake into hepatocytes for gluconeogenesis or glycerolipid synthesis and storage depending on the energy metabolic state. Cells of the intestine also express the aquaglyceroporins AQP7 and AQP10. Glycerol further fulfills a moistening function in the skin via AQP3 and contributes to wound healing (Agre 2004).

Other permeants of aquaglyceroporins are produced by metabolic degradation of amino acids, namely urea from the conversion of arginine to ornithine by arginase, and ammonia from the oxidative deamination of glutamate to α-ketoglutarate or the deamination of glutamine to glutamate. Ammonia is also a product of purine and pyrimidine metabolism. There are, however, dedicated transporters for urea (solute carrier family members SLC14A1 and SLC14A2) and ammonia (Rhesus proteins, Rh) encoded in the human genome that are probably of higher relevance than AQPs.

Ammonia, NH_3, is the neutral form of positively charged ammonium, NH_4^+, and both molecules are linked by a pH-dependent protonation equilibrium. At neutral pH, 99% are present as protonated NH_4^+ leaving only 1% of neutral NH_3. As a small, not too polar molecule, NH_3 exhibits substantial background diffusion across lipid bilayers even in the absence of a suitable channel or transporter. This property is even more pronounced in the permanently neutral and more lipophilic gas molecule carbon dioxide, CO_2. Nevertheless, a role of AQPs in CO_2 transmembrane permeability has been proposed for lipid bilayers with high membrane protein content, e.g., urothelial cells mainly of the bladder expressing uroplakins (Endeward et al. 2017).

Hydrogen peroxide, H_2O_2, is quite similar to the water molecule in terms size, polarity, and hydrogen bonding capability (Almasalmeh et al. 2014). Accordingly, several AQPs conduct hydrogen peroxide, which is produced during aerobic energy metabolism by chemical reduction of oxygen. Recently, a role as a signaling molecule has been attributed to hydrogen peroxide affecting many cellular processes such as proliferation, differentiation, inflammation, circadian rhythm, and aging (Sies 2004).

Pharmacological Intervention

The many basic physiological functions of orthodox aquaporins and aquaglyceroporins, and their roles in diseases render AQPs attractive drug targets (Beitz et al. 2015). One can think of brain and lung edema, glaucoma, Menière's disease, tumors, fatty liver disease (steatosis) to name a few. However, direct modulation of AQP functionality by drug-like small molecules is difficult due to the narrow transport path in which inhibitors would need to bind to gain sufficient affinity (Beitz et al. 2015). Until today, there are experimental, preclinical compounds that block aquaglyceroporin permeability; however, suitable inhibitors of orthodox aquaporins are still to be found.

There are two pharmacological scenarios in which AQPs are indirectly involved in pharmacotherapy. First, the aquaglyceroporin AQP9 serves as an entry pathway for arsenic trioxide, As_2O_3, which is used as an antineoplastic agent in acute promyelocytic leukemia. Arsenic trioxide, when dissolved and absorbed into body fluids, forms very weak arsenous acid, $As(OH)_3$. In its mostly protonated form, it resembles glycerol in shape and size, and therefore is a permeant of AQP9. Once inside the cytosol, arsenic drives the malignant promyelocytes into apoptosis (Beitz et al. 2015). Second, inhibition of the kidney vasopressin receptor by the "vaptan" drug class act as "aquaretics" that selectively increase the secreted water volume (Beitz et al. 2015). Different from "diuretics," salt secretion remains unaffected. Consequently, vaptans are used to treat euvolemic and hypervolemic hyponatremia. Other clinical conditions associated with water retention accompanying congestive heart failure or liver cirrhosis can be addressed as well. Vaptans have further been beneficial in animal models of surgically induced inner ear volume increase, which is responsible for the symptoms of Menière's disease, hearing loss, and severe vertigo attacks.

Cross-References

- ▶ Adenylyl Cyclases
- ▶ Glucocorticoids
- ▶ G-Protein-Coupled Receptors
- ▶ Na^+/K^+-ATPase
- ▶ Protein Phosphatases
- ▶ Vasopressin

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Arginine Vasopressin

► Vasopressin

Argipressin

► Vasopressin

Arteriosclerosis

► Atherosclerosis

Aryl Hydrocarbon Receptor

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Definition

Ah receptor complex: The AhR is a basic helix-loop-helix transcription factor that forms an active complex with the AhR nuclear translocator (Arnt) protein. The AhR-Arnt complex binds dioxins/xenobiotic response elements in target gene promoters to activate transcription. In addition to this classical mechanism, other AhR-mediated pathways have been identified. AhR-mediated downstream responses are dependent on multiple factors including ligand structure and cell context.

Basic Characteristics

AhR-Mediated Toxicity

The toxicities associated with exposures to the herbicide 2,4,5-trichlorophenoxy acetic acid were linked to a contaminant in the production of the herbicide which was identified as 2,3,7,8-tetrachlordibenzo-p-dioxin (TCDD) (Hites 2011). Early studies on TCDD and structurally related polychlorinated dibenzo-p-dioxins (PCDDs) dibenzofurans (PCDFs) and biphenyls (PCBs) showed that these compounds caused a familiar pattern of biochemical and toxic responses that were age, sex, organ, animal species, and strain dependent (Poland and Knutson 1982). Well-characterized effects caused by TCDD included a rabbit ear chloracnegenic response which is also observed in human skin and induction of cytochrome P4501A1 (CYP1A1) and other drug-metabolizing enzymes (Poland and Knutson 1982). It was also observed that the potency of individual PCDD and PCDF and PCB congeners was structure dependent, and maximal activities/toxicities were observed for compound such as TCDD which contained four

laterals (2, 3, 7, and 8) chloro substituents and potency decreased with increasing chlorination and loss of one or more lateral chlorine substituents (Poland and Knutson 1982). These observations led to the hypothesis that the toxicity of TCDD and structurally related chlorinated aromatic industrial by-products was due to an intracellular receptor protein since a hallmark characteristic of most receptors is their structure-dependent effects of ligands. In a landmark publication, Poland and coworkers identified an intracellular protein called the aryl hydrocarbon receptor (AhR) that bound TCDD with high affinity (K_D-sub nM) and exhibited structure-dependent binding affinities with other chlorinated aromatics and polynuclear aromatic hydrocarbons (PAHs) (Poland et al. 1976). Moreover, there was a rank order correlation between the receptor binding affinities versus response potencies (e.g., CYP1A1 induction) for the compounds suggesting that the AhR was necessary for mediating the toxicities of toxic chlorinated aromatics and some of the effects induced by PAHs (Poland et al. 1976).

The AhR Nuclear Translocator: A Partner for the AhR

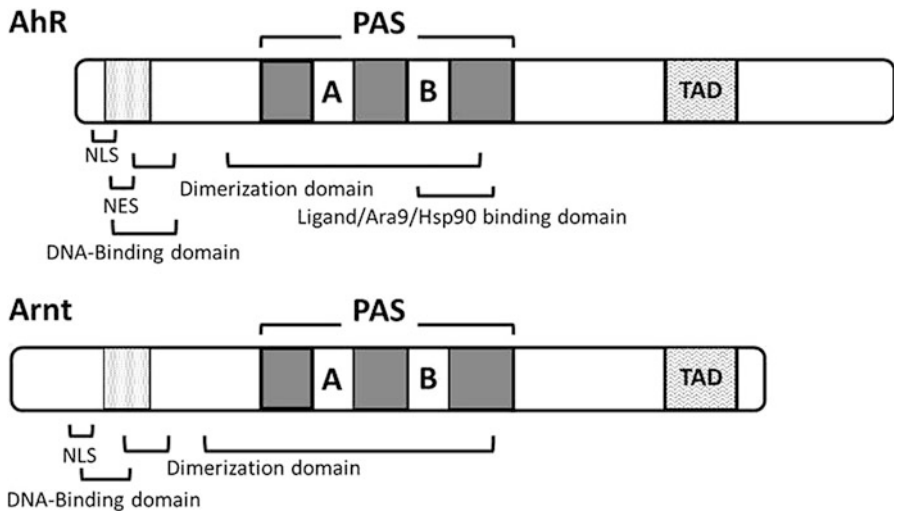
The molecular biology of AhR-mediated response was initially investigated using multiple approaches which included ligand-induced effects on receptor interactions and cellular location molecular weight studies, and the use of genetically modified cells, and identification of nuclear/gene target sites (i.e., cis-elements) (Probst et al. 1993). It was observed that in many cell lines, TCDD and related compounds induced cytosol to nuclear translocation of the AhR complex, and the estimated molecular weight of the complex indicated that at least one other protein was associated with the AhR. Moreover, the nuclear AhR complex exhibited increased DNA-binding affinity to putative dioxin or xenobiotic response elements (DREs/XREs) identified in promoter regions of AhR-responsive genes such as CYP1A1. Pioneering work by Hankinson and coworkers (Hoffman et al. 1991; Probst et al. 1993; Hankinson 1995) used genetic approaches to identify the AhR nuclear translocation (Arnt) protein which was necessary for nuclear uptake and DNA binding of the AhR-Arnt complex. The identification of Arnt as the AhR partner protein (Hoffman et al. 1991) led to the subsequent discovery that Arnt or hypoxia-inducible factor β (HIF1β) was also a partner for HIFs and other proteins that play integral roles in maintaining cellular homeostasis.

The AhR: Knockout Mice and Cloning

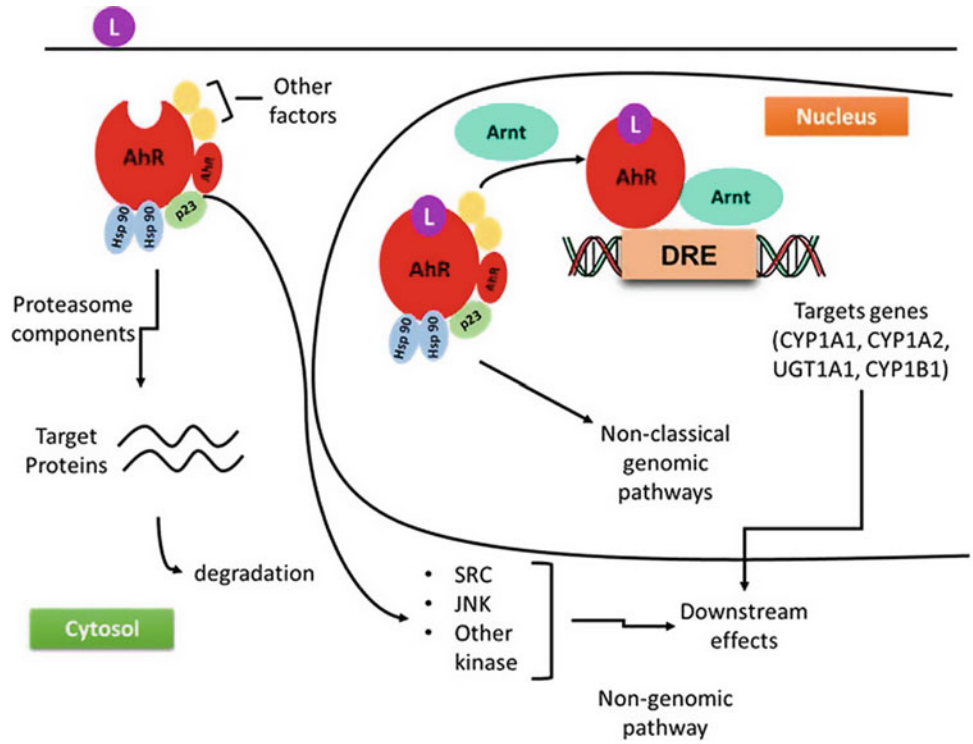
The next advance in understanding the function and structure of the AhR was dependent on molecular advances in gene cloning and development of knockout mouse models. The AhR knockout (AhRKO) mice were generated in three different laboratories and although their phenotypes varied, in all cases treatment with TCDD and related toxic compounds did not induce CYP1A1 and the typical AhR-mediated toxic responses (Schmidt et al. 1993; Fernandez-Salguero et al. 1995; Mimura et al. 1997). These results confirmed that the toxicity of TCDD was mediated through the AhR. The AhRKO mice were viable; however, comparable studies could not be performed with Arnt since Arnt knockout mice are embryolethal, and this is not surprising since Arnt interacts with and is necessary for the function of multiple members of the Per-Arnt-Sim (PAS) family of proteins. The AhR and Arnt genes were also cloned and sequenced and shown to be basic-helix-loop-helix (bHLH)-PAS proteins (Fig. 1) (McIntosh et al. 2010). The structural organization includes bHLH (DNA binding), PAS, and transactivation domains; an acidic D/E region (for AhR), Q and P/S/T regions within the transactivation domains of the AhR and Arnt gene, and a nuclear localization signal (Arnt). The bHLH-PAS genes are members of the PAS superfamily which encompasses genes that act as environmental sensors that play an increasingly broad role in biology (McIntosh et al. 2010) and the AhR-Arnt complex senses and responds not only to TCDD and related toxicants but many other endogenous and dietary AhR ligands.

AhR: Classical and Non-classical Mechanisms of Action

Figure 2 illustrates the classical mechanism of action of the AhR which is bound in the inactive



Aryl Hydrocarbon Receptor, Fig. 1 Domain structure of the human AhR and Arnt genes



Aryl Hydrocarbon Receptor, Fig. 2 Mechanisms of action of the ligand-activated AhR complex

form in the cytosol with Hsp90, ARA9, p23, and other proteins. Addition of ligand induces nuclear uptake of the complex, formation of a nuclear AhR-Arnt heterodimer which interacts with cis elements (DRE/XRE) in target gene promoters to activate gene transcription. However, there is also evidence for non-classical pathways in which the AhR can act independent of Arnt and also function outside the nucleus (Fig. 2). For example, there is evidence for ligand-induced functions of the cytosolic AhR which activates Src, JNK, and other kinase pathways (Enan and Matsumura 1996); AhR also acts as an E3 ubiquitin ligase (Ohtake et al. 2007). The ligand-activated nuclear receptor interacts with proteins other than Arnt and also binds cis-promoter elements that are not DREs. For example, TCDD suppresses liver regeneration, and this is due, in part, to induction of p21 by the AhR-KLF6 complex bound to a non-consensus DRE in the p21 gene promoter (Jackson et al. 2014). Thus, the AhR can function in both classical and multiple non-classical pathways in modulating genotypic and phenotypic changes in target cells/tissues.

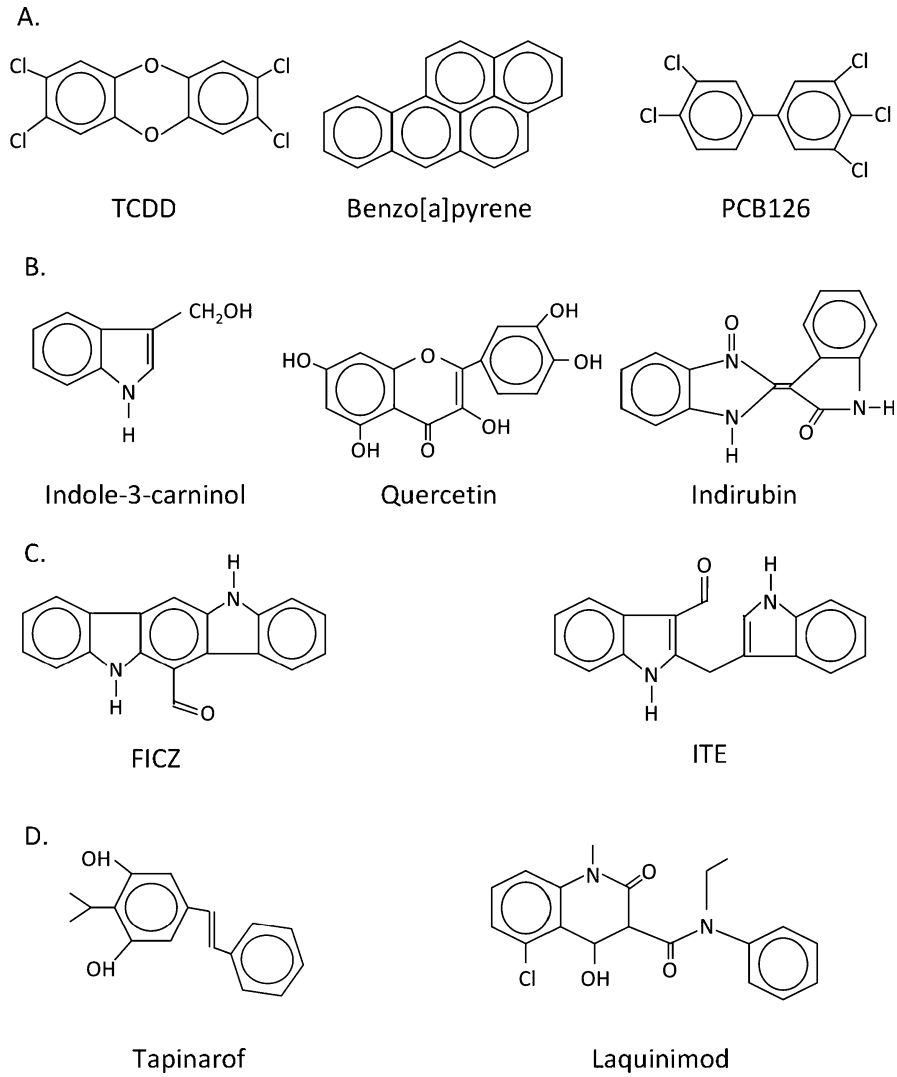
AhR: Role in Multiple Endogenous Functions

After generation of AhRKO mice, it became evident that although loss of the AhR was not embryolethal the mice exhibited multiple abnormalities indicating that the AhR was required for important physiological roles which were independent of the effects of AhR "toxic" ligands. For example, AhRKO mice exhibit some of the following differences from their wild-type counterparts; decreased liver size due, in part, to defects in fetal vascular structure and failure of ductus venosis closure, cardiovascular and reproductive deficits immune response problems, and many other tissue specific effects (Esser and Rannug 2015; Rothhammer and Quintana 2019). Many of these responses were observed during the initial generation of AhRKO mice; however, in the last 10–15 years the linkage of the AhR with tissue-/organ-specific phenotypic effects has expanded significantly. The AhR plays an important role in immune responses, infection autoimmune diseases, stem cell development cancer, inflammation, and antimicrobial resistance and mediates ligand-dependent crosstalk between the gut and distal organs including the brain (Esser and Rannug 2015; Rothhammer and Quintana 2019). The functions of the AhR in cancer are highly complex and tumor specific, and for some tumor types the results are contradictory. Studies in head and neck cancer clearly show that the AhR play a pro-oncogenic role, whereas for intestinal cancers the AhR acts as a tumor suppressor (Safe et al. 2013; Murray et al. 2014).

Drug

AhR: Ligand Multiplicity and Selectivity

The AhR was initially identified as the receptor that binds and mediates the toxic effects induced by TCDD and related compounds. Early studies also showed that PAH also bound the AhR, and like TCDD, PAHs induced CYP1A1 but did not induce the typical pattern of TCDD-induced toxic responses including chloracne dysregulation of the immune system, porphyria, bodyweight loss, cleft palate, and other developmental reproductive toxicities, and this may be due, in part, to their rapid metabolism. In addition, AhR ligands such as 6-methyl-1,3,8-trichlorodibenzofuran MCDF) and 3-methoxy-4-nitroflavone which exhibit partial antagonist/agonist activities (Astroff et al. 1988) were identified, and although these compounds inhibited some tissue/species-specific TCDD-induced responses, they also induced some TCDD-like activities. Subsequent and ongoing studies have identified several different structural classes of compounds that bind the AhR and activate/inhibit AhR-dependent effect, and these compounds include diverse aromatic industrial and non-industrial compounds, endogenous biochemicals including bilirubin, tryptophan-derived metabolites and photoproducts, plant extracts and phytochemicals, and diverse pharmaceuticals (rev. in Denison and Nagy 2003). Figure 3 illustrates some examples of the structurally diverse AhR ligand which include the highly toxic TCDD which binds the AhR with high affinity and other diverse structural classes of AhR ligands which exhibit lower AhR binding affinity. This pattern of structurally diverse



Aryl Hydrocarbon Receptor, Fig. 3 Structurally diverse AhR ligands including, (**a**) toxic [TCDD, benzo [a]pyrene and 3,3′,4,4′,5-pentachlorophenyl (PCB126)], (**b**) phytochemical derived (indole-3-carbinol, quercetin and indirubin), (**c**) endogenous (FICZ and ITE), and (**d**) pharmaceutical (Tapinarof and Laquinimod) ligands

compounds binding the AhR is paralleled by similar binding data for other receptors including estrogen receptor α which binds 17β-estradiol (E2) and diethylstilbestrol with high affinity as well as structurally diverse ligands with lower binding affinity (Kuiper et al. 1998). The structural diversity of ER ligands is paralleled by their functional diversity, and this has led to development of selective ER modulators (SERMs) such as tamoxifen which is an ER antagonist (breast) and agonist (uterus) and has been extensively used as an antiestrogen for treating ER-positive breast cancer patients**.** There is also AhR binding, genomic and functional data indicating that AhR ligands are selective AhR modulators (SAhRMs) and exhibit tissue-specific AhR agonist or antagonist activities (Astroff et al. 1988; Denison and Nagy 2003). There are an increasing number of examples where SAhRMs have been used to target the AhR to provide health benefits in animal models of disease, and this include phytochemicals such as indole-3-carbinol for inhibiting colon cancer and tryptophan-rich diets for enhancing immune cell function (Esser and Rannug 2015).

The realization that the AhR is a potential target for therapeutics has been hindered in the past by the linkage of this receptor to TCDD. However, AhR ligand such as laquinimod and tapinarof are being developed for treating autoimmune disease and dermatitis, respectively (Kaye et al. 2016; Smith et al. 2017), and due to the expression and functions of the AhR in multiple diseases, there are many exciting opportunities for future development of SAhRMs as therapeutics.

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Assay Development

► High-Throughput Screening (HTS) Technology

Asthma

► Interleukin-5

Asthma Bronchiale

► Bronchial Asthma

Asthma Controllers

► Glucocorticoids

Atherosclerosis

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Synonyms

Arteriosclerosis (see Definition)

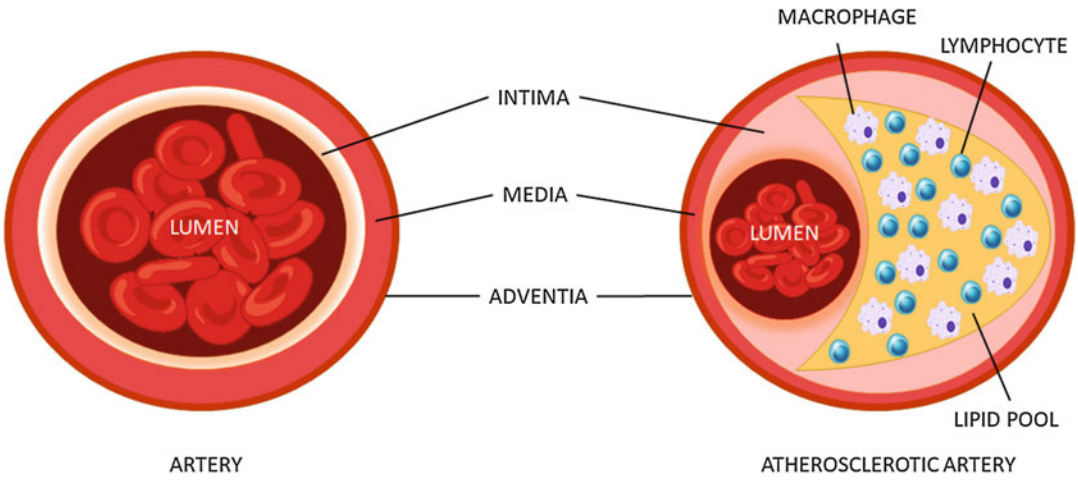
Definition

Atherosclerosis is an arterial disorder that comes from Greek words *athera* which means porridge and *sclereni* which means hardening of arteries. It is an inflammatory disorder, in which the lipid plaques along with inflammatory responses accumulate inside the arteries resulting in hardening and narrowing of the arteries and therefore restricting blood flow. Plaques are waxy substances that consist of lipids and oxidized lipids along with many inflammasomes. Plaques are complex, heterogenous composition which include fibrous caps, necrotic cells, and inflammatory responses (Barrett et al. 2019). Blockade of coronary artery (that supplies blood to the heart) with plaques hampers the blood flow to the heart, and the cardiac cells become deprived of oxygen. Symptoms of atherosclerosis in coronary artery include angina, vomiting, coughing, and anxiety, which eventually leads to myocardial ischemia. Plaques causing atherosclerosis of carotid artery eventually leads to brain stroke and cause symptoms like weakness, headache, facial numbness, and paralysis.

Basic Mechanisms

Atherosclerosis is a chronic condition that progresses silently for decades. The progression of the lesion initiates with the formation of thrombus which obstructs the blood flow (Bentzon et al. 2014) (Fig. 1).

Atherosclerosis grows in specific regions of artery. The initial changes that take place are morphological changes in the intima where the lipoproteins aggregate. The aggregated lipoproteins influence chemotaxis of leukocytes and other inflammasomes through multiple mechanisms which include oxidation and enzymatic and non-enzymatic proteolysis. At the initial stage, the monocytes or the macrophages adhere to the arterial endothelium which is accompanied by accumulation of macrophages in the intima (Ardies and Roberts 2014). More macrophages adhere to the area and these cells that modify the lipoproteins into lipid droplets which are filled with

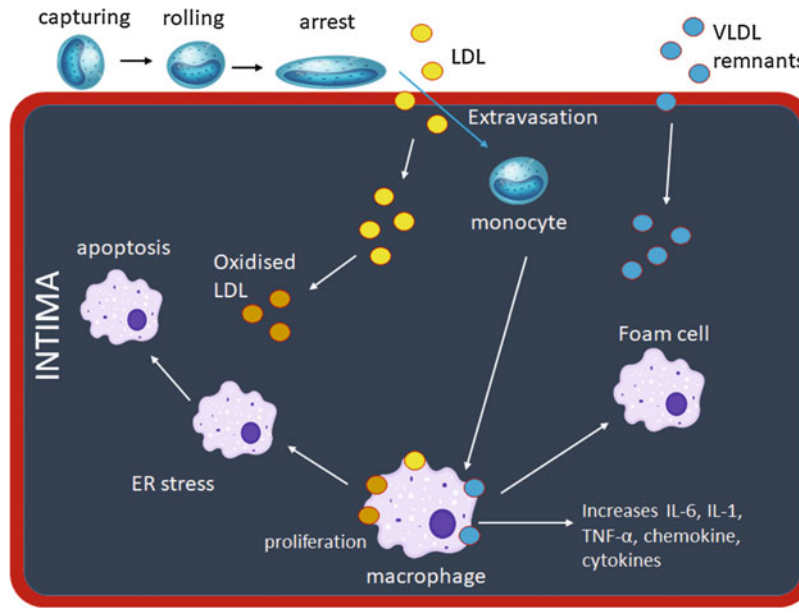


Atherosclerosis, Fig. 1 Nonatherosclerotic artery and atherosclerotic artery

cholesteryl ester; this entire process is known as foam cell formation. The disturbed metabolism of lipid alters the functioning of the cells. The foam cell that is aggregated in the lesion decreases the migration from the lesion that results in chronic inflammation and lesion progression. These complex plaques comprise of immune cells and vascular smooth muscle, and the macrophages within it secrete proinflammatory mediators, nitrogen species, reactive oxygen, and matrix-degrading proteases leading to apoptosis or necrosis of the cells. The dead macrophages secrete lipid and form prothrombotic necrotic core and form unstable plaques. Thin fibrous cap of these unstable plaques may get ruptured, forms thrombus which results in acute myocardial infarction or stroke. The formation of foam cells is the major and the central cause for the pathophysiology of atherosclerosis.

Cells Involved in Atherosclerotic Lesions

Multiple cell types along with various signalling pathways are involved in inflammation and atherosclerosis. Modified lipoproteins activate endothelial cells which secrete adhesion molecules like vascular cell adhesion molecule 1 (VCAM-1), intracellular adhesion molecule 1 (ICAM-1), and selectins. These molecules with chemoattractant mediators along with complement factors and MCP-1 help monocyte adhesion to the impaired endothelial into the intima. Monocytes are activated by macrophages that modify the lipoprotein to form foam cells. Macrophages are the major cell types that contribute to progression of atherosclerotic lesion. Lipoproteins that are accumulated in the intima release various cytokines like IL-1, IL-6, and TNF-α (Fig. 2). T lymphocytes are found in the atherosclerotic lesion and get accumulated in early lesion (Libby 2012). LDL stimulates T lymphocytes and produces interferon-γ. Atherosclerotic lesions contain minimal B lymphocytes. Oxidized LDL produces antibodies from B lymphocytes that get accumulated in atherosclerotic lesions. T lymphocytes are known to increase the progression of atherosclerosis, whereas antibodies secreted by B lymphocytes called as B1 cells protect against formation and development of atherosclerosis. Neutrophils are the most abundant leukocyte type in blood and are present for only 1–2 days. Neutrophils are the first line in acute inflammatory responses. The neutrophils get activated and reach to the area of lesions, but the mechanisms are still unknown (Weber and Noels 2011). VSMCs (vascular smooth muscle cells) are the most abundant cell type in arteries. Inflammation leads to VSMC



Atherosclerosis, Fig. 2 Process of formation of foam cells

proliferation and gets migrated into the atherosclerotic lesion. VSMCs stabilize the lesion by secreting extracellular matrix which forms a fibrous cap and transforms them into foam cells. VSMC foam cells lose its capacity of secreting extracellular matrix. Oxidized LDL increase the smooth muscle cell proliferation.

Process of Formation of Foam Cells

Recruitment of Circulating Monocytes

Endothelium dysfunction leads to recruitment of circulating monocytes to the site of injury (Moore et al. 2013) (Fig. 2). Monocytes in atherosclerotic lesions undergo processes like capture, rolling, and transmigration. The surface molecules of activated endothelial cells capture and roll the monocytes. This process depends on their immobilization by chemokines, p-selectin, VCAM1, and ICAM1 and interacts with chemokine receptor and integrins. The monocyte chemokines interact with chemokine PECAM1 or VCAM1 and facilitate the removal of monocytes across the endothelium and into plaque. PECAM1 or VCAM1 are secreted by endothelial cells, macrophages, and smooth muscle cells.

Foam Cell Formation

Macrophages forming foam cell are one of the earliest stage in progression of lesion and continue throughout its progression (Moore and Tabas 2011) (Fig. 2). Even though macrophages take apoB-containing lipoprotein from the LDL receptor, the receptor gets downregulated by increased cellular cholesterol. Scavenger receptors take up the modified lipoprotein and help in foam cell formation. Pathways related to foam cell formation are still unknown. The oxidative stress in the intima modifies the lipoprotein and produces damaged signals; these signals are then identified by the scavenger receptor. This scavenger receptor includes SR-A1, SR-A2, SR-B1, CD36, LOX1, SREC1, and CXCL16, and majorly SR-A1 and CD36 help in uptake of the oxidized LDL. These all receptors bind to oxidized LDL and help in formation of foam cell. During oxidation, various lipoprotein mediators like 12/15-lipoxygenase, myeloperoxidase, and free radicals including superoxide, hydrogen peroxide, and nitric oxide had been found in the walls of artery. The lipoproteins get attributed by these receptors into the late endosome and lysosomes. The lysosomal acid lipase hydrolyzes the lipoprotein cholesteryl ester into fatty acids and cholesterol. Cholesterol undergoes

reesterification and gets converted to cholesteryl ester and forms foam. Proteases and lipases in the intima modify the lipoproteins and accumulate LDL (Moore et al. 2013). Extracellular matrix glycoproteins in the intima retain apoB-containing lipoproteins and modulate the activity of enzymes. These enzymes include group IIA secretory phospholipase A2 (PLA2G2A), PLA2G5, and PLA2G10 and secretory sphingomyelinase and produce modified forms of LDL which are taken up by macrophages. Cholesterol gets delivered to the endolysosomal compartment and stimulates ACAT1-mediated cholesterol via receptor-independent endocytic pathway. They stimulate ACAT1-mediated cholesterol esterification. LDL and other apoB-containing lipoproteins form foam cell by multiple pathways. Excessive lipoprotein uptake results in defective lipid metabolism within macrophages and leads to increased lesion complexity. Cholesteryl ester in lipids is inert, but cholesterol inside the cell membrane can be toxic. Macrophages enriched with cholesterol suppress the esterification of lipoprotein by ACAT1 which results in increased accumulation of cholesterol, and the plasma membranes become enriched with cholesterol that increases the inflammatory signalling through Toll-like receptors. Macrophage cholesterol export gets hampered due to trafficking of cholesterol from lysosomes and further increases the inflammatory response. This dysregulated lipid metabolism, if prolonged, can lead to apoptosis and cell death. Clearance of apoptotic cells from lesions is known as efferocytosis that engulfs the cells to metabolize lipids inside the apoptotic cells. Excessive lipoprotein uptake by macrophages affects the cellular lipid metabolism and increases apoptosis and suppresses efferocytosis that leads to secondary necrosis and a necrotic core; this necrotic core with thinned fibrous cap is more prone to rupture.

Macrophage Polarization

Monocytes when entering intima get polarized to M1 macrophages (classically activated inflammatory cells) or M2 macrophages (alternately activated cells) that are known to resolve inflammation (Moore et al. 2013). M1 macrophages are derived from Ly6Chi monocytes, and M2 macrophages are from Lys6Clow monocyte precursors. The factors in lesion microenvironments that promote macrophage polarization remain incompletely defined. M1 macrophages are known as foam cell precursors, and in plaques they are enriched in lipids and localized to areas distinct from the less inflammatory M2 macrophages.

Inflammatory Responses

Activation of innate immunity is known to be the main system in progression of atherosclerosis and is mainly affected due to dysregulated lipid metabolism that causes accumulation of lipids and oxidized lipid in the lesion (Moore et al. 2013). This triggers various receptors like scavenger receptor, TLR, and nucleotide-binding oligomerization domain (NOD)-like receptors which results in activation of various inflammatory responses. In early and advanced lesions, cholesterol crystals are found in the extracellular spaces and in macrophages; these cholesterol crystals activate the NLRP3 inflammasome which secretes many proinflammatory cytokines. The remnant of VLDL and fatty acid also activates pro-inflammatory cytokines, independent of TLRs, which are a class of membrane-spanning, non-catalytic receptors that have a significant role in innate immune responses. Oxidized LDL along with oxidized phospholipids activate TLR2- and/or TLR4-related signalling cascades and increase inflammation. The lipid ligands along with CD36 and SRA react with TLRs and activate myeloid differentiation factor (MyD) 88 and c-Jun N-terminal kinases (JNK) and are responsible for apoptosis.

Macrophage Retention and Emigration

In the pathogenesis of atherosclerosis, macrophages aggregate in the lesion and are

accompanied by monocyte recruitment, proliferation of macrophages, formation of foam cell, and finally macrophage emigration and death (Moore et al. 2013) (Fig. 2). Lipoproteins in the intima causing pathogenesis of atherosclerosis are responsible for the recruitment of macrophages. Macrophage emigration occurs in the early stage of the plaques formation, but its rate of withdrawal decreases with the progression of lesion. The macrophages in the lesion do both retention and emigration of signals. Macrophage foam cells formed due to an increased expression of the neuro-immune guidance cues, netrin 1 and semaphorin 3E on macrophages, which promote its chemostasis. Deficiency of netrin 1 decreases the progression of lesion and increases emigration of macrophages. The CC-chemokine receptor CCR7 and CC-chemokine ligands CCL19 and CCL21 are responsible for the signalling of macrophage emigration from the endothelium to the lumen. As the progression of lesion continues, the foam cells enriched with lipid cause cytotoxicity induced by cholesterol and saturated fatty acid, which further causes ER stress and therefore increases apoptosis (Fig. 2). Tyrosine protein kinase MER (MERTK) and LRP1 are the receptors involved in efferocytosis that removes the apoptotic cells, but as these receptors get damaged due to accumulation of cholesterol, they continue as secondary necrosis and increase the lipid-enriched necrotic core that causes the lesion to rupture.

Pharmacotherapy for Atherosclerosis

Dyslipidemia is one of the common reasons that promote the progression of atherosclerosis; the lifestyle changes have increased the risk for atherosclerosis (Chan et al. 2014). Statins are considered as first-line lipid regulator. Patients resistant to statin are advised for alternative drugs like cholesterol absorption inhibitor or bile acid sequestrant. Various other approaches like targeting other lipid-regulating agents can be opted to treat dyslipidemia and ultimately for the treatment of atherosclerosis.

Statins (3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Inhibitor)

It is a lipid-lowering class of drugs that lowers the LDL levels by inhibiting HMG-CoA reductase. HMG-CoA is a rate-controlling enzyme that produces cholesterol. Inhibitors of HMG-CoA induce LDL expression which results in the reduction of intracellular cholesterol that induces an increase in SREBP-2-mediated hepatic LDL receptor synthesis (Sahebkar and Watts 2013). This results in increased clearance of LDL along with remnants of VLDL and chylomicron (Fig. 2). They lower the plasma concentration of LDL and apoB100 and increase HDL level. Clinical trials have consistently demonstrated that statin therapy reduces cardiovascular events. They also inhibit the mevalonic acid formation which shows anti-inflammatory activity. It reduces leukocyte adhesion, macrophage aggregation, and various other inflammasomes.

Fibrates (Peroxisome Proliferator-Activated Receptor-α Agonist)

Fibrates are nuclear hormone receptor agonists and activate PPAR-α, which decreases plasma TAG, LDL, and small-dense LDL particles and increases HDL levels (Do et al. 2014). They increase the oxidation of fatty acid due to which VLDL secretion decreases in the liver and decreases the availability of TAG substrate. They also increase the lipolysis of VLDL by LPL (lipoprotein lipase) activation and by decreasing the gene expression of apoc-III. They increase the reverse cholesterol transport by increasing apoA1 and ABCA1 expression by LXR activation. They repress the VCAM1 expression and inhibit inflammatory activation of vascular SMCs. Hence, several trials show that fibrates decrease atherosclerosis.

Niacin

Niacin has shown theoretical potential for treating atherosclerotic diseases (Do et al. 2014). It

decreases the TAG and LDL cholesterol levels in plasma and increases HDL cholesterol level. It also decreases lipoprotein a level in plasma. It decreases the secretion of VLDL TAG in the liver by inhibiting fatty acid mobilization and also decreases IDL and LDL. It decreases the clearance of HDL and apoA1 thereby increasing its plasma level. Despite of having these many desirable properties, yet it's not proven to be beneficial in cardiovascular disease.

Cholesterol Absorption Inhibitors

Ezetimibe is used to decrease the amount of cholesterol and other fatty substances in the blood. It also decreases the LDL cholesterol levels in the blood (Do et al. 2014). It works by inhibiting the function of Niemann-Pick C1-Like 1 protein (NPC1L1) and decreases the intestinal cholesterol absorption, its transport, and cholesterol content in liver (Fig. 2). It decreases the LDL particle number but does not affect the particle size of LDL. It decreases TAG in the liver and is not affected by body weight, visceral fats, and sensitivity of insulin. Ezetimibe when combined with statin shows beneficial effects and inhibits the absorption of cholesterol and shows fractional catabolism of apoB-containing lipoprotein. Ezetimibe when combined with low dose of statin decreases LDL cholesterol level more effectively as compared to higher dose of statin itself.

ω-3 Polyunsaturated Fatty Acids

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are ω-3 polyunsaturated fatty acids that are mostly found in fish oils. These fish oils reduce VLDL TAG levels (Rached et al. 2014). Its supplementation reduces VLDL production in the liver and reduces VLDL TAG levels accordingly. It inhibits the activity of diacylglycerol acyltransferase, fatty acid synthase, and acyl-CoA carboxylase enzyme which decreases the TAG levels and inhibits synthesis of fatty acid. It induces PPAR-α which enhances β-oxidation of fatty acids, but these are less effective than fibrates.

Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) Inhibitors

PCSK9 was found to be involved in autosomal dominant hypercholesterolemia and is known to degrade the LDL. Few mutations of this target increase hypercholesterolemia, but some loss of mutation shows hypocholesterolemia which decreases the risks related to CVS. PCSK9 increases LDL levels in plasma by degrading the LDL in the liver. Statins reduce LDL cholesterol in plasma and upregulate PCSK9 via SREBP2 which limits the efficacy of statin in reducing plasma LDL cholesterol level. Antibodies against PCSK9 increase the numbers of LDL receptors at the cell surface and reduce plasma LDL cholesterol (Rached et al. 2014). Antibodies like monoclonal or humanized antibodies are injected intravenously or subcutaneously. Other approaches to inhibit PCSK9 also include ASO therapies, and this target can be promising new approach for treatment of hypercholesterolemia and potentially atherosclerosis.

Antisense Oligonucleotide (ASO) Therapies

ASO sequences, when injected, bind to the mRNA and promote selective complex degradation by endogenous nucleases and also inhibit the functioning and processing of mRNA. These are short, single-stranded, synthetic analogues of natural nucleic acids that bind to mRNA in a specific manner. Mipomersen, an advanced ASO, inhibits apoB100 in the liver (Rached et al. 2014; Do et al. 2014) (Fig. 2). It reduces apoB, LDL, non-HDL, and TAG levels in plasma in different types of dyslipidemia. It decreases lipoprotein a levels by inhibiting its synthesis. It also targets other apoC-III and lipoprotein a, in which apoC-III blocks LPL-mediated lipolysis of VLDL and chylomicrons and reduces clearance of their remnant from the liver (Fig. 2). Decrease in apoC-III levels

in plasma decreases TAG concentration and reduces the risk of cardiovascular events.

Microsomal Triacylglyceride Transfer Protein (MTP) Inhibitors

This protein is useful in formation and helps in secreting apoB-containing lipoprotein from the liver and intestine (Do et al. 2014). It helps in transferring TAG, cholesteryl ester, and phospholipid to apoB in the cell during the lipoprotein assembly process. Abetalipoproteinemia is a disorder of lipoprotein assembly and is a rare condition that occurs due to mutation of this protein, in which apoB-containing lipoproteins become undetectable in plasma. Lomitapide is a MTP inhibitor that reduces LDL level in condition like hypercholesterolemia and can be given alone or in combination with ezetimibe, which is a cholesterol absorption inhibitor. Side effects like minor elevation in transaminase level in the liver, side effects in GIT, and increases in fats in the liver were reported. This drug is approved for treating homozygous FH as this drug is proven to be beneficial for cardiovascular disorders. MPT inhibitors that target the intestine promote weight loss without elevating enzymes in the liver or increases in fats in the liver and are suggested for treating broader range of lipid disorder (Rached et al. 2014).

ACAT and DGAT Inhibitors

Esterification of cholesterol in macrophages helps in formation of foam cells and is an important step in pathogenesis of atherosclerosis due to which there is accumulation of cholesterol in the intima which on esterification forms foam cells; this can be prevented by targeting ACAT (Acyl-CoA/cholesterol acyltransferase) using ACAT inhibitors. Early versions inhibited both ACAT isoforms like ACAT-1 and ACAT-2 (Rached et al. 2014). K-604 is an ACAT-1 selective inhibitor which is under development by Kowa Pharmaceuticals. ACAT-2 is an isoform that esterifies hepatic and intestinal cholesterol along with cholesteryl ester for the synthesis of lipoprotein. Inhibiting ACAT-2 using ASOs was found to inhibit secretion of VLDL and chylomicron in plasma and reduces atherosclerosis in mice. Therefore, inhibitors of ACAT-2 are under development. Diacylglycerol acyltransferase (DGAT) esterifies the third fatty acid to DAG to form TAG in hepatic, intestinal, and adipose tissue. In mice DGAT-1 inhibitor reduces TAG levels in plasma, steatosis in the liver, and obesity. Studies in mice with DGAT-1 deficiency or mice treated with DGAT-1 inhibitors demonstrated reduced plasma TAGs, hepatic steatosis, and obesity. It also improved insulin resistance. DGAT-1 inhibitor decreased fasting TAG levels in plasma in patients with hypertriglyceridemia. DGAT-2 isoform helps in synthesis of TAG in the intestine and liver and the provision of CE for lipoprotein synthesis. Specific DGAT-2 inhibitors reduce the TAG levels in the liver and in plasma and are under development for clinical use.

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ATP Binding Cassette Proteins

► ABC Transporters

ATP Pyrophosphate-Lyase

► Adenylyl Cyclases

ATP-Dependent K^+ Channels

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William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, Queen Mary, University of London, London, UK

Synonyms

ATP-sensitive potassium channels

Definition

ATP-dependent potassium (K_{ATP}) channels are potassium selective ion channels present in a range of tissues. The defining property of K_{ATP} channels is their sensitivity to intracellular nucleotides. The channels are inhibited by ATP and activated by ADP and thus couple cellular metabolism to membrane excitability. The opening of K_{ATP} channels results in membrane hyperpolarization and reduces cellular excitability. The channel is a member of the inwardly rectifying family of potassium channels though the rectification it displays is relatively weak and thus K_{ATP} channels can significantly influence excitability whatever the resting membrane potential. The channels were first described in cardiac tissue (Noma 1983) but subsequently isolated in pancreatic β cells, neurons, and skeletal and smooth muscle (Tinker et al. 2018). In this essay we consider only those channels unequivocally constituted of Kir6.0 subunits; a large number of other ion channels are ATP-responsive, but we will not review these. We also do not discuss the mitochondrial K_{ATP} channel given the controversy over its molecular composition. The following is based on a number of recent and historical reviews, and the citation to the primary literature can be found in those (Ashcroft and Rorsman 2013; Ashcroft and Ashcroft 1990; Babenko et al. 1998; Flagg et al. 2010; Foster and Coetzee 2016; Nichols 2016; Seino and Miki 2004; Tinker et al. 2014, 2018).

Basic Characteristics

Subtypes Defined by Functional Properties

K_{ATP} channels were first recognized by their electrophysiological and pharmacological properties. The channel has a single-channel conductance of 60–80 pS depending on the ionic conditions and is selective for potassium over sodium (permeability ratio P_{Na}/P_K~0.01). The nucleotide regulation of K_{ATP} channels can be characterized in inside-out patch clamp recordings. ATP inhibits K_{ATP} channels with a K_i (the concentration required to bring half maximal inhibition) of 10–500 μM and a Hill coefficient between 1 and 2. There are variations with differences between channels from one tissue to another: for example, pancreatic and neuronal channels are thought to be more metabolically sensitive than cardiac channels with a lower numerical Ki for ATP inhibition. The inhibition is not dependent on ATP hydrolysis, and non-hydrolyzable derivatives can substitute for ATP.

In solutions containing magnesium, MgADP is able to activate the channels generally in the low mM range and relieve the inhibition by ATP. There is another important property related to ATP provision: the activity of K_{ATP} channels progressively declines in ATP-free solutions, and this activity can be restored by the addition of low μM concentrations of MgATP. This phenomenon is present in a number of potassium channels and results from an unqualified dependence on phosphatidylinositol (4,5) bisphosphate in the membrane. Thus phosphatidylinositol (4,5) bisphosphate is an essential cofactor for K_{ATP} channel activity, but it is less clear if this is an important pathway for cell regulation. Thus K_{ATP} channels respond to metabolic perturbations within the physiological range: a process that may be tuned by anionic phospholipid regulation.

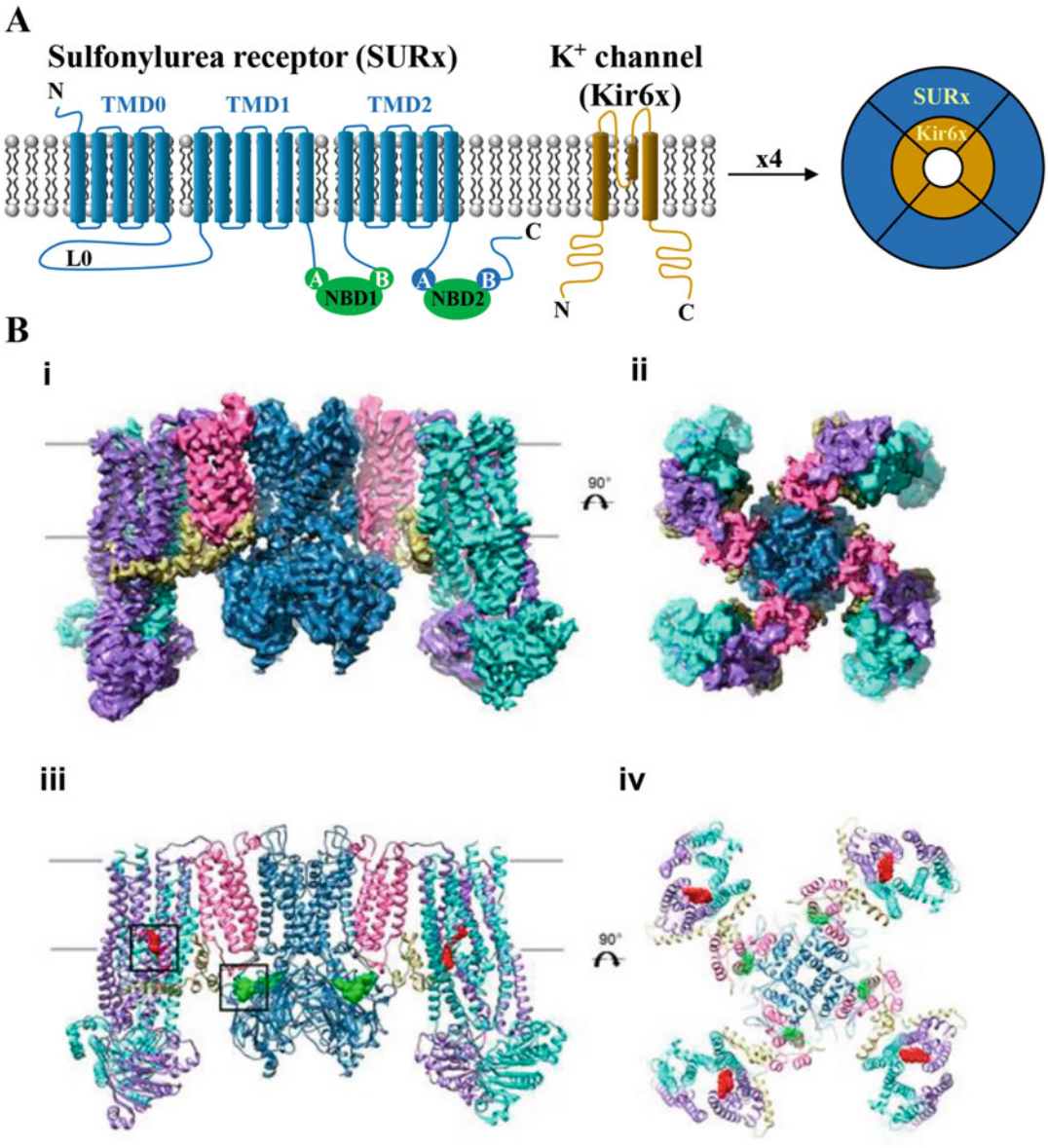
The above description defines the "classical" K_{ATP} channel; however particularly in smooth muscle, there exist channels with similar but also some distinct properties. These channels have a lower single-channel conductance (30–35 pS), and channel activity is absolutely dependent on cytosolic nucleotide diphosphates. The current is more aptly described as a "K_{NDP}" current. This is accompanied by reduced sensitivity to ATP and prominent regulation by cell signalling pathways particularly involving protein kinase A and protein kinase C.

K_{ATP} channels have a distinctive pharmacology: they are inhibited by sulfonylureas including the earlier low-affinity reagents such as tolbutamide and chlorpropamide and a second-generation group including glibenclamide, gliclazide, and glipizide. Newer agents which are based on the non-sulfonylurea component of glibenclamide ("glinides") include meglitinide, mitiglinide, and repaglinide. In general these agents show selectivity for the K_{ATP} channel present in the pancreatic β cell, but at higher concentrations they can inhibit channel populations in other tissues. Synthetic efforts have also led to the development of drugs with a different pattern of selectivity. For example, HMR-1098 preferentially inhibits cardiac over pancreatic beta cell K_{ATP} channels. Furthermore, PNU99963 and PNU37883A are non-sulfonylureas and selectively block the vascular K_{ATP} channel. They were initially developed as potentially selective agents for the vascular channel.

K_{ATP} channels can be activated by a number of drugs with a variety of chemical structures. These include diazoxide, minoxidil, cromakalim, nicorandil, pinacidil, aprikalim (and analogues such as rilmakalim), WAY-151616, ZM-244085, and ZD-6169. Initially these drugs were developed for their selective actions on smooth muscle and in particular hypotensive effects. However diazoxide is more potent in activity for the pancreatic channel and can cause hypoglycemia. Diazoxide, minoxidil, cromakalim, nicorandil, pinacidil, and aprikalim all have significant additional activity toward the cardiac channel, while WAY-151616, ZM-244085 and ZD-6169 seem to be more smooth muscle specific.

Subtypes Defined by Molecular Cloning

A major advance in understanding of the molecular physiology of K_{ATP} channels came with the cloning of their component subunits. The channel is an octameric complex consisting of four Kir6.0 subunits and four sulfonylurea receptors (Fig. 1a). The coexpression of both proteins is necessary for the expression of membrane currents, and only correctly folded and assembled octamers are exported from the endoplasmic reticulum to the plasma membrane. Incorrectly folded subunits are recognized in the Golgi and retro-translocated to the endoplasmic reticulum for degradation. There are two Kir6.0 subunits, Kir6.1 and Kir6.2, encoded by the genes *KCNJ8* and *KCNJ11*. There are two sulfonylurea receptor genes, *ABCC8* and *ABCC9*, and these generate the proteins SUR1 and splice variants of *ABCC9*, SUR2A, and SUR2B. Other splice variants have been suggested, but their significance is debated. Kir6.0 subunits are a member of the inwardly rectifying family of potassium channel and have two transmembrane segments, a pore-forming H5 domain and an intracellular N and C-terminus. SURs belongs to the superfamily of ATP-binding cassette (ABC) proteins with 17 transmembrane segments grouped into 3 domains comprised of 5,



ATP-Dependent K^+ Channels, Fig. 1 Molecular composition and structure of a K_{ATP} channel. (**a**) Schematic of the molecular composition of a K_{ATP} channel. K_{ATP} channels are an octameric complex consisting of four pore-forming Kir6.x subunits and four regulatory sulfonylurea receptor (SUR) subunits. Kir6.x belongs to the inward-rectifying K^+ channel family (Kir); it is composed of two transmembrane domains (M1 and M2), a pore-forming region (H5) with the K^+ selectivity sequence and intracellular N and C termini. SUR belongs to the ATP-binding cassette (ABC) family of proteins. It consists of three transmembrane domains (TMDs) composed of five, six and six transmembrane segments, respectively. Two nucleotide-binding domains (NBD1 and NDB2) comprised of Walker A and B nucleotide-binding motifs provide the binding sites for magnesium-complexed adenine nucleotides. (**b**) The high-resolution structure of the pancreatic K_{ATP} channel. (i) Cryo-EM density map (side view) of the K_{ATP} channel (3.63 Å resolution). Gray lines indicate the position of the membrane. (ii) The K_{ATP} channel complex when viewed extracellularly. (iii) A model of the K_{ATP} channel complex with various ligands bound as indicated (ATP in green and glibenclamide in red). (iv) The model viewed from the extracellular side of membrane. This figure is reproduced from Martin et al. (Martin et al. 2017)

6, and 6 transmembrane helices (TMD0, TMD1, and TMD2, respectively). The N-terminus is extracellular with cytoplasmic linkers connecting the other domains to one another and nucleotide-binding domains in the second linker (NBD1) and C-terminus (NBD2). As the name suggests, the nucleotide-binding domain protein modules bind and hydrolyze adenine nucleotides.

The specific combinations of Kir6.0 and SUR subunits determine the particulars of the current in each tissue. In pancreatic β cells, SUR1 and Kir6.2 underlie the current and lead to 70 pS single-channel conductance, a high sensitivity to ATP inhibition (Ki ~ 10–30 μM), inhibition by tolbutamide, and activation by diazoxide. In many smooth muscles, SUR2B and Kir6.1 constitute the channel with a 35 pS single-channel conductance, an absolute prerequisite for provision of nucleotide diphosphates for activity, low-affinity inhibition by glibenclamide, and activation by levcromakalim. The contents of this section are summarized in Table 1.

The Relationship of Structure to Function

A great deal is understood about how modulators of K_{ATP} channel function from mutagenesis studies and recent high-resolution cryo-EM structures (Fig. 1b). The channel, in the closed state, assembles with four SUR1 subunits attached at the periphery of the tetramer of Kir6.2 subunit. The N-terminus and proximal C-terminus of Kir6.2 link to the SUR1 TMD0 and linker L0, and TMD1 and TMD2 lie away from the channel subunit. The inhibition of the channel by ATP is governed by amino acids in the pore-forming Kir6.0 subunit: a horseshoe-shaped binding pocket is defined by adjacent N and C domains from separate Kir6.2 subunits, and the complex can bind up to four molecules of ATP. Activation by MgADP is determined by the sulfonylurea subunit in particular interaction with residues in the NBDs. The binding site is made up of residues from both NBD1 and NBD2, and the two binding sites formed in an individual SUR are asymmetrical with respect to structure and function with only one able to hydrolyze ATP rapidly. However ATP hydrolysis is not key for activation as MgADP readily modulates the channel. In other ABC transporters, the NBDs dimerize on activation though this has not yet been unequivocally demonstrated for SUR. Sulfonylureas bind to SUR, but at high concentrations some may also directly interact with the pore. The binding site is defined by the transmembrane segments in TMD2, particularly the transmembrane domains 15 and 16, though the cryo-EM structures also indicate interaction with the linker between TMD0 and TMD1 and TMD1. There are indications from the structures that glibenclamide might affect the ability of the NBDs to align and potentially dimerize. The binding site for potassium channel openers is present on the SUR subunit but is less well defined, but the cytoplasmic linker between TM13 and TM14 and the last TM helices, TM16 and TM17, may be involved in activation by pinacidil. It is also plausible that different potassium channel openers may have different binding sites and it is clear that the sites don't overlap with that for sulfonylureas.

Physiology, Pathophysiology, and Therapeutic Potential

Insulin Secretion in Pancreatic β Cells

Insulin is synthesized, stored, and released from the β cells present in the islets of Langerhans in the pancreas. Insulin is key to maintaining blood glucose within the normal range by promoting glucose uptake in peripheral tissues and switching off glucose production. The process by which the blood glucose concentration is coupled to insulin secretion is known as stimulus-secretion coupling, and K_{ATP} channels are centrally involved. When blood glucose increases, pancreatic β cells depolarize and initiate increased firing of action potentials. The metabolism of glucose by β cells is tightly coupled to ATP production via mitochondrial oxidative metabolism. This increase in ATP leads to inhibition of K_{ATP} channels and is the main reason for membrane depolarization. The increased action potential firing leads to calcium entry into the β cell via voltage-dependent calcium channels which in turn promotes the exocytosis of insulin containing vesicles. This

ATP-Dependent K^+ Channels, Table 1 Tissue-specific location, function, and pharmacology of K_{ATP} channels

Subunit composition	Location	Conductance (pS)	ATP IC_{50} (μM)	Physiological function	Pharmacology	
					Openers	Blockers
Kir6.2/ SUR1	Pancreas	70–80	15–160	Regulation of insulin release	Diazoxide	Tolbutamide, Glibenclamide Glipizide Glimepiride Glipizide Meglitinide Repaglinide Nateglinide Mitiglinide
	aCMs	52–90	39–100	Repolarization of the atrial action potential	Diazoxide	Tolbutamide Glibenclamide Glipizide Glimepiride Glipizide Meglitinide Repaglinide Nateglinide Mitiglinide
	Brain	44–73	12–130	Neural protection Neurotransmitter release Perception of pain Regulation of excitability	Diazoxide	Tolbutamide Chlorpropamide Glibenclamide Glipizide Glimepiride Glipizide
Kir6.2/ SUR2A	vCMs	70–90	10–100	Adaptation response to stress Protection against Ca^{2+} overload during hypoxia	Pinacidil, P-1075 Cromakalim Levcromakalim Nicorandil Minoxidil Aprikalim	Tolbutamide, Glibenclamide Glipizide Glimepiride Glipizide HMR-1098 HMR-1883
Kir6.1/ Kir6.2/ SUR2B	CCS	52–60	16–120	Stress adaptation Regulation of pacemaker activity	Pinacidil P-1075 Cromakalim Levcromakalim	Tolbutamide Glibenclamide Glipizide Glimepiride Glipizide
Kir6.1/ SUR2B	Smooth muscle	15–50	29–200	Dilation of blood vessels Regulation of blood pressure	Pinacidil P-1075 Diazoxide Cromakalim Levcromakalim Aprikalim WAY-151616 ZM-244085 ZD-6169	Glibenclamide, Glipizide Glimepiride Glipizide PNU-37783A PNU-99963

vCMs, ventricular cardiomyocytes; aCMs, atrial cardiomyocytes; CCS, cardiac conduction system; ATP, adenosine triphosphate

process is mimicked by the application of sulfonylureas and their derivatives and is why they are used to treat type II diabetes mellitus. It is worth bearing in mind that they are prescribed less frequently due to issues with weight gain and as result drugs such as metformin are usually the

first-line therapy. Stimulus-secretion coupling in pancreatic β cells is shown in Fig. 2a.

Mutations in K_{ATP} channel subunits occur in rare hereditary diseases of insulin handling. Congenital hyperinsulinism is one such syndrome and refers to high and inappropriate secretion of insulin resulting in life-threatening hypoglycemia. The disease is rare and occurs in 1 in 50,000 births but is more common in areas where there are high levels of consanguinity. The commonest cause is missense mutations in ABCC8 and KCNJ11, and these lead to loss-of-function properties in the channel. The disease can be treated with K_{ATP} channel openers specifically diazoxide. Diabetes mellitus occurring in the neonatal population (up to 9 months) is rare occurring in 1 in 100,000 births but has a unique genetic basis. Mutations in ABCC8 and KCNJ11 are the commonest but not the only causes of neonatal diabetes mellitus. ABCC8 and KCNJ11 mutations are heterozygotic and generally occur de novo. In general, these result in quite subtle gain-of-function phenotypes with channels being less sensitive to ATP inhibition and thus inappropriately active at a given blood glucose concentration resulting in impaired insulin secretion. The patients used to be treated with insulin, but with these advances in understanding the pathogenesis of the disease, they are now given sulfonylureas often with dramatic responses.

The ability of K_{ATP} channels to act as metabolic sensors has led to studies in a number of related areas. Thus, K_{ATP} channels have been suggested to have a role in glucagon release from α cells in the islets of Langerhans and in secretion of various incretins from the enteroendocrine system in the gut. Furthermore, Kir6.2 and SUR1 are expressed in central neurons that are thought to influence feeding behavior and satiety. Thus there is active research in these areas with suggestive data, but there isn't a prevailing consensus yet.

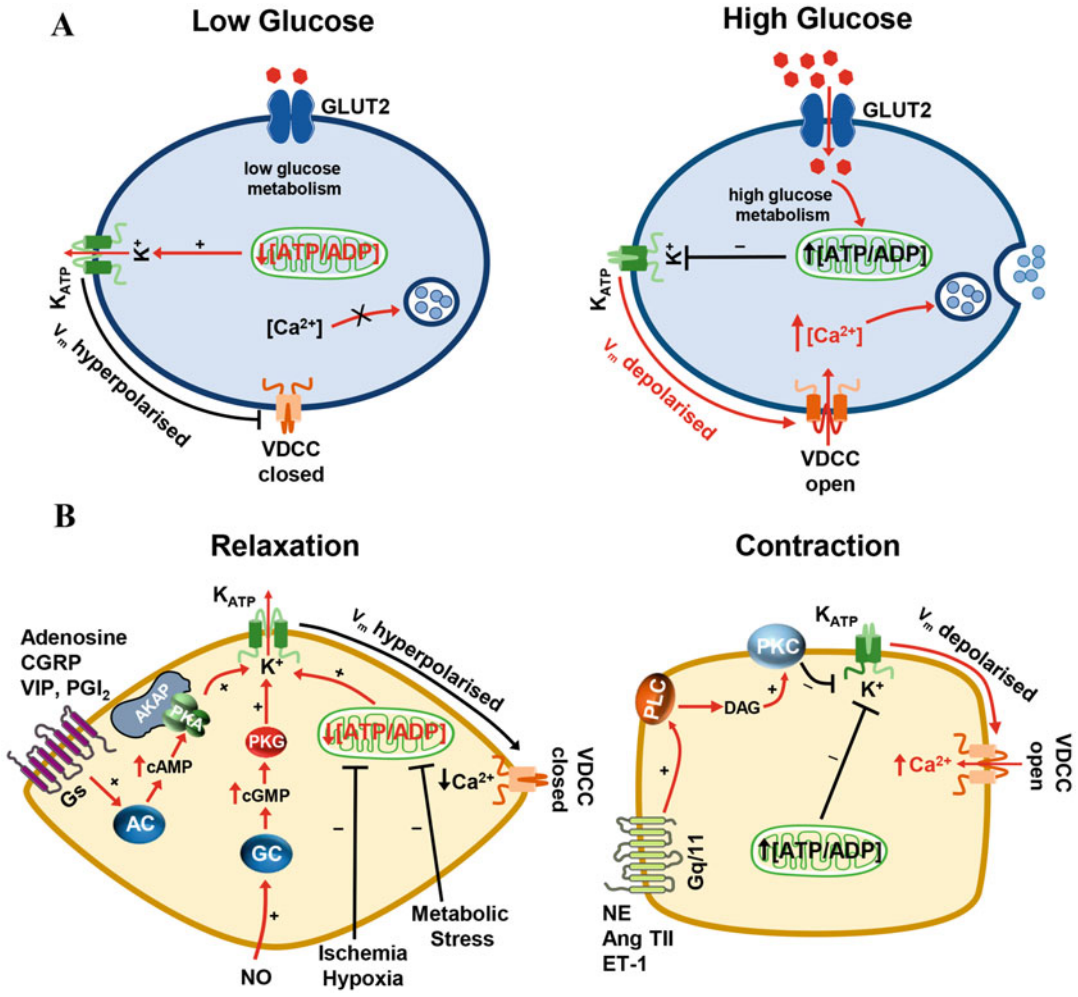
Cardiac Function

The expression of K_{ATP} currents in cardiac myocytes is robust and substantial though under resting metabolic conditions they are not active. Though the focus is usually on ventricular cells, these currents are present in atrial cells and cells of the conduction system. The most heavily investigated area is the role of K_{ATP} channels in cardiac protection which includes phenomena such as resistance to ischemic cell death, ischemia-reperfusion injury, and preconditioning, i.e., the ability to promote myocyte survival by prior application of short sublethal episodes of ischemia. The central idea is that opening of K_{ATP} channels shortens the cardiac action potential, reducing calcium entry through voltage-dependent calcium channels and reducing contractile work. This attenuates calcium overload, metabolic demands, and ultimately cell death. There is a substantial body of evidence supporting this which has been accrued in animals and humans based on pharmacological and more recently studies in genetically modified mice. There has been interest in exploiting this therapeutically, but to date no approach has been established in clinical practice.

The focus with cardiac K_{ATP} channels is usually on pathological scenarios in which channel opening may be protective. However this doesn't address the question of what physiological role the channel may play. For example, the channel contributes little to the cardiac action potential in the absence of metabolic challenge. The most convincing hypothesis, supported by work in genetically modified mice, is that the channel prevents myocardial damage under high workloads and accompanying adrenergic drive.

Control of Smooth Muscle Tone

The channel in smooth muscles has distinct properties as discussed above, and this is consistent with the channel being constituted of Kir6.1 and SUR2B. K_{ATP} currents are widely expressed in many vascular beds in both conduit and resistance vessels. Endothelial cells also express a K_{ATP} current; however this is much less studied. It may be that they perform complementary roles in regulation of vascular function. K_{ATP} channels are centrally involved in blood pressure control. If K_{ATP} channels open, then calcium entry through



ATP-Dependent K^+ Channels, Fig. 2 The role of K_{ATP} channels in pancreatic β cells and vascular smooth muscle. (**a**) K_{ATP} channels regulate insulin release by coupling cellular metabolism to electrical activity. ATP production is reduced when blood glucose is low, allowing K_{ATP} channels to open leading to membrane hyperpolarization thus preventing an increase intracellular Ca^{2+} and subsequent insulin release (left). Cellular metabolism increases when blood glucose is high, and ATP production increases leading to K_{ATP} channel inhibition and an increase in intracellular Ca^{2+} and insulin release (right). (**b**) Modulation of vascular smooth muscle K_{ATP} channels via vasodilators and vasoconstrictors regulates smooth muscle tone. The membrane potential of vascular smooth muscle (VSM) cells is determined by the opening/closing of K_{ATP} channels. Activation of K_{ATP} channels either directly or indirectly cause membrane hyperpolarization resulting in closure of voltage-dependent calcium channels (VDCC), reduced intracellular Ca^{2+}, and relaxation. Conversely, K_{ATP} channel inhibition causes depolarization of the membrane, opening of VDCC, increased intracellular Ca^{2+}, and contraction. Left, K_{ATP} channel activation initiated by vasodilators such as adenosine, calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP), and PGI_2 via the G-protein (Gs)/adenylate cyclase (AC)/protein kinase A (PKA) signalling pathway leads to the relaxation of VSM. Hypoxia, ischemia, and metabolic stress inhibit oxidative phosphorylation and therefore decrease the ATP/ADP ratio leading to activation of K_{ATP} channels. Right, endogenous mediators such as norepinephrine (NE), angiotensin II (ang II), and endothelin-1 (ET-1) inhibit K_{ATP} channels via the G-protein (Gq/11)/PKC signalling pathway leading to VSM contraction

voltage-dependent calcium channels is reduced, and cytosolic calcium falls leading to vascular relaxation. Furthermore, a number of vasodilators including adenosine, calcitonin gene-related peptide, vasoactive intestinal polypeptide, and prostacyclin can activate the channel. This occurs through activation of the relevant G-protein-coupled receptor, activation of the stimulatory G-protein, and then engagement of the adenylate cyclase, cAMP, and the protein kinase A pathway. Protein kinase A directly phosphorylates residues on both Kir6.1 and SUR2B, and this leads to an increase in channel open probability. Vasoconstrictors such as angiotensin II, endothelin, and norepinephrine acting via $G_{q\backslash 11}$ coupled receptors and protein kinase C can inhibit the K_{ATP} channel. Evidence from genetically modified mice lacking the Kir6.1 subunit in vascular smooth muscle shows that the K_{ATP} channel is predominantly involved in vasorelaxation as the mice are hypertensive. Furthermore, administration of many potassium channel openers leads to significant falls in blood pressure. Vascular K_{ATP} channels are also involved in local vascular reactivity to increase blood flow to tissues and cells with high metabolic demand. This occurs through their direct metabolic sensitivity but also and probably more critically via adenosine-mediated signalling. The role of the channel in vascular smooth muscle is illustrated in Fig. 2b.

K_{ATP} channels are also present in nonvascular smooth muscles such as the gastrointestinal tract and bladder. In a similar vein, the application of potassium channel openers can lead to muscle relaxation. There has been interest, for example, in developing such drugs to treat bladder instability.

Other Roles

The areas described above are those where the role of K_{ATP} channels has been best defined, and there is largely a scientific consensus. However K_{ATP} currents are prominent in a range of other tissues, and we discuss those here with suggestions about their functional role. There are prominent K_{ATP} currents in skeletal muscle which have similar properties to those in cardiac muscle. Their exact physiological role is not clearly defined, but they have been proposed to be involved in preventing muscle fatigue with exercise. However they may also have a broader metabolic function by influencing glucose uptake and cellular metabolism. K_{ATP} channels are present in a number of neuronal populations including peripheral nerves. In addition to influencing feeding behavior, they have also been proposed to have roles in regulating neuronal excitability and cellular protection. Specifically, they have been suggested to be involved in pain, Parkinson's disease, and epilepsy.

There is rare disease called Cantu syndrome. This is a multisystem disease and has a number of features including increased hair growth, dysmorphic facial features, skeletal abnormalities, cardiac hypertrophy, and heart failure. Novel sporadic missense mutations have been found in ABCC9 and KCNJ8 and result in increased K_{ATP} channel activity. Despite its rarity this disease suggests a role for K_{ATP} channels during development.

Drugs

The relevant drugs are discussed in prior sections and also listed in the table; however it is worth briefly reviewing the current use in clinical practice. In some of the rare genetic syndromes, specific agents targeting the channels are being used in a precision-based fashion to target the underlying defect. More broadly, sulfonylureas and derivatives are used to treat type II diabetes mellitus though they are now a second-line agent. Nicorandil is used to treat angina but again only as a second-line agent as it causes gastrointestinal and skin ulcers, and diazoxide and minoxidil are still available to use in hypertensive emergencies. Minoxidil is associated with increased hair growth possibly by increasing blood flow to the hair follicle and is used in a topical preparation for male pattern baldness (395).

Cross-References

- ▶ ABC Transporters
- ▶ Blood Pressure Control
- ▶ Diabetes Mellitus
- ▶ Inward Rectifier Potassium Channels

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ATP-Sensitive Potassium Channels

- ▶ ATP-Dependent K^+ Channels

Atrial and Brain Natriuretic Peptides

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Synonyms

Atrial natriuretic factor/B-type natriuretic peptide

Definition

Atrial natriuretic peptide (ANP) and brain (also known as B-type) natriuretic peptide (BNP) are biologically active molecules that belong to the natriuretic peptide family, which also includes a third member, C-type natriuretic peptide (CNP). ANP was first isolated from human atrial tissue and its complete amino acid sequence was determined in 1984 (Kangawa and Matsuo 1984). BNP was first identified in porcine brain tissue in 1988. It is now known, however, that both ANP and BNP are secreted by the heart and act as cardiac hormones. In response to stretching of the myocardium, the two peptides are secreted from cardiomyocytes into the circulation. Both ANP and BNP preferentially bind to natriuretic peptide receptor-A (NPR-A or guanylyl cyclase-A) and exert similar effects through increases in intracellular cyclic guanosine monophosphate (cGMP) within target tissues. Through this action, they induce a decrease in vascular tone, an immediate increase in electrolyte and water excretion via the kidney and antifibrotic and antihypertrophic effects in the heart, all of which functionally antagonize the renin-angiotensin-aldosterone system (RAAS) (Nishikimi et al. 2006).

Basic Characteristics

Characteristics of ANP and BNP Genes and Peptides and Their Regulation of Gene Expression and Secretion

The *Nppa* gene encoding human ANP contains three exons and its transcript is translated into a 151-amino acid precursor, preproANP. A 25-amino acid signal peptide is then removed, yielding 126-amino acid proANP, which is the tissue form of the hormone. ProANP is stored in secretory granules in the atrium and is thought to be proteolytically converted to the 28-amino acid peptide ANP (α-ANP) and 98-amino acid N-terminal proANP (NT-proANP) by the transmembrane enzyme corin during its secretion (Nishikimi et al. 2011). The major molecular form of circulating human ANP is α-ANP, which contains a ring structure with a disulfide linkage (Nishikimi et al. 2011). β-ANP, which is an antiparallel dimer of α-ANP, and proANP (γ-ANP) have also been isolated from the human failing heart, and plasma levels of both β-ANP and proANP are increased in cases of severe heart failure, though the precise mechanism regulating the formation of β-ANP remains unclear.

The *Nppb* gene encoding human BNP also contains three exons. Within the endoplasmic reticulum in ventricular myocytes, BNP mRNA is initially translated into a 134-amino acid precursor, preproBNP, and then converted to 108-amino acid proBNP through removal of a 26-amino acid signal peptide (Nishikimi et al. 2006, 2011). The N-terminal region of proBNP is glycosylated within the Golgi apparatus and then cleaved, presumably by the proteolytic enzyme furin, yielding 32-amino acid BNP and 76-amino acid N-terminal proBNP (NT-proBNP), which are secreted from the heart in equimolar fashion. Not all proBNP is cleaved into BNP and NT-proBNP, and the remaining proBNP is also secreted into the circulation, though it is less active than BNP. Whereas the amino acid sequence of ANP is well conserved among species, the amino acid sequences of BNP are diverse. The predominant circulating form of BNP is composed of 26-, 45-, or 32-amino acids in pig, rat, and human, respectively (Nishikimi et al. 2011).

In normal conditions, most all of ANP in the circulation are secreted from the atrium, whereas most of BNP in the circulation are derived from the ventricle. Within the atrium, ANP is stored in granules and is immediately secreted in response to a secretory stimulus. Within the ventricle, by contrast, BNP secretion is controlled through gene expression via the so-called constitutive pathway. It therefore takes time for blood BNP levels to increase, even in response to a strong stimulus.

Expression and secretion of ANP and BNP are increased under pathological conditions in the heart. Mechanical stress, for example, stimulates the synthesis and secretion of both peptides in atrial and ventricular cells. Neurohumoral factors, such as endothelin-1, α-adrenergic agonists, and angiotensin-II, as well as various growth factors and cytokines, including interleukin (IL)-1β, IL-18, leukemia inhibitory factor (LIF), cardiotrophin (CT) -1, tumor necrosis factor (TNF) α, and transforming growth factor (TGF) -β, all stimulate ANP and/or BNP synthesis in cultured cardiomyocytes. ER stress also induces BNP gene expression in cardiomyocytes (reviewed in Nakagawa et al. 2019).

Characteristics of Action

The natriuretic peptide system also includes three membrane-bound natriuretic peptide receptors: NPR-A (or guanylyl cyclase-A), NPR-B (or guanylyl cyclase-B), and NPR-C (or clearance receptor). The three natriuretic peptides preferentially bind to NPR-A with a rank order potency of ANP $\geq$ BNP $>>$ CNP. NPR-A is linked to guanylyl cyclase, leading to production of the intracellular messenger cGMP in target cells. By contrast, the three peptides bind to NPR-B with a rank order potency of CNP $>$ ANP $\geq$ BNP. The three peptides also bind to NPR-C, or clearance receptor, which possesses no known intrinsic enzymatic activity, with a rank order potency of ANP=CNP>BNP.

The physiological actions of ANP and BNP at target cells and organs are similar and commonly mediated via NPR-A. Binding to NPR-C ultimately leads to internalization and lysosomal

degradation of the peptides and their clearance from the circulation. Another important mechanism by which natriuretic peptides are cleared is via membrane-bound metalloendopeptidase, also known as neprilysin, which is broadly distributed among tissues, including the lung and kidney, and acts on a variety of peptides (Volpe et al. 2016).

The effects of ANP/BNP include diuresis, natriuresis, vasodilation, and inhibition of aldosterone synthesis and renin secretion, which enable these hormones to play key roles in regulating blood pressure and fluid volume (reviewed in Nishikimi et al. 2006). After secretion from the heart, ANP/BNP immediately affect electrolyte and water excretion by the kidney and functionally antagonize the renal actions of the RAAS. ANP/BNP affect blood pressure acutely by acting on vascular smooth muscle cells and chronically by affecting vascular endothelial permeability. They also reportedly prevent vascular smooth muscle cell growth and proliferation and vascular fibrosis. In addition, ANP/BNP act on the heart itself to suppress cardiac hypertrophy and pathological cardiac remodeling at least in part by antagonizing signaling from the RAAS (Nishikimi et al. 2006). Like cardiomyocytes, cardiac fibroblasts also express NPR-A, NPR-B, and NPR-C; in fact, cardiac fibroblasts show larger increases in intracellular cGMP in response to NPR-A or NPR-B stimulation than do cardiomyocytes. Moreover, ANP/BNP-NPR-A signaling is an important regulator of the growth and proliferation in cardiac fibroblasts (Volpe et al. 2016).

Genetically modified animals have proven to be highly useful for studying ANP/BNP-NPR-A signaling. Marked cardiac hypertrophy with interstitial fibrosis is observed in NPR-A knockout mice (Oliver et al. 1997), and pressure overload in these mice leads to reduced left ventricular systolic function (Knowles et al. 2001). On the other hand, although ANP knockout mice exhibit salt-sensitive hypertension, there is no obvious cardiac hypertrophy under basal conditions (John et al. 1995). However, pressure overload leads to robust interstitial and perivascular fibrosis with cardiac hypertrophy and reduced fractional shortening in ANP knockout mice (Franco et al. 2004). BNP knockout mice exhibit multifocal fibrotic lesions within the cardiac ventricles with no signs of systemic hypertension or ventricular hypertrophy (Tamura et al. 2000). In response to ventricular pressure overload, the focal fibrotic lesions are worsened in BNP knockout mice than those in wild-type mice, despite similar increases in ventricular weights in the two groups (Tamura et al. 2000). These results indicate that ANP and BNP exert anti-fibrotic effects in vivo and serve as important local regulators of ventricular remodeling. The difference in the phenotypes of ANP and BNP knockout mice may highlight the predominant paracrine effects of BNP within the ventricles.

Drugs

ANP and BNP as Diagnostic Agents

Listed in guidelines published worldwide, BNP and NT-proBNP are diagnostic indicators for patients with heart failure (Yancy et al. 2017; Ponikowski et al. 2016; Tsutsui et al. 2019). Plasma BNP and NT-proBNP levels are established biomarkers that increase in proportion to disease severity and are used for diagnosis and estimation of prognosis, and for evaluating treatment efficacy in patients for heart failure (Yancy et al. 2017; Ponikowski et al. 2016; Tsutsui et al. 2019). Meta-analyses have shown that BNP- or NT-proBNP-guided therapy reduces all-cause mortality in patients with chronic heart failure, as compared to usual clinical care, especially in patients <75 years of age (Porapakkham et al. 2010). As mentioned above, proANP is enzymatically converted to ANP and NT-proANP. Because NT-proANP is further cleaved into smaller fragments in vivo, the mid-region of proANP (amino acids 53–90; MR-proANP) is the preferred detection site of this peptide, and it too is available for diagnosis of heart failure. However, there are also noncardiovascular conditions that can affect plasma levels of natriuretic peptides, including age, renal failure, and obesity, which may affect their diagnostic utility in heart failure (Yancy et al. 2017).

According to the ACC/AHA/HFSA guidelines in the USA, measurement of BNP or NT-proBNP is useful for diagnosis of heart failure and for establishing prognosis or disease severity in cases of chronic heart failure (recommended class I, level of evidence A). BNP- or NT-proBNP-guided heart failure therapy can be useful to achieve optimal dosing of guideline-directed medical therapy (recommended class IIa, level of evidence B) (Yancy et al. 2017). In the ESC European guidelines, measurement of plasma BNP, NT-proBNP, or MR-proANP is recommended for diagnosis in all patients with acute dyspnea or suspected acute heart failure as a means of differentiating acute heart failure from noncardiac causes of acute dyspnea (recommended class I, level of evidence A) (Ponikowski et al. 2016). In the JCS/JHFS guidelines in Japan, measurement of plasma BNP or NT-proBNP is recommended for diagnosis in acute heart failure and for evaluating the severity and prognosis of heart failure (recommended class I, level of evidence A), for screening (recommended class IIa, level of evidence C) and for evaluating the therapeutic effect of treatment (recommended class IIa, level of evidence B) (Tsutsui et al. 2019).

ANP/BNP System as a Therapeutic Target

Natriuretic peptides themselves or drugs that enhance their actions are being used for treatment of heart failure and other cardiovascular conditions.

Carperitide is a recombinantly produced intravenous formulation of human ANP, which is administered to patients with acute decompensated heart failure to improve their hemodynamic condition. Several studies have reported its beneficial effects in a variety of contexts. For example, continuous infusion of low-dose carperitide from the start of cardiopulmonary bypass effectively maintains postoperative renal function (Sezai et al. 2009). In addition, patients with acute myocardial infarction administered carperitide had smaller infarcts, fewer reperfusion injuries, and better outcomes than controls (Kitakaze et al. 2007). Finally, low-dose carperitide infusion for 72 h as the initial treatment improved the long-term prognosis of patients with acute decompensated heart failure (Hata et al. 2008). Carperitide is now available in Japan.

Nesiritide is an intravenous formulation of recombinant human BNP. The US Food and Drug Administration approved nesiritide for use in acute decompensated heart failure in 2001. Nesiritide reduces LV filling pressure, but has variable effects on cardiac output, urinary output, and sodium excretion. Several clinical trials have reported beneficial effects of nesiritide, which reduces the severity of dyspnea more rapidly than diuretics alone (Colucci et al. 2000). Nesiritide was associated with significantly lower in-hospital mortality than positive inotropic therapy (milrinone and dobutamine) in patients with acute decompensated heart failure listed in the ADHERE registry (Abraham et al. 2005). The NAPA trial showed that nesiritide improves postoperative renal function and may improve survival (Mentzer et al. 2007). However, the ASCEND-HF study, a large randomized trial in patients with acute decompensated heart failure, reported that nesiritide had no impact on mortality, rehospitalization, or renal function, and it increased the risk of hypotension, though it showed a small but statistically significant impact on dyspnea (O'Connor et al. 2011). Because nesiritide has a longer effective half-life than nitroglycerin or nitroprusside, two vasodilating agents, adverse effects such as hypotension may persist longer (Yancy et al. 2017). The effective half-life of nesiritide is also longer than that of carperitide. On February 5, 2018, the US Food and Drag Administration reported that pharmaceutical companies were discontinuing the manufacture of nesiritide.

Neprilysin, or neutral endopeptidase 24.11, degrades vasodilating peptides such as natriuretic peptides, adrenomedullin, and bradykinin as well as vasoconstrictors such as angiotensin II and endothelin-1, among others. Neprilysin cleaves ANP and CNP, breaking the ring and inactivating the peptides. In contrast to ANP or CNP, the primary cleavage site of human BNP is within

the N-terminal region, making BNP relatively resistant to degradation by neprilysin in humans (Bayes-Genis et al. 2016). By inhibiting neprilysin, degradation of natriuretic peptides is slowed. The resultant increases in ANP and BNP levels would be expected to enhance their physiologic effects by increasing binding to NPR-A and generation of cGMP, thereby enhancing their beneficial cardiovascular effects (Ponikowski et al. 2016). Moreover, because neprilysin is reportedly elevated in heart failure (Bayes-Genis et al. 2016), its inhibition may be an effective therapeutic strategy for heart failure and other cardiovascular conditions.

Candoxatril is a pure neprilysin inhibitor that was shown to elevate plasma ANP concentrations in patients with essential hypertension, though levels of angiotensin II and aldosterone were also significantly increased (Richards et al. 1993). Moreover, candoxatril failed to exert favorable clinical effects in patients with hypertension or heart failure (Bevan et al. 1992; Kentsch et al. 1996). These results suggest that although neprilysin inhibition suppresses the degradation of natriuretic peptides, it also suppresses degradation of angiotensin-II, resulting in RAAS activation. Thus, to produce the clinical benefits of neprilysin inhibition, it is necessary to also suppress the RAAS.

Omapatrilat is an inhibitor of both neprilysin and angiotensin converting enzyme (ACE), a so-called vasopeptidase. In the IMPRESS trial of 573 patients with NYHA class II to III heart failure, omapatrilat decreased the rate of a composite endpoint (death, hospitalization, or discontinuation of treatment for worsening heart failure) as compared with lisinopril, an ACE inhibitor (Bayes-Genis et al. 2016). However, in the OVERTURE trial of 5770 patients with NYHA II to IV heart failure and LVEF$\leq$30%, omaptrilat did not show superiority to enalapril with respect to a primary endpoint (death from any cause or admission for heart failure). In addition, angioedema was reported in 24 (0.8%) omapatrilat-treated patients by only 14 (0.5%) enalapril-treated patients (Bayes-Genis et al. 2016). And in the OCTAVE trial of 25,302 patients with hypertension, angioedema was reported in 2.2% of omapatrilat-treated patients but only 0.7% of enalapril-treated patients. The high rate of angioedema in patients with hypertension and the nonsuperiority of omaptrilat compared to an ACE inhibitor in patients with heart failure led to interruption of the clinical development of omapatrilat and cessation of further development of this class of drugs (Volpe et al. 2016). The increased incidence of angioedema is possibly due to the presence of excess bradykinin caused by dual inhibition of neprilysin and ACE, both of which degrade bradykinin.

Angiotensin receptor neprilysin inhibitors (ARNIs) have been developed as a therapeutic class of agents acting on the RAAS and neprilysin. The first in this class is LCZ696 (sacubitril/valsartan), which is a molecule that combines moieties of valsartan, an angiotensin II receptor type 1 blocker (ARB), and sacubitril, a neprilysin inhibitor, in a single substance. By inhibiting angiotensin II type 1 receptors with valsartan, ARNIs inhibit the action of angiotensin II without affecting bradykinin degradation. A randomized double-blind clinical trial showed that LCZ696 significantly reduced death from cardiovascular causes and hospitalization for heart failure as compared to enalapril, an ACE inhibitor, in patients with NYHA class II to IV heat failure and ejection fractions of 40% or less (PARADIGM-HF) (McMurray et al. 2014). Thus, according to the ESC guidelines, LCZ696 is recommended as a replacement for an ACE inhibitor to further reduce the risk of heart failure hospitalization and death in ambulatory patients with heart failure with reduced ejection fractions who remain symptomatic despite optimal treatment with an ACE inhibitor, a beta-blocker, and a mineralocorticoid receptor antagonist (Ponikowski et al. 2016). Similarly, the AHA/ACC guidelines recommend an ARNI as an alternative to ACE inhibitors or ARB to reduce morbidity and mortality in patients with chronic heart failure with reduced ejection fraction and as replacement of an ACE inhibitor or ARB in

patients with chronic symptomatic NYHA class II or III heart failure with reduced ejection fraction to further reduce morbidity or mortality (Yancy et al. 2017).

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Atrial Natriuretic Factor/ B-Type Natriuretic Peptide

► Atrial and Brain Natriuretic Peptides

Atypical Antipsychotics

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Synonyms

Second-generation antipsychotics

Definition

The introduction of atypical antipsychotics (AAPs), since the discovery of its prototypical drug clozapine in the 1970s, has been a milestone in psychopharmacology for treating schizophrenia, bipolar disorder, and other brain diseases that are characterized by psychotic features, as these drugs allow a significant recovery not only in terms of hospitalization and reduction in symptoms severity, but also in terms of safety, socialization, and better rehabilitation in the society. Compared to typical antipsychotics (TAPs), AAPs produce minimal motor side effects, such as parkinsonism and tardive dyskinesia, and seem to be more effective in improving cognitive symptoms (Meltzer 2013). Unfortunately, AAPs may cause weight gain and metabolic adverse reactions; hence, patients should be constantly monitored. Besides, each AAP has a unique pharmacological profile, which explains the clinical diversity between them.

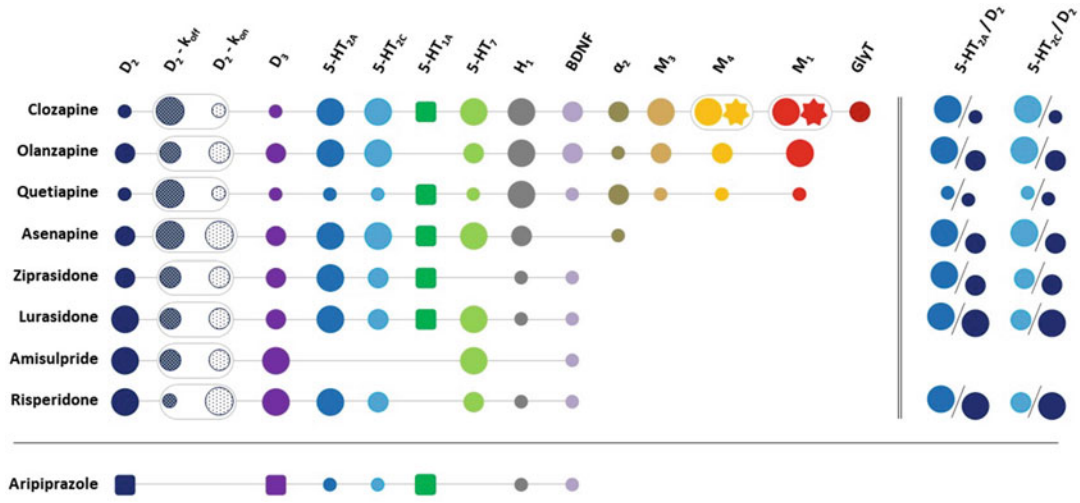
Mechanism of Action

AAPs are characterized by a wide array of receptor and molecular targets (Fig. 1).

Dopaminergic system and dopamine receptors. The dopaminergic hypothesis for schizophrenia is still the leading theory of the disease, and this is confirmed by the fact that the vast majority of APs are antagonists at D_2 dopamine receptors. However, if D_2 blockage is the only mechanism of action of TAPs, AAPs are weak D_2 antagonists, and they act beyond this target involving other receptors that can regulate the dopaminergic system. Regarding TAPs, it was shown that D_2 receptor occupancy between 65% and 80% seems optimal for clinical efficacy, while above 80% the occurrence of EPS increases. Conversely, D_2 occupancy is low for clozapine and quetiapine and never reaches the 80% threshold. In respect to clozapine, higher level of occupancy can be found for olanzapine and risperidone, particularly at higher doses, with the possible occurrence of EPS. New AAPs such as brexpiprazole and cariprazine act as D_2 receptor partial agonists, similar to aripiprazole.

In addition, AAPs have fast dissociation kinetics (K_{off}) from the D_2 receptor, and this is believed to contribute to their low propensity to EPS. Recently, it was also underlined the importance of the association constant (K_{on}) together with the K_{off} for determining EPS, while K_{off} seems predominant for inducing increase of prolactin secretion.

D_3 receptor is another important target of AAPs, and it is not easy to differentiate the effect of these drugs on D_2 versus D_3. Selective D_3 antagonists seem to enhance dopamine and acetylcholine secretion in the PFC, and this could



Atypical Antipsychotics, Fig. 1 Molecular targets of AAPs. List of the most relevant targets involved in the mechanism of action of AAPs based on receptor occupancy. Values are reported as high (●), medium (●), and low (●). ●, ■ and ✹ represent receptor antagonism, partial agonism, and positive allosterism, respectively. ◉ and ◉ represent K_{off} and K_{on} values for the D_2 receptor and ● represents BDNF production, while ✹ represents positive allosterism by the clozapine metabolite, norclozapine, at M_1 and M_4 receptors. Aripiprazole is shown at the bottom for its different mechanism of action. Finally, the 5-HT_{2A}/D_2 and 5-HT_{2C}/D_2 receptor affinity ratios are included on the right. Clozapine covers a wide range of molecular targets among all AAPs, while risperidone and amisulpride are mostly limited to just a few, and this might explain clozapine's superiority among AAPs

have a pro-cognitive effect (Aringhieri et al. 2018).

Regarding D_4 receptors, recently, Cardozo et al. (2017) employed an innovative in silico approach and demonstrated that the D_4 receptors in the pineal gland can be a unique target for clozapine.

Serotoninergic (5-HT) system and serotonin receptors. Serotonin, via its 15 receptors, is capable of having a profound impact on dopaminergic, glutamatergic, and GABAergic neurons and other neurotransmitters in the human brain. Besides the hypothesized 5-HT_{2A}/D_2 ratio-based mechanism of AAPs, other serotonin receptors have also been considered as potential targets of different AAPs. In fact, today it is clear that receptors such as 5-HT_{2C} and 5-HT_{1A} have an important role similar to that of 5-HT_{2A} in the mechanism of action of AAPs. In addition, other serotonin receptors such as 5-HT_6 and 5-HT_7 seem potential targets for old and new AAPs such as amisulpride and others. The general idea is that the dopamine efflux in the mesolimbic and mesocortical pathways is regulated by 5-HT_{2A} and 5-HT_{2C} receptors, mostly expressed in the glutamatergic and GABAergic cortical neurons. 5-HT_{2A} receptor antagonism seems relevant to reduce the dopamine release in the mesolimbic areas, while blockade of 5-HT_{2C} should increase dopamine efflux in the cortex. Besides improving negative symptoms of schizophrenia, 5-HT_{2C} antagonism might be responsible for some antidepressant activity, and this is shared with some atypical antidepressants like mirtazapine, trazodone and nefazodone.

In addition, clozapine, quetiapine, aripiprazole, and ziprasidone also act as 5-HT_{1A} partial agonists, and this in turn determines the increase in dopamine release in the PFC and hippocampus. Intriguingly, the modulation of the serotoninergic receptors alone is not sufficient to exert a full antipsychotic activity, while combined with other receptor targets (e.g., D_2 receptors), it finds its optimal therapeutic effect.

Glutamatergic system and glutamate receptors. The glutamatergic system has a prominent role in the pathogenesis of schizophrenia and other psychoses. In fact, in drug-resistant schizophrenic patients or in patients with very limited

response, abnormalities in the glutamatergic system may be particularly relevant and the use of NMDA antagonists in animals mimics some aspects of the human schizophrenic disease. Importantly, in non-responders, only clozapine seems to be effective, at least to a certain degree, while in few cases, olanzapine shows partial efficacy at higher doses. This evidence suggests that the superiority of clozapine is partially explained by its activity on the glutamatergic system. It is also true that the glutamatergic, dopaminergic, and serotoninergic systems are strongly interconnected, which further complicates the analysis of the mechanism of action of AAPs on the glutamatergic system. Noteworthy, the activity of serotonin 5-HT_{2A} and 5-HT_{1A} receptors seems to be relevant for controlling the glutamatergic system, and this may explain the superiority of AAPs over TAPs. In addition, effects on NMDA receptors and glycine transporters (GlyTs) that contribute to homeostasis in the glutamatergic system seem important for controlling glutamate hyperactivity, a feature which may be relevant particularly for clozapine's action. Interestingly, agonists at the glycine site or glycine transporter inhibitors, in monotherapy or in association with AAPs, have demonstrated some promising results in schizophrenic patients.

Cholinergic system and muscarinic receptors. Decreased M_1 receptor signaling has been linked to cognitive impairment associated with schizophrenia (CIAS), and, thus, enhancement of M_1 receptor signaling has been postulated to be a therapeutic target for CIAS. Clozapine was the first AAP reported to improve CIAS in schizophrenia. Notably, even though clozapine is an antagonist at M_1, M_3, and M_5 receptors, its principal metabolite norclozapine behaves as positive allosteric modulator of the M_1 receptor. Patients under clozapine treatment with high norclozapine/clozapine ratio showed higher improvement in memory tasks and reduced learning impairment. Notably, clozapine therapy usually lacks the traditional anticholinergic side effects like dry mouth, and it could instead promote sialorrhea.

A recent preclinical work suggests that the M_4 receptor also has an important role in cognitive function. Regarding AAPs, clozapine behaves as M_4 receptor antagonist in the rat striatum, while its metabolite norclozapine behaves as M_4 receptor agonist, an effect that can be relevant in the human neocortex. Olanzapine appears to be a weak partial agonist at the M_4 receptor, but in some experimental conditions, it behaves as an antagonist instead.

Histaminergic system and histamine receptors. Clozapine, olanzapine, quetiapine, and asenapine show high occupancy values for the H_1 receptors in human brain at minimum clinical doses. In fact, weight gain is one of the major side effects of these AAPs. Instead, risperidone and ziprasidone have low to medium affinity for this receptor, and this might explain the reduced weight gain in patients treated with them. H_1 receptor antagonism is also responsible for sedation, a side effect that may be helpful in acute psychoses, particularly in agitated patients. The H_3 receptor is mainly a presynaptic autoreceptor, and it acts as a presynaptic heteroreceptor on non-histaminergic neural systems (such as ACh, noradrenaline, dopamine, or glutamate). Some preclinical studies have highlighted the possible role of H_3 receptor antagonism in treating schizophrenia, particularly its role in cognition, which was confirmed in schizophrenic patients by a mild effect to improve cognition. Thus, AAPs acting on the H_3 auto- and heteroreceptor, such as clozapine, facilitate the release of histamine, noradrenaline, ACh, and serotonin, and these neurochemical changes might be responsible for increase in food intake and improved cognition.

Noradrenergic system and noradrenergic receptors. Many AAPs have an affinity for the noradrenergic α_1 and α_2 receptors as antagonists, and in spite of limited clinical evidence, a role for the noradrenergic system has been proposed in schizophrenia. The antagonism of many AAPs and in particular clozapine on α_1 receptor might contribute to the control of positive symptoms and modulate the correct firing of mesolimbic dopaminergic neurons. The concomitant block of α_1 and D_2 receptor may synergistically contribute to clinical efficacy. Clozapine and norquetiapine also act as α_2 receptor antagonists, and this can be important for their antidepressive characteristics. α_2 receptors modulate the firing of

noradrenergic and dopaminergic neurons, and a strong α_2 antagonism with a moderate D_2 antagonism could contribute to a good receptor profile for an ideal antipsychotic.

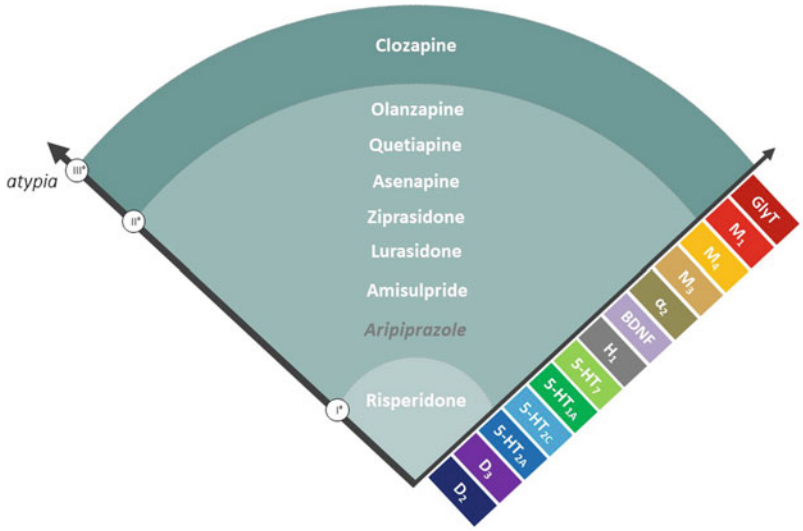
Neurotrophic factors, synaptogenesis, and neurogenesis. AAPs can increase neurotrophic factors production in many areas with relevant consequences in the long-term treatment of schizophrenia. Different studies reported an increase in BDNF, NGF, and FGF2 levels in preclinical models following the treatment with different AAPs, particularly with clozapine, olanzapine, risperidone, and lurasidone. However, olanzapine and risperidone lose this property at higher doses. As a consequence, AAPs seem to induce neurogenesis, synaptogenesis, and neuroplasticity, a peculiar characteristic that distinguish them from TAPs.

Biased agonism. The capacity of a ligand to preferentially activate either G protein-dependent signaling or G protein-independent signaling (e.g., β-arrestin pathway) is called "biased agonism" or "functional selectivity." This innovative new concept reflects the heterogeneity and complexity of the different receptor conformations in the active state depending from the stimulant (Kenakin 2013). In addition, recent data have demonstrated how receptor functional selectivity is a dynamic and adaptable process, which can also be modified by physiopathological conditions. Apparently, downstream D_2 receptor signaling, AAPs antagonize strongly β-arrestin pathway, while TAPs are strong antagonists on both G_i and β-arrestin pathways. This difference could be important even if the clinical implications are still unknown. In addition, some β-arrestin biased agonists derived from aripiprazole have been synthetized, and they have shown antipsychotic activity in vivo with fewer adverse events. Recently, it has been found that clozapine, which is classically considered an antagonist on 5-HT_{2A} receptor, can activate this receptor and promote ERK and Akt kinases phosphorylation via a G protein-independent pathway (Aringhieri et al. 2017). Olanzapine and quetiapine showed a similar effect but with less intensity.

Receptor dimers. There is a large amount of evidence that shows how GPCRs, apart from being monomers, form homo- and heterodimers. The action of AAPs on several receptor dimeric complexes has been demonstrated especially in vitro; however the confirmation in vivo in animal models or humans is still lacking. Among the many receptor dimers analyzed, the most convincing evidence refers to 5-HT_{2A}/$mGlu_2R$ heteromer in the somatosensory cortex in mice (Fribourg et al. 2011). In normal conditions, this receptor complex enhances 5-HT_{2A} activity toward G_i, while in animal models of schizophrenia, this complex switches mostly toward a G_q activity. In these pathological conditions, clozapine can restore the physiologic equilibrium in favor of G_i coupling. Importantly, in schizophrenic patients, it has been found a downregulation of $mGlu_2R$ and 5-HT_{2A} upregulation that may lead to an increase of a G_q signaling.

Clinical Use

AAPs are generally used in several mental disorders such as schizophrenia, bipolar disorder, obsessive-compulsive disorder, dementia with agitated patients, and autism spectrum disorder. Schizophrenic patients should be treated with AAPs in the acute phase and in the long-term maintenance. Bipolar mania as well benefits from the use of these drugs together with mood stabilizers. With the discovery of AAPs, great expectations raised on their claimed superiority in treating positive, negative, and mood symptoms of schizophrenia compared to TAPs. Several reviews on this topic and few meta-analysis have systematically compared TAPs and AAPs, and they have found data supporting AAPs superiority for certain aspects, where clozapine, olanzapine, risperidone, and amisulpride have resulted better than TAPs in terms of efficacy, quality of life, tolerability, dropout, and side effects (Huhn et al. 2019). However, the clinical differences are less evident when TAPs are used at lower doses, particularly in comparison with risperidone. For these reasons, it has been proposed a continuum spectrum of atypia that ranges from risperidone, the least atypical, to clozapine, the most atypical, while all other



Atypical Antipsychotics, Fig. 2 Continuum spectrum of atypia: the three levels of atypicality. Classification of AAPs in three categories, where risperidone is least atypical (Level I) and clozapine is most atypical (Level III), while all others fall within these two extremes of the spectrum (Level II). The molecular targets shown on the right add up, beginning with the D_2 and 5-HT_{2A} receptors that are common targets for all AAPs, extending to additional mechanisms such as M_1 positive allosterism and GlyT activity that seem specific to clozapine. Further targets, such as H_1 and α_2 receptors and BDNF, are relevant to both Level II and III of atypia. The clinical characteristics of each AAP are well explained according to their molecular profile on different targets

AAPs fall within these extremes of the spectrum (Fig. 2).

In the first episode of psychosis, generally AAPs and TAPs have similar efficacy. However, as it has been demonstrated in several clinical studies, in terms of relapse and treatment discontinuation, AAPs seem more successful since they show a better tolerability profile.

On negative symptoms (apathy, anhedonia, asociality) of schizophrenia, AAPs perform better than TAPs for two main reasons in relation to their different mechanism of action: (1) TAPs strongly antagonize D_2 receptors in the prefrontal cortex (PFC), and this may worsen these symptoms; (2) AAPs improve dopamine, serotonin, and acetylcholine transmission in the PFC with positive clinical consequences. On this aspect, differences within the AAP family are small, with clozapine being the most effective. In addition, the reduction of negative symptoms may be influenced in part by elimination of positive symptoms, making the comparison among APs not so obvious.

On cognitive symptoms, AAPs may obtain a mild improvement apparently in specific domains. Clozapine seems to improve verbal fluency, while olanzapine and quetiapine show some effect on attention and processing speed. The cholinomimetic properties of the clozapine metabolite norclozapine on M_1 receptor may contribute to its cognitive activities.

Apart from clinical differences, AAPs have a more favorable profile compared to TAPs in terms of severe adverse reactions (Table 1). In fact, regarding motor side effects, AAPs have a strong reduction of extrapyramidal symptoms (EPS). On this topic, within the AAP family, there are differences where clozapine practically never shows parkinsonism, while risperidone sometimes can have this side effect, especially at higher dose. As clozapine, quetiapine also never induces EPS, and for olanzapine this adverse event is rare. A second motor adverse reaction that concerns psychiatrists in the long-term use of APs is the occurrence of tardive dyskinesia (TD). The prevalence of TD in patients treated with APs is about 20% after 1 year, with a cumulative increase of 5% per year during drug exposure. The introduction of AAPs has determined a strong

reduction of TD; however, TD can still be caused by AAPs, and this possibility should not be underestimated. The incidence of TD is about two to ten times lower for AAPs compared to TAPs, and for this reason, the second class should be preferred for long-term treatment. Among the AAPs, clozapine has the least propensity to induce TD and may have beneficial effects on patients who develop this long-term motor adverse reaction. In fact, clozapine should be considered for patients who develop TD caused by the other APs (Table 2).

Despite a better profile in terms of motor side effects, AAPs cause weight gain and other metabolic adverse reactions (Table 1). Olanzapine and clozapine treatments are associated with the greatest risk of weight gain, whereas quetiapine, risperidone, and amisulpride show low-to-moderate levels of this undesired effect. Interestingly, the new molecules such as ziprasidone, lurasidone, and asenapine seem more tolerable on the metabolic profile. However, this advantage in terms of adverse effects has to be combined with their therapeutic efficacy, as these new drugs need more data to show similar efficacy compared to the other AAPs. The mechanisms of action related to weight gain are complex, and many receptors may be involved such as H_1, 5-HT_{2C}, and D_2 receptors, either expressed in the hypothalamus or in the peripheral tissues. Nutritional intervention and psychoeducational programs are a good tool to minimize this tedious side effect caused by AAPs. In addition, clozapine, and especially olanzapine, are associated with hyperglycemia and dyslipidemia; hence patients receiving these treatments should be cautiously monitored.

Atypical Antipsychotics, Table 1 Principal side effects among AAPs. Most relevant side effects recurring in patients undergoing AAPs therapy. Values are reported as high (+++), medium (++), low (+), and very rare (+/−)

Drug	EPS	Hyperprolactinemia	Weight gain	Risk of diabetes	Hyperlipidemia	Sedation	QTc prolongation
Clozapine	+/−	+/−	+++	+++	+++	+++	+
Olanzapine	+/−	+	+++	+++	+++	+++	+
Quetiapine	+/−	+/−	++	++	++	+++	+
Asenapine	+	+	+	+	+	++	++
Ziprasidone	+	+	+	+	+	++	++
Lurasidone	+	+	+	+	+	+	+/−
Amisulpride	+	++	+	+	+	+	++
Risperidone	++	+++	++	+	+	+	+
Aripiprazole	+	+	+	+	+	+	+/−

Atypical Antipsychotics, Table 2 Clozapine, the gold standard AAP: pros and cons

Pros	Cons
Efficacy in treatment-refractory schizophrenia	Agranulocytosis (0.7–1%)
Efficacy on negative and cognitive symptoms (improved verbal fluency)	Weight gain
Efficacy in psychoses associated with PD	Hyperglycemia, increase in triglycerides
Efficacy in patients who develop TD	Sialorrhea
Only FDA-approved AAP to lower suicide risk and to exert some antidepressant properties	Risk of epileptic seizure
Diminished aggressive behaviors	Risk of myocarditis
No EPS	Sedation
No TD	
No increase in serum prolactin	

These adverse reactions arise partly as a consequence of weight gain but also for AAP activity on different molecular targets present in different organs, such as pancreas, liver, and adipose tissue. Another possible adverse effect of AAPs, in particular for sertindole, is the alteration of the electrocardiogram, like QTc prolongation, requiring patients monitoring before and after AP introduction (Table 1).

Besides the overall effectiveness of APs in schizophrenia, about 30% of patients are pharmacoresistant, defined as an insufficient clinical response with at least two or more drugs. In this subset of individuals, clozapine is superior to all the other drugs and is considered the gold standard for treatment-refractory schizophrenia (Table 2). Clozapine superiority compared to the others is also evident for patients with violent behavior and with high suicide risk.

The benefits of clozapine are unfortunately outweighed by the risk of agranulocytosis, which is rare (0.7%), but potentially life-threatening, and patients should be monitored on white blood cell counts weekly for the first 6 months. For this reason, usually clozapine is not the first choice among AAPs, even if many authors have underlined how this drug is underused considering its clinical superiority in different medical conditions.

Cross-References

- ▶ Dopamine System
- ▶ G-Protein-Coupled Receptors
- ▶ Muscarinic Receptors
- ▶ Neurotrophic Factors
- ▶ Serotoninergic System

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Autoinflammation

- ▶ Interleukin-1 (IL-1)

Autophagy

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Synonyms

Macroautophagy

Definition

Cells are continuously recycling constituent materials. For this, they have available two degradative systems, the proteasome and the lysosome (Liebl and Hoppe 2016). Degradation through the ubiquitin-proteasome system (UPS) is mainly used for small and short-lived proteins, whose degradation in the 26S proteasome is highly selective. It is macroautophagy which is the high capacity degradative pathway, enabling a massive

catabolic switch in cell metabolism upon demand. Especially larger proteins and protein aggregates, as well as bulk "gulps" of cytoplasm, invading microorganisms and even whole cellular organelles such as dysfunctional mitochondria, go to the lysosome via macroautophagy after the formation of a double-membrane vesicle around the target or "cargo" (Yu et al. 2018). Autophagy can initiate large-scale degradation and can produce metabolic intermediates on a large scale (Mizushima and Komatsu 2011).

Common to both proteasomal and macroautophagic degradation is the process of ubiquitination which puts a tag on cargo, labeling it for disposal (Liebl and Hoppe 2016). The choice of appropriate cargos, small or large, random or selected, and their transport to lysosomes where they can be degraded, represents the domain of autophagy.

The broader term, autophagy (from the ancient Greek for "self-eating"), refers to three forms of vesicular transport ending with the incorporation of contents into the lysosome (Mizushima and Komatsu 2011): microautophagy, chaperone-mediated autophagy, and macroautophagy. With microautophagy, late endosomes invaginate and engulf small elements of the cytoplasm and then, by fusing their membranes, complete the enclosure; these vesicles then transport their contents to the lysosome. With chaperone-mediated autophagy, as is apparent from the name, cytoplasmic elements are selected by specific chaperones having affinity for their targets, which then are taken up into the lysosome for degradation (Mizushima and Komatsu 2011).

Macroautophagy, the main subject of this article, is the best investigated form of autophagy and, for simplicity, will be referred to hereafter just as autophagy. This major catabolic pathway exists in practically all cells, having been conserved in evolution back to the yeasts. Autophagy has essential roles in maintaining cell survival, in meeting nutritional needs, and responding to stresses, in selective degradation of particular macromolecules, but also for selective degradation of whole organelles and invading parasites. Peculiar to macroautophagy is the concentration of cargo on an isolation membrane which forms at modified regions of endoplasmic reticulum (ER). As indicated above, these cargos can be large. They can also be complex, containing proteins, lipids, and nucleic acids. Cargos can be very specifically selected, or they can be just neighboring cytosolic elements. The isolation membrane grows and closes around the cargos, forming the extended double-membrane autophagosome which then fuses with lysosomes, taking advantage of special proteins, called SNARES [soluble *N*-ethylmaleimide-sensitive factor attachment receptors (Yu et al. 2018)]. These bring the membranes into close proximity, thus promoting fusion events. In this way, a mature, actively degradative autolysosome is formed (Mizushima and Komatsu 2011; Yu et al. 2018).

Basic Characteristics

All forms of autophagy are essentially pathways leading to the lysosome. The lysosome must be seen not just a degradative organelle with numerous potential hydrolytic enzymes, but as a platform for the regulation of the metabolism required for cell survival, including the regulation of autophagy (Inpanathan and Botelho 2019). This means overseeing nutrient availability and supplying large amounts of amino acids which can be used as an energy source as well as for new protein synthesis. These same degradative processes allow for safe disposal of toxic cell materials such as misfolded proteins, for malfunctioning whole organelles, and for defense against microbial invasion. The garbage disposal role is essential; thus, basal autophagy at a low level is continuous in almost all cells (Mizushima and Komatsu 2011). Upregulation of autophagy in response to stresses leads to induction of genes required for increased autophagic and lysosomal activity. This induction is controlled by complex, multiple interacting networks acting mainly on two enzyme complexes, the mammalian target of rapamycin complex 1 (mTORC1) and the AMP-activated protein kinase (AMPK), both of which are located on the lysosome and are central for control of cell growth and proliferation (Inpanathan and Botelho 2019).

Autophagy Is Important

The Belgian scientist Christian de Duve was awarded the Nobel Prize in 1974, for the discovery of lysosomes and implicitly, the process of autophagy. An understanding of the molecular events in autophagy began to emerge through work of Ohsumi and colleagues, which led to his receiving the Nobel Prize finally in 2016. Working with yeast mutant screens, Ohsumi's laboratory was able to define numerous autophagy-related (*ATG*) genes encoding the so-called autophagy-related (ATG) proteins for which functions were gradually defined (Yang and Klionsky 2010). A pathway for the interactions of the different ATG proteins contributing to autophagy was worked out taking advantage of the unique opportunities provided by the yeast system. Mizushima, working in Ohsumi's laboratory, then proceeded to identify the mammalian autophagy genes encoding ATG5 and ATG12 and was able to demonstrate their conjugation to a complex (Yang and Klionsky 2010). This work was subsequently extended in a worldwide effort which now defines a collection of *Atg* genes numbering more than 35.

Serious diseases are associated with dysfunctional autophagy (Mizushima and Komatsu 2011). Mutations in autophagy-related genes are either causal or represent risk factors for development of numerous diseases. Prominent are the neurodegenerative diseases, for example, Parkinson's disease (van Beek et al. 2018), cancer (Byun et al. 2017), and diseases of innate immunity, leading to inadequate responses to infection (van Beek et al. 2018; Germic et al. 2019a), but also to autoimmune diseases such as Crohn's disease (van Beek et al. 2018). Natural processes such as ageing also exhibit a correlation with declining autophagic activity (Mizushima and Komatsu 2011). Thus, pharmacological targeting of autophagy may eventually lead to important therapeutic innovations.

Outline for a Canonical Autophagic Pathway

The stepwise events in the process of autophagy have been illustrated in excellent review articles (Yang and Klionsky 2010; Mizushima and Komatsu 2011; Simon et al. 2017; Yu et al. 2018). Thus, the pathway will be described here only briefly.

The most important, immediately proximal regulators of autophagy are two cellular regulatory enzyme complexes acting in opposition. These are mTORC1, a central regulator of cell growth, and AMPK, a kinase activated by declining energy charge, i.e., increasing AMP levels. Both AMPK and mTORC1 are serine/threonine protein kinases, and both are localized on the lysosome. The mTORC1 is bound to Ragulator in a complex with the Rag proteins, small GTPases serving jointly as sensors for amino acid levels, not just in the cytoplasm but also across the membrane in the lysosome. The mTORC1 is displaced from the lysosome and inactivated when stress conditions such as amino acid or glucose starvation, or hypoxia, mandate a shift to catabolism (Inpanathan and Botelho 2019). Under these conditions, AMPK may also be activated, phosphorylating ULK1 or ULK2 directly and initiating autophagy. Most of the more distal inducers of autophagy also act indirectly via mTORC1 or AMPK.

The initiation of autophagy represents a collaboration between two kinase complexes, the first a protein kinase and the second a class 3 phosphatidylinositol 3-kinase (PI3K). The protein kinase complex that initiates autophagy is made up of ULK1 or ULK2, ATG13, FIP200, and ATG101 (Galluzzi et al. 2017). Both the ULK proteins and ATG13, like mTORC1, are localized on the lysosomal surface, and both are maintained inactive owing to mTORC1-dependent phosphorylation (Inpanathan and Botelho 2019). As indicated above, declining proportions of ATP to AMP lead to an AMPK activation with subsequent ULK1 or ULK2 phosphorylation, overriding mTORC1 inhibition. Similarly, following any stress which leads to mTORC1 displacement and inactivation, the ULK protein kinase can activate by autophosphorylation, then phosphorylating ATG13, after which both translocate, and together with FIP200 and ATG101 assemble an autophagy initiating complex at special sites on the ER (Galluzzi et al. 2017; Yu et al. 2018). These sites are believed to be enriched for ATG9, the only ATG which is a membrane protein and is subject to

phosphorylation by the activated ULK protein. These will make up the phagophore, which is then elongated following continuing ATG9 and membrane enrichment, believed to be brought in by fusion with endosomes originating in the Golgi (Yu et al. 2018).

At this point, the second multiprotein kinase, the components of the class 3 phosphatidylinositol 3-kinase (PI3K) complex are recruited to the phagophore. This complex is composed of vps34 (the catalytic subunit), vps15 (the regulatory subunit), Beclin-1 (BECN1), ATG14, and the nuclear receptor-binding factor 2 (NRBF2). ULK kinases phosphorylate both BECN1 and ATG14, activating the local production of phosphatidylinositol 3-phosphate [PI3-P (Galluzzi et al. 2017; Simon et al. 2017)]. Important is the availability of BECN1, which exists in the cell in complexes, largely with BCL-2, an inhibitor of apoptosis. For PI3K function, BECN1 must be released from these complexes. This can be accomplished by phosphorylation with, among other kinases, the activated ULK kinase, thus freeing both partners to act, respectively, stimulating autophagy and potentially inhibiting apoptosis (Simon et al. 2017).

Further phagophore development requires recruitment of several additional ATG proteins which constitute two ubiquitin-like conjugation systems: the first producing complexes of ATG5-ATG12-ATG16L1 which localize at the phagophore; the other conjugation takes place with ATG8, which after its proteolytic processing by ATG4, is then conjugated to phosphatidylethanolamine (PE). There exist at least six mammalian orthologues of yeast ATG8, which owing their conjugation to PE, can localize at the phagophore membrane. Prominent among these is microtubule-associated protein 1 light chain 3 beta (LC3B), that after its lipidation is designated LC3-II in consequence of its altered electrophoretic mobility (Mizushima and Komatsu 2011; Galluzzi et al. 2017).

Initially, the phagophore represents two extended, neighboring protrusions of the ER, cargo must now be recognized and concentrated within the arms of these protrusions as they extend and enlarge.

As with a 26S proteasome, cargo recognition for autophagy depends on the presence of ubiquitin chains tagged onto targets. Ubiquitin, a 76-residue, highly conserved protein, can be conjugated to proteins using one of its seven lysines or N-terminal methionine (Liebl and Hoppe 2016). A large selection of more than 600 different E3 ubiquitin ligases provides a very high versatility as well as specificity in this ubiquitin conjugation process. Interestingly, almost all proteins, protein aggregates or organelles must be conjugated to ubiquitin at its Lys63 in order to undergo autophagy, whereas "for proteasomal degradation, all non-Lys63 ubiquitin linkages were found to facilitate proteasomal degradation in vivo" (Liebl and Hoppe 2016).

How are targets which have been conjugated with Lys63-polyubiquitin chains recognized? The prototype autophagy receptor or adaptor is the multifunctional sequestosome 1 protein (SQSTM1), more commonly designated simply p62. P62 binds to Lys63-polyubiquitin chains with its ubiquitin-binding (UBA) domain, and, by virtue of an LC3 interacting region (LIR domain), is able to bind lipidated LC3 on the membrane of the phagophore. In this way, the localization of cargos is achieved prior to membrane closure and the formation of the autophagosome. Just as there exist several LC3 orthologues [e.g., LC3 or γ-amino butyric acid receptor-associated proteins (GABARAP), etc.], there exist also several functionally analogous proteins which like p62 can serve to bridge Lys63-polyubiquitinated cargos to LC3 at the phagophore membrane [(examples, p62, NDP52, or optineurin) (Galluzzi et al. 2017)].

The choice of targets for autophagy is accomplished in a process of selective ubiquitination which is possible owing to the versatility of the huge number of selective E3 ubiquitin ligases. Appropriate levels of certain individual cellular regulatory proteins such as transcription factors and enzymes are maintained with the help of autophagy (Jiao et al. 2018; Flynn et al. 2019; Sánchez-Martin and Komatsu 2019). Recognition of and selective autophagic degradation of larger structures such as organelles [e.g., *mitophagy* (removal of mitochondria) or *pexophagy*

(targeting peroxisomes)], or *aggrephagy (*aimed at degradation of protein aggregates) or *lipophagy* (needed for lipid homeostasis)] are described in a comprehensive review (Galluzzi et al. 2017). The various processes are generally analogous to the canonical pathway described above, with additional highly specialized features associated with the recognition of dysfunctional organelles or threats like intracellular parasites.

Networks Regulating Autophagy

There are networks regulating autophagy activation in response to low ATP or amino acid levels, loss of growth factor receptor activation, hypoxia, oxidative stress, or DNA damage. These, through various intermediate signaling steps as indicated in Fig. 1, activate autophagy mainly through AMPK or mTORC1 (Inpanathan and Botelho 2019). Figure 1 illustrates these networks in a simplified form as well as others responding to DNA damage and furthermore indicates some of the overlaps. It is important to realize that the same regulatory enzymes can be activated by signals coming from many directions, many contradictory when they finally come to mTORC1 or AMPK.

Significantly increased demands on autophagy and lysosomal degradation also require increased gene expression in the nucleus to produce the proteins needed in autophagosome formation and lysosomal expansion. Thus, as shown in Fig. 1, several transcription factors may be activated; examples are tumor suppressor p53, involved in responding to DNA damage, or TFEB (transcription factor EB) regulating expression of many lysosomal proteins, or transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) which is important for the response to oxidative stress (Sánchez-Martin and Komatsu 2019). Another interesting example is transcription factor p73 which regulates the transcription of ATG5 at times when the whole organism is subjected to starvation. Livers of animals deficient in p73, but starved, show huge numbers of accumulated cytoplasmic lipid droplets, presumably owing to the blocked triglyceride hydrolysis into the fatty acids which would be needed to mobilize energy (He et al. 2013).

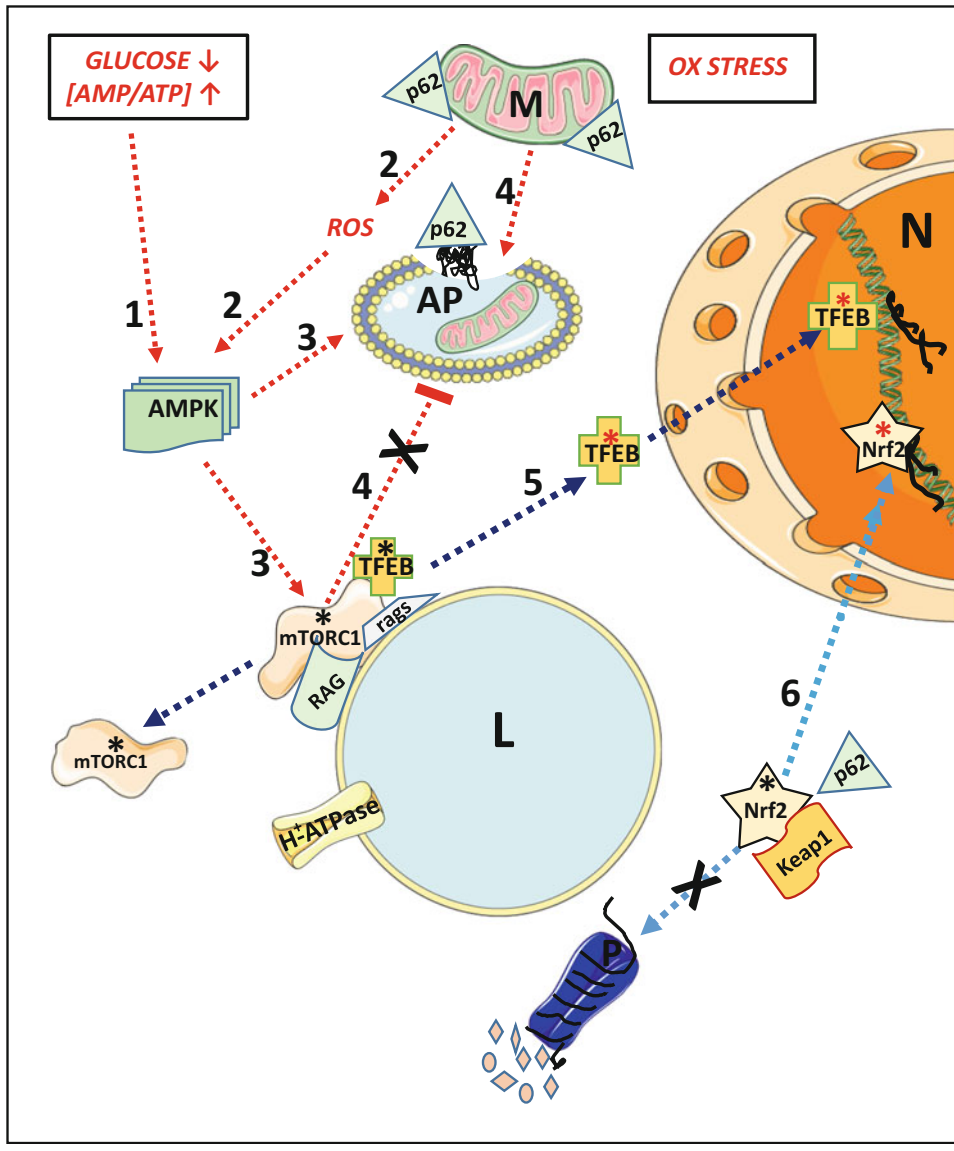
Drugs

Autophagy Activators Versus Inhibitors: Which and When, a Quandary

The regulation of autophagy is complicated, as can be seen from the examples presented in Fig. 1. While a pharmacological intervention with autophagy is clearly possible, our present knowledge is insufficient to predict all the potential side effects. Even so, empirical trials with different drugs are in progress. What are the predictable risks?

***Neurodegenerative diseases and ageing*:** These conditions seem to have in common an inadequate autophagy leading to the accumulation of misfolded proteins, protein aggregates, and damaged mitochondria. Apparently, these also slow the flow along the narrow axons of neurons. The accumulation of protein aggregates is toxic and dysfunctional mitochondria produce insupportable levels of reactive oxygen species (ROS); in consequence neurons die. Thus, one hopes that an additional activation of autophagy might be able to alleviate the progression of diseases like Parkinson or Alzheimer, or even, in principle, ageing. Experimental efforts to increase autophagic activity seem appropriate and in fact trials have been performed with impressive initial results (Byun et al. 2017; Moors et al. 2017; Thellung et al. 2019). Some of the relevant drugs and their targets are summarized in Tables 1 and 2 below. Because of the central roles which most of the targets play in the regulatory processes of the cell and because the drugs are still at an early stage of development, lacking the desired specificity, drugs (Tables 1 and 2) need to be evaluated empirically, under the closest clinical scrutiny. The greatest risk with all of these drugs is obviously the impact of such drugs on different organ systems.

***Cancer*:** Dysfunctional autophagy favors genomic instability, allowing the accumulation of p62 together with toxic protein aggregates and damaged mitochondria producing high ROS levels. Thus, a deficient autophagic response contributes to an accumulation of mutations, some of which are likely to be oncogenic. A widely held opinion is that a capability for an effective



Autophagy, Fig. 1 Pathways leading to the activation of autophagy are illustrated as simplified activation networks numbered from one to six. On the left side metabolic stresses like starvation, i.e., low glucose and a rising AMP/ATP quotient, are indicated. On the right are depicted the consequences of oxidative stress (OX) arising from excessive levels of reactive oxygen species (*ROS*) produced by a damaged mitochondrion (M). Both stresses lead to an activation of autophagy. A developing autophagosome (AP) is shown at midline, containing a dysfunctional mitochondrion and in the process of taking up protein aggregates (black squiggles) together with the SQSTM1 (p62) ubiquitin adaptor. P62 is shown acting as an adaptor at several points in this illustration. The numbered arrows indicate the several different networks activating autophagy. (**1** and **2**) show responses to starvation and oxidative stresses, both typically via activation of AMPK. The kinase activity of AMPK phosphorylates mTORC1, causing its inactivation and displacement from its binding to Ragulator on the lysosome (L), but in (**3**) AMPK also directly phosphorylates sites on ULK1 and 2, thereby initiating the first steps in the formation of an autophagosome. The black asterisk on mTORC1 designates an inactive kinase, ending its role in many anabolic functions together with its inhibitory role (**4**) on ULK1 and 2. The transcription factor TFEB, which is maintained inactive by phosphorylation via mTORC1, then translocates to the nucleus (**5**) and initiates transcription of several ATG genes and genes encoding lysosomal proteins. The activation of another transcription factor

autophagic response plays an early protective role, preventing tumor initiation (Byun et al. 2017; Moors et al. 2017). Moreover, in tumor progression, tumor cells showing deficient autophagy are common and probably very relevant for the course of the disease. The role of autophagy in cancer progression seems to be context dependent. While an active autophagy clearly can contribute positively to mobilization of the metabolic intermediates needed for rapid tumor cell growth (Byun et al. 2017; Moors et al. 2017), it is selective autophagy which is normally responsible for regulating levels of enzymes like phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 and hexokinase 2, which, if they accumulate because of deficient autophagy, provide a huge impetus to glycolysis, possibly explaining the famous "Warburg effect" (Jiao et al. 2018; Flynn et al. 2019). That would suggest that loss of functional autophagy could spur tumor progression. In fact, it is possible sensitize tumors to chemotherapy if at the same time, autophagy is inhibited. On the other hand, there are potential effects of autophagy on cell differentiation which could channel populations of tumor cells into an end-differentiated or dormant state. Thus, one must surmise that with different tumors, disease progression can potentially benefit either way, from active autophagy or from a deficiency of autophagy, presumably in a tumor type-dependent fashion. What will be needed in the future is a personalized molecular analysis of individual tumors in order to decide which, if any interventions in autophagy offer promise. Hence, for innovative cancer therapies with autophagy drugs, right now a real quandary exists, and concerns about risk are self-evident.

***Diseases of dysfunctional immunity*:** Autophagy is central for immunity for several reasons. Neutrophils, important as a first line of defense against microbial infection, depend on autophagy and certain ATG proteins for essential neutrophil functions including differentiation, phagocytosis, cytokine production, degranulation, and cell death (Germic et al. 2019a). Moreover, in some little understood way, autophagy is also able to limit inflammatory responses and to a degree to protect normal tissues (Byun et al. 2017; Germic et al. 2019b).

Some intracellular parasites are able to develop strategies to overcome phagocytic killing, achieving a release from phagocytic vacuoles into the cytoplasm. Recognition of such free, invading microbes by pathogen-recognition receptors such as Toll-like receptors or NOD-like receptors induces autophagy, stimulates their inclusion in autophagosomes, and facilitates finally their degradation in lysosomes (Byun et al. 2017).

Moreover, autophagy is involved in development, survival, and differentiation of T lymphocytes (Byun et al. 2017) and as a degradative pathway, autophagy also plays a significant role in antigen processing and presentation on MHC class II molecules (Byun et al. 2017; Germic et al. 2019b). This multiplicity of immune functions which show a dependence on the process pf autophagy suggests that autophagy modulation with inflammatory or autoimmune diseases must present therapeutic opportunities. Here again, the potential risks are self-evident.

Perspective

It is evident from Fig. 1 and Tables 1 and 2 that numerous potential drug targets suitable for modulating autophagy exist. In fact, many autophagy-inhibiting (Pasquier 2016) or activating drugs (Byun et al. 2017; Moors et al. 2017; Thellung et al. 2019) are known and have been subjects of investigation for years. Because most of these targets belong to networks that are central in

Autophagy, Fig. 1 (continued) (Nrf2) is illustrated in (**6**). Nrf2 is normally bound to the ubiquitin ligase Keap1, which targets it to the proteasome (P), thus maintaining Nrf2 levels very low. SQSTM1 (p62) binding to Keap1 targets its degradation via uptake into an autophagosome, freeing Nrf2 to translocate to the nucleus where it stimulates transcription of numerous antioxidative genes and, interestingly, also the SQSTM1 (p62) gene itself. This figure was prepared using several Servier Medical Art Templates, which are licensed under a Creative Commons Attribution 3.0 Unported License; https://smart.servier.com

Autophagy, Table 1 Some drugs inhibiting autophagic activity[a]

Drug	Network	Target	Specificity
3-Methyladenine	PI3K class 3 and autophagy	Vps34	Inhibits Vps34 and autophagy, but also PI3K class 1, hence indirectly mTORC1
Wortmannin	PI3K class 3 and autophagy	Vps34	Pan-PI3K inhibitor, inhibits autophagy, but also other kinases including mTORC1
LY294002	PI3K class 3 and autophagy	Vps34	Synthetic inhibitor for Vps34 and autophagy; inhibits also PI3K class 1 and mTORC1
Compound 31	PI3K class 3 and autophagy	Vps34	Optimized inhibitor for Vps34 and autophagy for oral application. Specific
Pepstatin A	Lysosomal proteases	Lysosomal cathepsins D, E, and BHL	Protease and autophagy inhibitor, blocking lysosomal degradation of cargos. Specific
Bafilomycin A1	Lysosomal acidification and hydrolase activation in the lysosome	Vacuolar lysosomal H^+-ATPase	Inhibits autophagy by preventing lysosomal acidification, hence lysosomal hydrolase activities. V-ATPases are present in endosomes and secretory vesicles. Unspecific
Chloroquine and hydroxychloroquine	Lysosomal acidification and hydrolase activation	Lysosomes	Lysosomotropic drugs inhibiting lysosomal acidification and autophagy. Relatively specific

[a]Modified from Pasquier (2016)

Autophagy, Table 2 Some drugs activating autophagic activity[a]

Drug	Previous applications in the clinic	Target	Mechanism
Rapamycin, tacrolimus, etc.	Antifungal, immunosuppressive, anticancer	*Raptor*	Prevents raptor assembly in mTORC1, hence inhibiting the mTOR kinase and allowing autophagy initiation
Torin	Anticancer	*Blockade of mTOR kinase ATP-binding site*	Blocks the ATP binding site of mTOR, hence allowing autophagy initiation
Lithium	Mood stabilizer	*Inositol mono-phosphatase inhibitor*; causes inositol depletion	Blocks inositol mono-phosphatase, reducing levels of free inositol, for example, needed for synthesis of phosphatidylinositol 3,4,5 trisphosphate
Valproic acid	Antiepileptic, mood stabilizer	*Inositol mono-phosphatase inhibitor*; causes inositol depletion	Acts like lithium; blocks inositol mono-phosphatase, limiting free inositol levels, for example, needed for phosphatidylinositol 3,4,5 trisphosphate synthesis
Metformin	Antidiabetic	*AMPK activation*, hence, UNK 1 and 2 activation	Activated AMPK phosphorylates ULK1 and 2, resulting in autophagy initiation and mTOR inactivation, blocking its anabolic kinase activity
Resveratrol	Antioxidant	*AMPK activation*, hence, UNK 1 and 2 activation	Activates AMPK (after targeting SIRT1) like metformin. It also activates transcription factors responsible for ATGs and antioxidant proteins
Verapamil	Anti-arrhythmic and antihypertensive	*Ca^{2+} channel blocker*; also calpain inhibitor	Protects cells from oxidative damage by inducing Keap1 degradation and activating transcription factor Nrf2 to transcribe antioxidant proteins. See Fig. 1

[a]Modified from Thellung et al. (2019)

regulating cellular homeostasis, the main concern at this early stage of clinical study and drug development for autophagy modulators is the lack of specificity.

As a fundamental housekeeping and survival program, autophagy modulation promises huge benefits for the management of several different diseases. What will be needed for therapeutic applications, however, will be drugs showing a fine, incremental impact allowing a favorable intervention in a specific setting, but without too many negative systemic side effects. As with practically all applications in pharmacology, future efforts will have to focus on the refinements in drug structure which can provide the increased specificity needed for treatment of different pathologies.

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Bacterial Resistance to Antibiotics

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Introduction

Antibiotics have revolutionized the therapy of infectious diseases nearly a century ago. For the first time with penicillin, a selective therapy targeting the bacterial cell synthesis was available. However, concomitantly with the detection of the antibacterial activity of penicillin, Sir Alexander Fleming reported on the phenomenon of bacterial resistance to penicillin. Since then, several strategies have been applied to overcome this problem and conserve antibiotic activity for future therapeutic options:

Different strategies for the development of new antibiotics

- By isolation from natural sources antibiotics belonging to different molecular classes (e.g., aminoglycosides, tetracyclines, cephalosporins)
- By chemical modification known antibiotics (e.g., aminopenicillins, doxycycline, amikacin)
- By total chemical synthesis of novel drugs (e.g., sulfonamides, fluoroquinolones)
- By a combination of antibiotics with inhibitors of known resistance enzymes (e.g., amoxicillin with the β-lactamase inhibitor clavulanic acid)

Although all approaches have proven to be successful, this was only for a short time period due to the ability of bacterial cells to rapidly adapt to novel drugs by the development of potent mechanisms of resistance. Intensive research on the underlying molecular mechanisms of antibiotic resistance have revealed several contributing factors: These include the following:

Bacterial factors contributing to antibiotic resistance

1. A haploid genome, so that a genetic alteration immediately results in a new phenotype
2. High numbers of pathogen cells either due to a high replication rate or to successful escape from the immune response, thereby increasing the probability for the development of resistant mutants which can be selected in the presence of antibiotics
3. The existence of efficient mechanisms of horizontal gene transfer allowing for simultaneous spread even of multiple resistance determinants encoded by extrachromosomal elements.

Human economic and ecologic factors contributing to antibiotic resistance

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4. A continuing decrease of the number of novel antibiotics developed during the last two decades which restricts the therapeutic options and simultaneously favors the selection and spread of resistant mutants.
5. Inappropriate use of antibiotics in agriculture and livestock breeding as well as insufficient treatment of wastewater from hospitals or antibiotic manufactures resulting in a wide disposal of antibacterial compounds in the environment. Though the resulting antibiotic concentrations are in the sub-inhibitory range, this condition induces stress responses in bacteria resulting in the acquisition of genetic alterations associated with resistance in existing DNA or by horizontal transfer of DNA (Fig. 1).

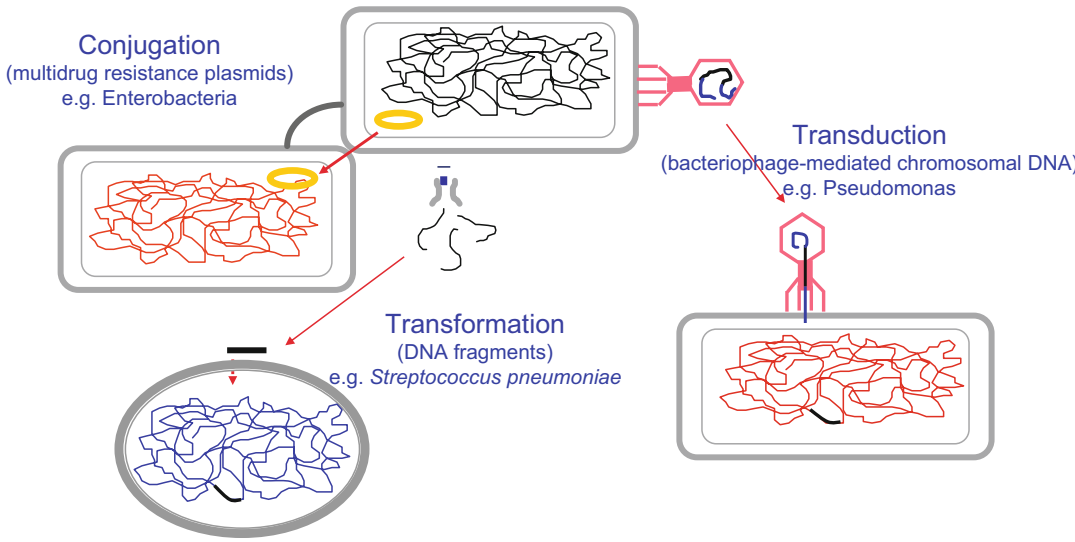
Antibiotic Resistance: Definitions

Biological Resistance

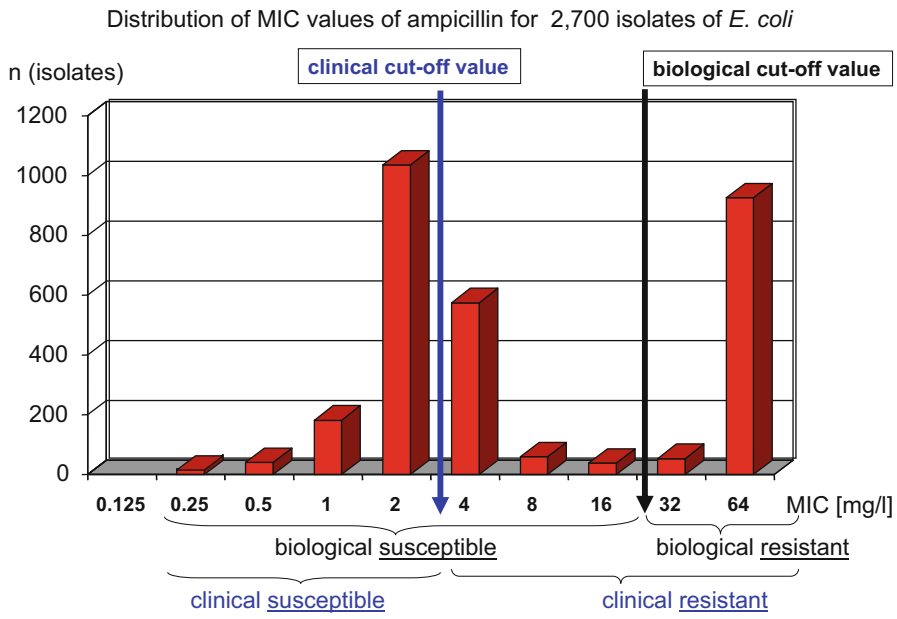
From the biological point of view, antibiotic resistance is the relative reduction of the susceptibility to a specific antibiotic either of all strains of a species compared to the strains of another species (primary or natural biological resistance) or of a bacterial strain compared to other strains of the same species (secondary or acquired biological resistance). For the identification of the respective type of biological resistance, the antibiotic susceptibilities of a set of different strains of a species are determined, and each strain is assigned to a category of antibiotic susceptibility (e.g., given as drug concentration in μg/ml capable of inhibiting the growth of these strains: minimal inhibitory concentration, MIC). The resulting diagram (Fig. 2) typically yields a bimodal distribution of the susceptibilities obtained for strains of a single species. The main peak at the lower drug concentration represents the majority of strains and defines the naturally susceptible population, while the minor peak at higher drug concentrations corresponds to strains expressing an acquired mechanism of resistance (secondary/acquired resistance).

Clinical Resistance

From the clinically point of view, the drug concentrations capable of inhibiting bacterial isolates from patients are compared to the drug concentrations typically achievable at the site of infection under conditions of a standard dose and normal metabolic activity of the patient. Depending upon



Bacterial Resistance to Antibiotics, Fig. 1 Mechanisms of horizontal transfer of antibiotic resistance genes



Bacterial Resistance to Antibiotics, Fig. 2 Definition of biological and clinical resistance

the type and location of infection, the type of pathogen, and its metabolic activity, the drug concentration obtained in vivo is required to exceed the inhibitory drug concentration for the given pathogen by a factor of 10 for a specific time period. This pathogen is said to be clinically susceptible, while pathogens showing lower susceptibilities are clinically resistant. Since this rule of thumb is not valid for any type of infection, several additional factors have to be considered for choosing the right antibiotic in the most effective dose for a specific patient. Nevertheless, one crucial factor is the degree of antibiotic susceptibility of the causative pathogen. Antibiotic susceptibility can easily be determined by one of two principle test procedures: agar diffusion and broth dilution.

Methods for the Determination of Antibiotic Resistance In Vitro

To determine the susceptibility of a pathogen for a specific antibiotic the first step is to isolate bacteria from the site of infection in pure culture and identify the species of the suspected causative pathogen. Typically a characteristic fingerprint of the molecular masses of different ribosomal proteins using matrix-assisted laser desorption ionization time-of flight (MALDI-TOF) mass spectrometric analysis is determined. Depending upon the species of the respective pathogen, the knowledge of its primary, i.e., natural resistance to some antibiotics, allows to exclude these drugs as therapeutic options.

The next step is the determination of the antibiotic susceptibility. The underlying principle is to expose a defined number of bacterial cells (inoculum) to various concentrations of antibiotics and incubate these mixtures under defined conditions (time, temperature, growth medium). For the *broth dilution test*, one volume of inoculated broth is added to one volume each of stepwise 1:2 dilutions of the antibiotic. After a defined incubation time, the minimal inhibitory concentration (MIC in μg/ml) of the respective antibiotic is determined as the lowest drug concentration showing no visible growth.

For the second method, the *agar diffusion test*, a defined number of cells (inoculum) is evenly

spread over an agar plate before paper discs containing defined amounts of different antibiotics are evenly distributed on the surface of the agar. During the incubation time, the antibiotics diffuse from the disc into the agar generating concentric gradients of decreasing antibiotic concentrations. According to the respective drug susceptibility, bacteria grow around the disc until they encounter the inhibitory concentration forming an inhibition zone (Fig. 4). The exact inhibitory concentration in μg/ml can be determined by using a linear regression obtained from a correlation of known reference concentrations with the corresponding zone diameter in mm. Based on these reference correlations, the zone diameter can be used directly for identifying susceptible and resistant pathogens (Tenover 2010).

Mechanisms of Antibiotic Resistance: Variations of a Theme

For an antibiotic to act, it has to interact with a bacterial cell and bind to an essential target structure, e.g., a protein, a nucleic acid, or a cell wall or membrane component. The immediate result of this interaction is the impairment of the target's activity or function. However, this primary effect usually triggers subsequent reactions which ultimately result either in the reversible inhibition of cell growth (bacteriostatic activity) or in an irreversible reaction leading to cell death (bactericidal activity). Many secondary bactericidal effects have been demonstrated to be associated with the induction of stress responses, e.g., formation of reactive oxygen species (ROS) which directly destroy essential cellular structures (DNA, RNA, proteins, membrane lipids) or induction of DNA damage response (SOS response) introducing mutations.

Genetic Basis of the Transfer of Antibiotic Resistance

The acquisition of antibiotic resistance results in a genetically stable phenotype. This is in contrast to the typically transient alteration of the phenotype of bacteria in biofilms which also show a reduced susceptibility to antibiotics. This phenotype, however, is due to the metabolic state of a subpopulation of cells in cell colonies, which do not carry a stable genetic alteration.

The genetic alterations associated with acquired resistance include either *mutations* which alter the existing genetic information or *uptake of additional genetic material (e.g., as transposable element or plasmid) by one of* three basic mechanisms (Fig. 1) (Courvalin 2008):

- *Transformation*, i.e., the uptake of naked DNA either as autonomously replicating extrachromosomal plasmid or as linear DNA fragment which is integrated into the existing chromosome via homologous recombination
- *Transduction*, i.e., the transfer of genetic material by bacteriophages which incorporate fragments of chromosomal DNA from a host cell to a newly infected cell followed by integration of the piece of foreign DNA into the chromosome of this cell via homologous recombination
- *Conjugation*, i.e., the direct transfer of DNA such as extrachromosomal plasmid DNA from a donor cell to a recipient cell mediated by a cell-to-cell contact via so-called sex or fertility (F) pili.

Molecular Basis of Antibiotic Resistance Mechanisms

These genetic alterations result in at least one out of three different principle types of antibiotic resistance mechanism:

1. Alteration of the target structure which subsequently reduce the affinity for the antibiotic
2. Reduction of the concentration of the antibiotic at the target
3. Inactivation of the antibiotic

The current problems with higher numbers of antibiotic resistant bacteria are exacerbated by:

- The *increasing variability of these three basic mechanisms* affecting even newest antibiotics
- The *combination of different genetic determinants* of resistance on extrachromosomal genetic elements (plasmids)

– The *wide distribution of such multi-drug resistant (MDR) plasmids* by transfer even between bacteria belonging to a different species

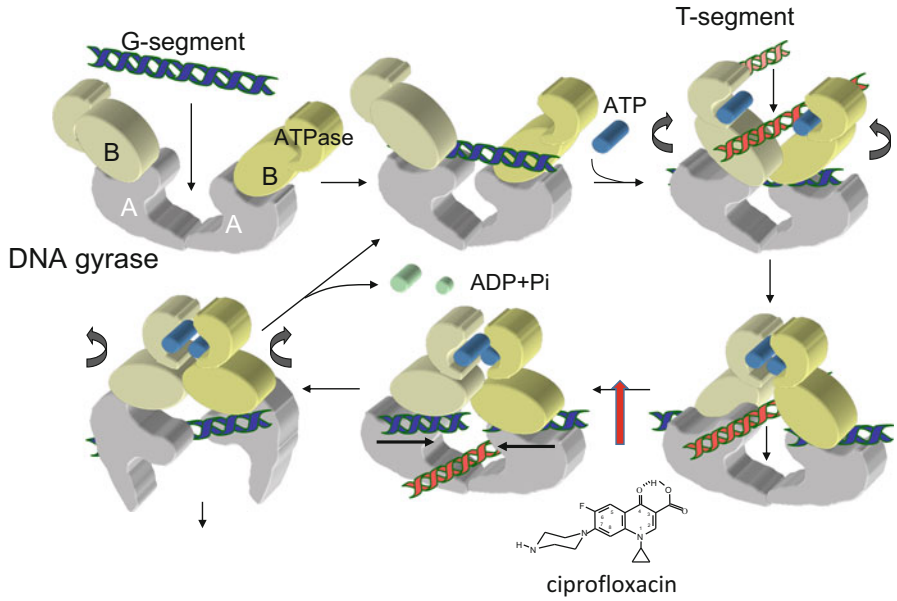
Alteration of the Target Structure

Bacterial targets of antibiotics typically are partial structural regions of a nucleic acid (e.g., ribosomal RNA), a protein (e.g., RpoB subunit of RNA polymerase holoenzyme), or a membrane component (e.g., anionic regions of phospholipids in the cytoplasmic membrane) which are essential for the cellular metabolism. Binding of an antibiotic to its target structure(s) affects the activity/function of the target resulting in reversible growth inhibition or even – irreversible – cell death. The binding site of a target typically is a three-dimensional pattern of functional chemical groups which covalently or non-covalently interact with complementary functional groups of the antibiotic molecule. Since the altered target is an essential compound of the cellular metabolism, only some specific alterations which retain the natural target function are tolerable for a cell. However, frequently, such alteration affects the fitness of the resulting mutant. Such mutant cells often acquire one or more additional mutations either in the respective target gene or in a different gene to compensate for this fitness deficit. Bacteria have evolved several mechanisms to alter the target susceptibility:

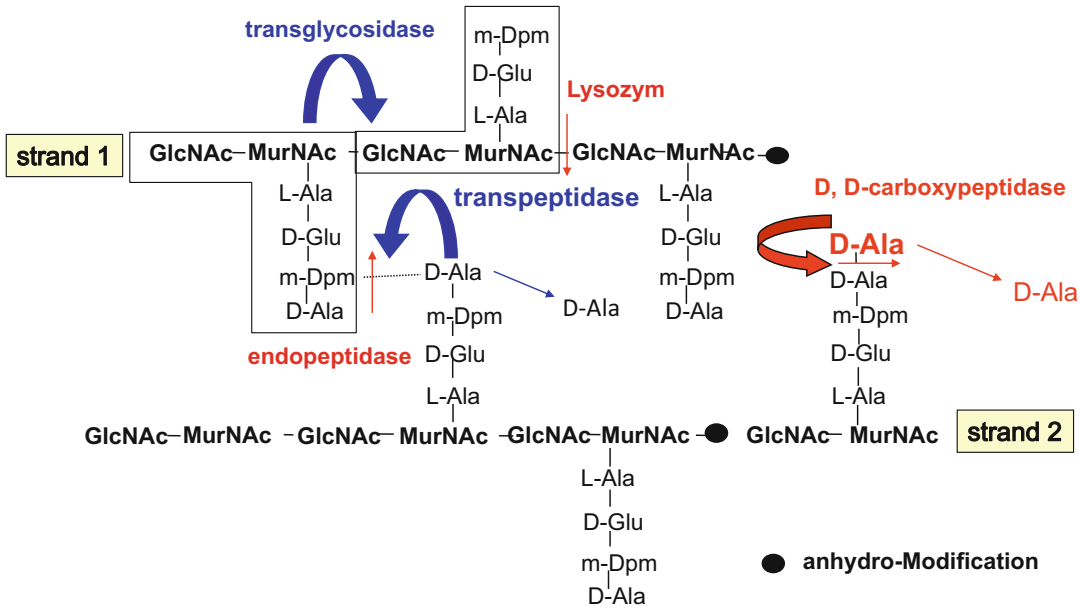
– Mutation resulting in amino acid substitution.
– Production of an alternate homologous target with adequate activity, but with reduced susceptibility for the antibiotic.
– Enzymatic modification of, e.g., the protein or RNA target. Thus, introduction of a single functional group (methyl, phosphate) interferes with the interaction between a functional group of the antibiotic and its corresponding binding partner (amino acid side chain, nucleotide) of the target.
– Overproduction of an pseudo-target which outcompetes the antibiotic for the target binding site.
– Overproduction of a protecting protein shielding the drug binding site of the target.

Target Alteration by a Mutation The easiest way to impair drug binding and thereby increase the inhibitory concentration of the antibiotic is to eliminate at least one of the functional groups at the binding site of a target. This can occur by mutational exchange of a respective binding partner, i.e., a nucleotide or an amino acid of the respective target structure. A prominent example is fluoroquinolone resistance which is primarily due to the acquisition of mutations in a conserved region of the *gyrA* gene encoding subunit A of the fluoroquinolone target DNA gyrase. Beside the homologous topoisomerase IV, DNA gyrase is an A_2B_2 tetrameric type II topoisomerase, which together with topoisomerase I (*topA* gene product) controls the global negative supercoiling degree of DNA in bacterial cells. This assures the continuous progress of replication and transcription. At the molecular level, gyrase acts by transiently introducing a DNA double-strand break with both GyrA subunits covalently bound to the free 5′-phosphate ends of the broken DNA (Fig. 3). The molecular mechanism of fluoroquinolone action is the stabilization of this reaction intermediate resulting in a blockage of DNA replication. A single point mutation in a highly conserved α-helical region (part of the so-called quinolone resistance-determining region, QRDR) affecting either a serine (aa 83 in *E.coli*) or a nearby aspartate (aa 87 in *E. coli*) residue reduces the binding affinity of the drug by a factor of about 20, a combination of both residues even by a factor of more than 2000 (Heisig et al. 1993).

Target Alteration by Producing an Alternate Homologous Target An alternative strategy to achieve antibiotic resistance by target alteration is to (over)express a functionally homologous but resistant target structure, as is seen in methicillin-resistant *Staphylococcus aureus* (MRSA) isolates. Methicillin belongs to the important group of β-lactam antibiotics, which act by imitating an intermediate of the natural reaction of transglycosidase/transpeptidase enzymes (an essential representative of the so-called penicillin binding proteins, PBPs) with the cell wall precursor disaccharide-pentapeptide. This essential PBP

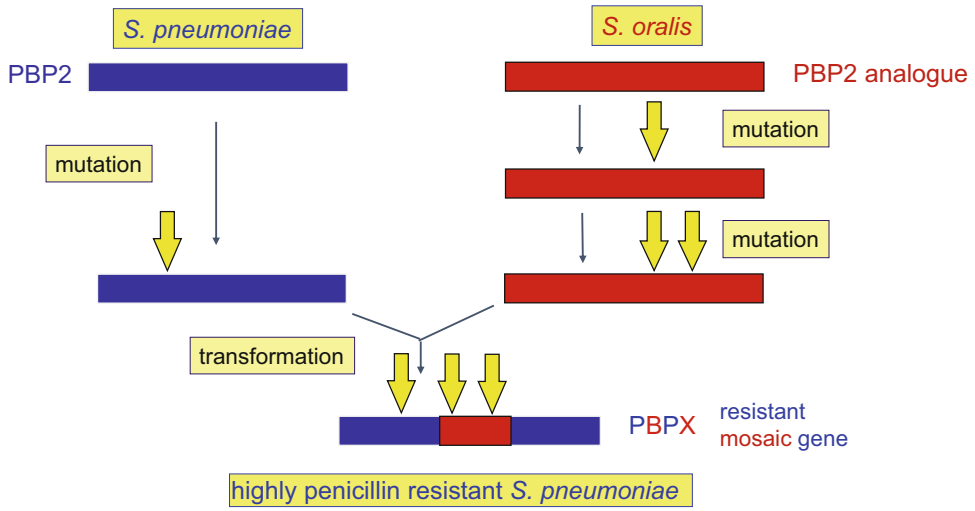


Bacterial Resistance to Antibiotics, Fig. 3 Steps of DNA gyrase-mediated DNA supercoiling and mechanism of quinolone action



Bacterial Resistance to Antibiotics, Fig. 4 Enzymes involved in regulation of peptidoglycan biosynthesis

catalyzes the cross-linking of growing glycan strands of the peptidoglycan layer in the bacterial cell wall by forming isopeptide bonds between two neighboring pentapeptide side chains. The different reaction steps involve (I) binding of the transpeptidase to its natural substrate, the terminal D-alanyl-D-alanine bond of the disaccharide-pentapeptide, (II) cleavage of this D-ala-D-ala peptide



Bacterial Resistance to Antibiotics, Fig. 5 Evolution of penicillin-resistant penicillin binding proteins in *Streptococcus pneumoniae*

bond by formation of a transient reaction intermediate between the transpeptidase covalently bound to the penultimate D-alanine, and (III) cross-linking of the carboxy-terminus of this D-alanine residue via an isopeptide bond to a basic amino acid (e.g., L-lysine) in an adjunct pentapeptide by transpeptidase (Fig. 4). The β-lactam core structure of a respective antibiotic mimics the structure of D-alanine covalently bound to the active site serine residue of the transpeptidase. However, the resulting β-lactam-enzyme-complex is extremely stable and permanently blocks cell wall synthesis. MRSA isolates have captured a gene complex presumably from another *Staphylococcus* species encoding a naturally β-lactam-resistant variant of a PBP, the *mecA* gene product, which can substitute for the activities of all *S. aureus* PBPs required for cell wall synthesis (Johnson et al. 2013).

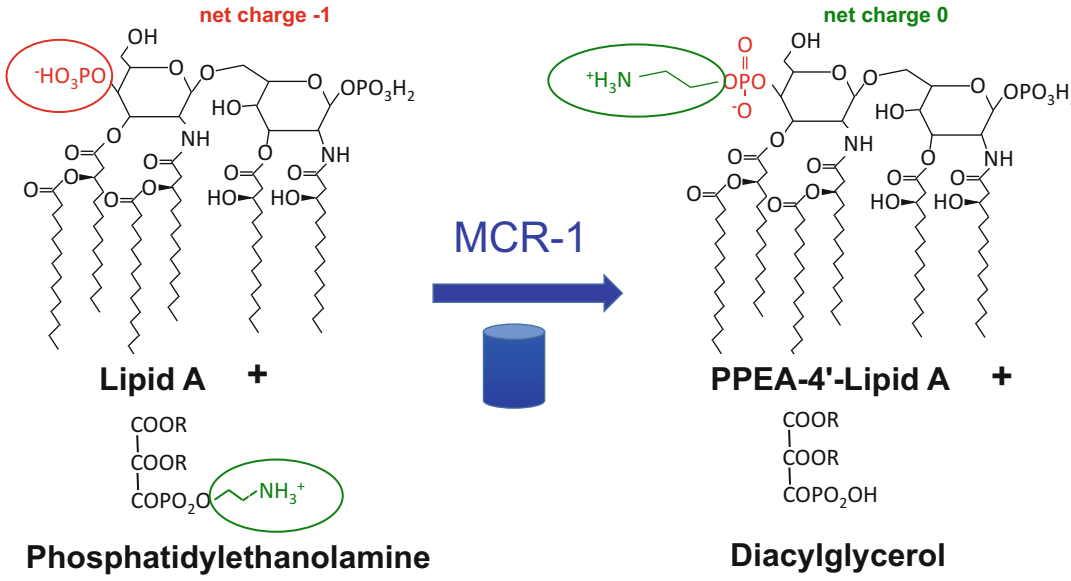
Isolates of *Streptococcus pneumoniae* share stretches of homology in genes encoding PBPs with many other streptococcal species of the oral microbiota. But these PBPs are naturally less susceptible to β-lactam inhibition due to genetic variations in the penicillin-binding regions of the PBP-encoding genes. Due to their natural competence, i.e., the intrinsic ability to take up linear DNA fragments from dead cells in the immediate environment, pathogenic *S. pneumoniae* strains in the upper respiratory tract can capture foreign gene fragments encoding less susceptible PBPs from oral streptococci. Following the uptake of such DNA fragments by *S. pneumoniae* regions of high homology in the PBP, gene sequences shared with naturally less susceptible oral streptococci can be exchanged by homologous recombination with the chromosome of *S. pneumoniae*. By this strategy, a set of resistance-associated mutations are transferred en bloc and give rise to novel PBP variants designated as PBPX (Fig. 5) (Sibold et al. 1994).

Target Alteration by Enzymatic Modification The enzymatic modification of structural elements of the target participating in drug binding often affects all antibiotics of one class. As a consequence, the number of functional groups available for drug binding is significantly reduced or even eliminated. Thus, a higher drug concentration is required for effective antibiotic activity.

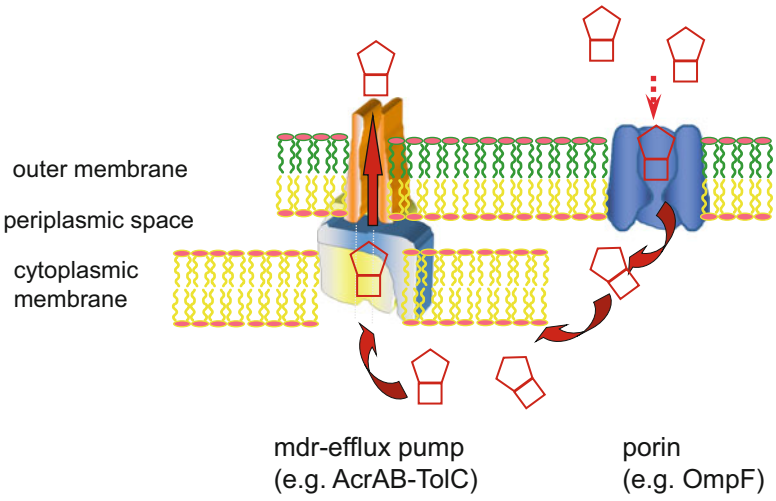
An example of a target modification reaction by a single enzyme is the resistance to all aminoglycoside antibiotics by the *armA* gene product. ArmA methylates a specific ribonucleotide in the aminoglycoside binding region. This

region is created by combining two three-dimensional folds of the 16 S RNA. After methylation the clustering of the two-folded regions is impaired resulting in a significant reduction of the aminoglycoside binding (Galimand et al. 2003). The *armA* gene and its homologues (*rmt*) are encoded on different plasmids, which can be transferred by conjugation or transformation between different bacterial cells even belonging to different species. In contrast, the most widely distributed mechanism of resistance to aminoglycoside antibiotics is due to the production of enzymes which very specifically modify a functional group of some, but not all aminoglycosides (see below).

Another type of enzymatic target modification is found for colistin, a derivative belonging to the cationic polymyxin group of antibiotics. The positively charged functional groups of colistin tightly bind to negatively charged functional groups of the lipopolysaccharide (LPS) in the outer membrane, e.g., phosphate moieties. Consequently, the LPS structure is disturbed allowing access of colistin to the cytoplasmic membrane which also carries anionic phosphate residues for colistin binding. Destruction of the cytoplasmic membrane is the final result leading to generation of reactive oxygen species and subsequently to cell death. To become resistant to colistin, Gram-negative bacteria can overproduce enzymes which are able to transfer building blocks containing positively charged functional groups (e.g., phosphoethanolamine or 4-amino-arabinose) to the phosphate residues of lipids. As a consequence, the negative charges of LPS, i.e., the binding sites of colistin, are eliminated either by conversion into positive charges or by neutralization. Many bacteria encode respective transferase enzymes, which are regulated to allow for a moderate transient LPS modification in response to changing environmental conditions. Exposing cells to colistin induces upregulation of genes encoding LPS-modifying enzymes by inducing two-component regulatory elements. The most widely distributed LPS-modifying enzymes are phosphoethanolamine transferases (e.g., plasmid-encoded MCR-1; see Fig. 6) and 4-amino-arabinosyl-transferases. Exposing cells for a longer period of time to higher colistin concentrations can select for genetically stable colistin-resistant mutants. Such mutants carry mutations in regulatory genes controlling the respective



Bacterial Resistance to Antibiotics, Fig. 6 Molecular mechanism of colistin resistance by enzymatic lipopolysaccharide modification



Bacterial Resistance to Antibiotics, Fig. 7 Antibiotic resistance by reduced influx combined with increased efflux of antibiotics

transferases on different levels (Wright et al. 2015).

Reduced Access of the Antibiotic to the Target Structure

For many classes of antibiotics, the respective target structure is located in the cytoplasm; however, for some others the target is associated with the peptidoglycan layer or even the outer membrane. Thus, for interacting with their target, most antibiotics have to pass the cytoplasmic membrane and the peptidoglycan layer. For Gram-negative bacteria additionally, the outer membrane is an effective barrier. Its unique structure with the inner layer resembling the phospholipid structure of the cytoplasmic membrane and the outer layer consisting mainly of lipopolysaccharides (LPS) makes the outer membrane nearly impermeable for hydrophilic antibiotics with a molecular weight above ca. 700 Da. Nevertheless, the controlled permeation of small hydrophilic molecules such as nutrients (influx) and some toxic compounds through the outer membrane (efflux) is essential for a living bacterial cell to adapt to changing environmental conditions. For these transport processes, different routes are accessible, and some participate in the uptake and export of antibiotics. For adapting to different environmental conditions, cells can alter the expression of transport-associated membrane proteins: (1) small hydrophilic molecules (e.g., many nutrients) passively diffuse through hydrophilic transmembrane pores in the outer membrane, called porins, and (2) energy-driven transmembrane efflux pump proteins incorporated in the cytoplasmic membrane to export hydrophilic toxic compounds, e.g., antibiotics (Fig. 7). Some efflux pumps act alone by shedding toxic molecules into the periplasm or as tripartite complexes with the pump docked to a specific outer-membrane protein (OMP) via a periplasmic membrane-fusion (MF) protein. Such a complex forms a hydrophilic channel through both membranes in Gram-negatives. Thus, to reduce the susceptibility for antibiotics, cells can use two complementary strategies which affect drug permeability of the cytoplasmic and/or outer membrane:

- Reduced influx
- Increased efflux

The underlying genetic mechanisms regulating these processes include inducible, i.e., reversible responses by transiently increased expression of efflux-associated and/or reduced expression of influx-mediating membrane components. This strategy enables the cell to transiently adapt to changing environmental conditions. Without genetic changes, bacteria can survive in the presence of high concentrations of toxic compounds (e.g., antibiotics). Mechanisms of induction often

include influx of a small amount of a key molecule (antibiotic) through one of the few porins produced at a low basal level. Inside the cell, these key molecules bind to a repressor controlling genes, e.g., for an efflux pump or for a negative regulator of porin gene expression. As a consequence, efflux pump expression is increased and/or porin protein expression is reduced resulting in a significant reduction of the intracellular antibiotic concentration (Fig. 7). Stable mutants inactivating global regulators showing this phenotype can easily be selected. The presence in most bacteria of more than one such efflux pump with partially overlapping substrate specificities reduces the impact of such alterations on antibiotic resistance. In contrast, in the absence of the respective antibiotic, the permanent inactivation of an efflux pump may reduce the adaptability of a mutant and cause a disadvantage for the cell in competition with wild-type cells.

Additionally, the broad substrate specificities of many efflux pumps mediate a multidrug resistance (MDR) phenotype. Consequently, the selection of efflux pump-associated mutants by one antibiotic frequently causes multiple resistance also to other structurally unrelated antibiotic substrates of the same MDR efflux pump.

Other strategies are:

- The overproduction of a target-protecting protein which shields the binding site sterically, thereby impairing the access of a drug to its target
- The production of a protein which can substitute for the activity of the original susceptible target protein due to its lower binding affinity for an antibiotic

Reduced Access to the Target due to Reduced Permeability of the Outer Membrane Two factors have an impact on the permeability of the outer membrane barrier function: (I) the number of outer membrane porins and (II) the specificity, number, and activity of efflux pumps. Mutations affecting either factor typically do not alter the structure but rather affect the amount of the respective membrane protein. In many Gram-negative pathogens, expression of major porins (e.g., OmpF, OmpC in *E. coli*) underlies several different regulatory circuits in response to varying environmental factors, such as temperature or osmolarity of the growth medium. Expression of major efflux pumps (e.g., the tripartite MDR efflux pump AcrAB-TolC in *E. coli*) often is regulated negatively by a local repressor (e.g., AcrR). In addition, at a higher level, the expression of efflux pump is controlled by the *marRAB* global regulatory system with MarR repressing the MarA gene activator. Any mutation inactivating the *marR* gene causes *marA* gene overexpression which in turn results in overexpression of both *acrAB* and tolC as well as the *micF* antisense RNA. *micF* functions as repressor of porin *ompF* gene expression by binding via complementary base pairing to its 5′ end. This block of the ribosomal binding site (rbs) prevents translation of the *ompF* mRNA. A *marR* mutation results in multiple drug resistance (MDR) to antibiotics of different classes (e.g., fluoroquinolones, chloramphenicol, and tetracyclines). The molecular basis for the broad substrate specificity is the existence of several only partially overlapping binding sites for the different substrates.

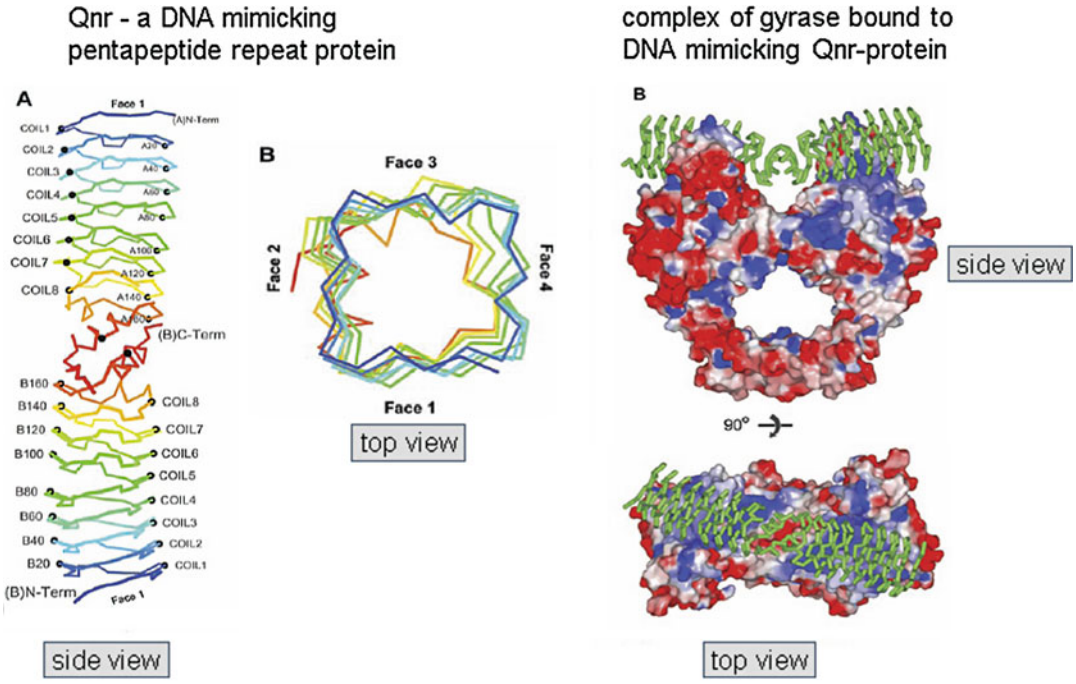
Expression of MDR efflux pumps is also a frequent cause for natural (i.e., primary) resistance of many Gram-negative pathogens (e.g., *P. aeruginosa*, *A. baumannii*) to different antibiotics. This has experimentally been demonstrated for mutants of *E. coli* lacking a functional AcrAB efflux pump: the MIC of vancomycin is reduced from 128 μg/ml to 1 μg/ml.

An important specific mechanism of resistance due to reduced intracellular concentration is tetracycline resistance by overexpression of a tetracycline-specific (Tet) efflux pump. Several different variants of Tet efflux pumps have been identified in both, Gram-positive and Gram-negative bacteria. The respective *tet* genes are typically encoded on plasmids and, therefore, are easily transferable between cells. The expression of some *tet* genes is inducible: after binding of a tetracycline molecule to a specific binding site of a TetR repressor, the conformational change results in a strong reduction of TetR binding affinity to DNA. In contrast to the moderate effect of overexpression of an MDR efflux pump, the induction of a Tet-specific

efflux pump significantly decreases tetracycline susceptibility. Tigecycline, a glycylcycline derived from tetracycline by introducing a glycyl-side chain to minocycyline, is refractory to Tet efflux pump-mediated resistance and also shows a strongly reduced binding affinity to TetR. As a consequence no relevant cross-resistance between tetracyclines and glycylcyclines is observed.

Reduced Access to the Target due to Production of a Target Protecting Protein Beside tetracycline-specific efflux pumps, another major mechanism of tetracycline resistance, which in contrast also affects glycylcycline susceptibility, is the expression of ribosomal protection proteins. These proteins show a high affinity to the ribosomal A-site. This site is the binding site of the aminoacyl-tRNA during the elongation reaction of protein biosynthesis and simultaneously the molecular target of tetracyclines. The sterical block by a ribosomal protection protein does not affect the vital ribosomal function. Ribosomal protection is found both in Gram-positive and Gram-negative bacteria.

Another mechanism of target protection has been identified as a very specific transferable mechanism of reduced susceptibility to fluoroquinolones. So-called quinolone resistance (Qnr) proteins contain a repetitive pentapeptide-repeat structure and fold into a DNA-like structure. These Qnr proteins imitate the natural reaction substrate DNA and compete with it for binding to the fluoroquinolone target topoisomerases, gyrase, and topoisomerase IV (Fig. 8) (Vetting et al. 2005). Unlike DNA, Qnr proteins cannot be cleaved by these enzyme. Instead, they bind to these enzymes in competition with DNA. This reduces the number of catalytically active topoisomerase-DNA complexes and thus



From Hegde, S.S. et al., (2005): A fluoroquinolone resistance protein from Mycobacterium tuberculosis that mimics DNA. Science 308:1480. Reprint with permission from AAAS

Bacterial Resistance to Antibiotics, Fig. 8 Quinolone resistance due to protection of DNA gyrase by a DNA mimicking Qnr protein

prevents the conversion of the complex to the true fluoroquinolone target, i.e., the topoisomerase covalently bound to DNA via two phosphodiester bonds (see above CCC). Since the Qnr-topoisomerase complex is non-covalent, the binding is reversible, and the number of complexes depends upon the amount of Qnr in the cell. Due to the essential function of DNA-topoisomerases for DNA metabolism (i.e., replication, transcription), it is obvious that not all enzymes can be protected by Qnr without killing the cell. Instead, few topoisomerase-DNA complexes are required to be active. Thus, fluoroquinolone resistance due to Qnr is only moderate, and the clinical importance of this mechanism alone is low.

Reduced Access to the Target due to Production of a Target-Substituting Protein with Reduced Binding Affinity for Antibiotics Sulfonamides are synthetic antibacterials and were the first antibiotics clinically used. Their target is the bacterial dihydropteroate synthetase (DHPS) required for the biosynthesis of the cofactor tetrahydrofolic (THF) acid. In essential biosynthetic pathways including biosynthesis of nucleotides and amino acids, THF is involved in the incorporation of C1-building blocks. Thus, inhibition of the THF biosynthesis finally causes cell death. The molecular structure of sulfonamides shares high homology to para-aminobenzoic acid (p-ABA) and, thus, can easily enter bacterial cells without being exported. Also single mutations altering the THF binding site of the susceptible DHPS target without affecting that of the natural substrate p-aminobenzoic acid are not found. However, in some bacterial species, variants of the DHPS target protein have evolved which show natural resistance to sulfonamides due to a set of variations in the sulfonamide/p-ABA binding region. Three such genes (*sul*-1, *sul*-2, and *sul*-3) encoding sulfonamide-resistant DHPS enzymes have been mobilized in ancient times by transposable genetic elements (integrons) from the chromosome to conjugative broad-host-range plasmids. These are easily transferable between different bacterial species and, thus, have spread from environmental bacteria to clinical isolates. During short-term evolution, the integron structures found in plasmids from clinical isolates have been expanded by acquisition of several additional antibiotic resistance genes to several different antibiotics, including e.g., different β-lactams, aminoglycosides, and tetracyclines) (Yun et al. 2012).

Enzymatic Inactivation of the Antibiotic

With the exception of sulfonamides and fluoroquinolones, which are purely synthetic drugs, antibiotics are of natural biosynthetic origin which are used unmodified or after partial chemical derivatization. The large group of β-lactam antibiotics originate from moulds (e.g., *Penicillium* spp., *Cephalosporium* spp.); most other biogenic antibiotics are produced by bacteria, presumably as a strategy to protect the producing cells in the microenvironment from other bacteria as competitors for nutrients. Consequently, for the antibiotic-producing bacterial cells, adequate mechanisms are required to self-protect them against the action of their own antibacterials. In bacteria production of secondary metabolites, such as antibiotics typically, is initiated in the late log phase. Simultaneously, expression not only of antibiotic biosynthesis genes but also of antibiotic resistance genes underlies a growth phase-dependent regulation in order to minimize potential impacts on the global fitness.

Among the three basic mechanisms of antibiotic resistance, mutational alteration of the target does not underlie a cell cycle-regulated expression, while upregulation of efflux pumps is (see above). A third basic mechanism of resistance – the expression of genes for antibiotic-modifying enzymes – is very efficient and can be regulated according to the cell cycle. Even a complete shutdown of the expression is not unusual in growth phases during which the enzyme is not required for the cell.

Enzymatic modification of antibiotics is a specific mechanism that has been found to affect several classes of antibiotics, especially of natural origin. Depending upon the antibiotic class, two types of enzymatic reaction mechanisms are known:

– Hydrolysis of a key chemical bond of an antibiotic (e.g., β-lactamase)
– Modification of a functional chemical group (e.g., O- or N-acetyltransferase)

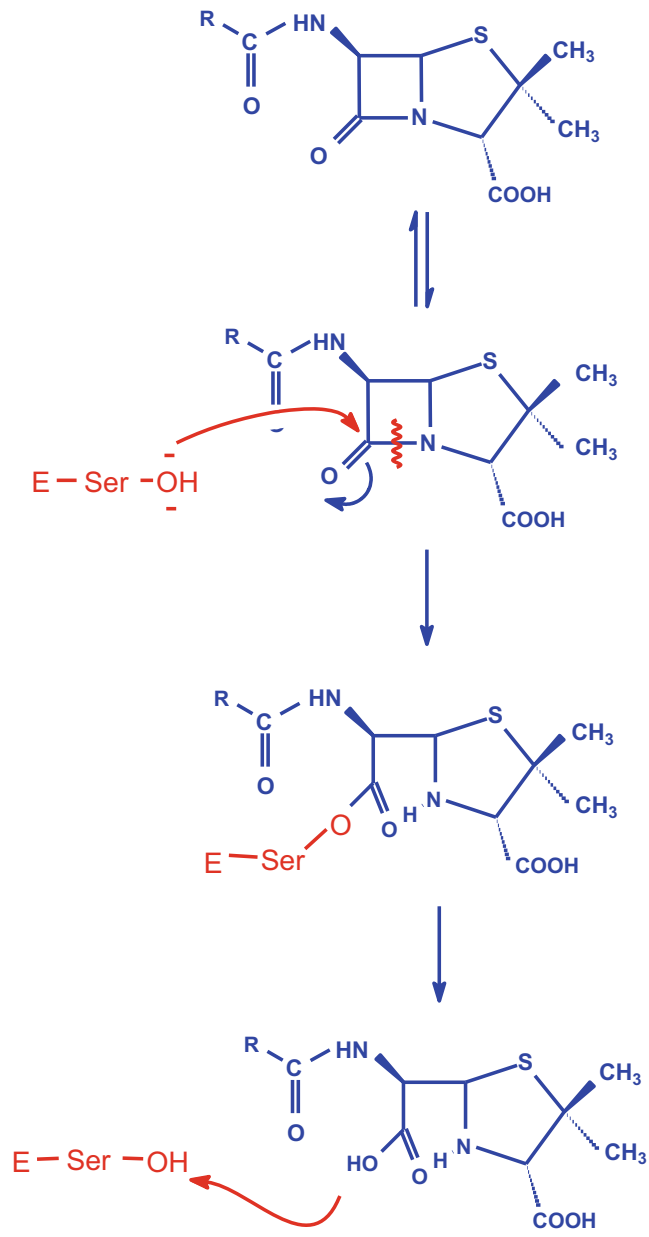
Resistance by Enzymatic Hydrolysis of β-Lactam Antibiotics The key element mediating antibiotic activity of β-lactam antibiotics is the four-membered β-lactam ring containing a peptide bond, which, however, is formed during non-ribosomal biosynthesis of a tripeptide (aminoadipyl-cysteinyl-D-valine, ACV). β-Lactam antibiotics imitate a reaction intermediate of the D-alanyl-D-alanine moiety of disaccharide-pentapeptide cell wall precursors as the natural substrate of cell wall-synthesizing transpeptidase/transglycosidase enzymes (see above). These so-called penicillin binding proteins (PBP) can react with either substrate, D-alanyl-D-alanine, and β-lactam and thereby introduce a covalent bond. However, while the natural reaction intermediate, i.e., a covalent complex of PBP and D-ala, is only transiently formed followed by a rapid release of a regenerated active PBP and a cross-linked disaccharide tetrapeptide in the growing cell wall once the peptide bond is cleaved, the PBP-β-lactam-complex is stable and blocks the essential PBP activity. Thus, a very effective strategy mediating β-lactam resistance in bacteria is to form and secrete β-lactamase enzymes. These very specific peptidases compete with the PBPs for cleaving the peptide bond in the β-lactam ring, thereby forming a covalent complex with the antibiotic molecule. However, a covalent complex of β-lactam with a PBP is highly stable, while that of the antibiotic with β-lactamase is rapidly resolved by deacylation releasing an inactive, i.e., hydrolyzed antibiotic (Fig. 9). Based upon their molecular mechanism of proteolytic cleavage, two different types of β-lactamases can be discriminated: (1) serine-type and (2) metallo-type β-lactamases. According to amino-acid sequence homology and substrate spectrum, serine-type β-lactamases are further subdivided (classes A, C, and D), while all metalloenzymes belong to class B (Fig. 10) (Bush and Jacoby 2010).

The serine-type enzymes share a common core structure of three sandwiched α/β/α domains which are linked by loops. The active site serine (Ser-70 in enzymes of the class A enzyme families TEM, SHV, and CTX-M) is part of one α helix and is located between this α-helical and the β-sheet domain. The important difference between both serine proteases – β-lactamases and PBP – is the presence in β-lactamases of a large flexible omega loop and the presence of a highly conserved glutamic acid residue (Glu-166). These two features reduce the affinity for peptidoglycan while favoring the binding to and hydrolysis of a β-lactam antibiotic by the active-site serine. Moreover, the Glu-166 supported by some other amino acid residues of the omega loop catalyzes the rapid deacylation of the β-lactamase enzyme. Due to the fact that only a small part of the enzyme molecule is involved in the formation of the active site and in β-lactam hydrolysis, the substrate binding pocket shows some flexibility which has favored the evolution of novel enzyme variants by acquisition of a few point mutations. The resulting so-called extended spectrum β-lactamases (ESBL) typically belong to class A and are able to hydrolyze also most novel β-lactams, with the exception of carbapenems. These antibiotics contain a β-lactam core structure as part of a penem moiety which is refractory to hydrolysis by many β-lactamases. Consequently, this results in a superior antibiotic activity. However, the metallo-β-lactamases belonging to class B contain a more flexible active site with a central Zn^{2+}-ion complexed by four histidine residues. These enzymes have the broadest spectrum of activity which also includes carbapenems and most other β-lactam antibiotics (Philippon et al. 2016).

Beside the production of novel, better adapted β-lactamase enzymes, two other relevant mechanisms can contribute to higher levels of β-lactam resistance against a broader spectrum of drugs in β-lactamase-producing bacteria: (1) overproduction of non-ESBL enzymes and (2) loss of outer membrane proteins (OMPs).

(1) ESBL-β-lactamases typically are encoded on small multicopy plasmids which are often

Bacterial Resistance to Antibiotics,
Fig. 9 Simplified scheme of the β-lactam hydrolysis reaction by a serine β-lactamase



transferable to other cells by conjugation. In contrast, non-ESBL-type β-lactamases (class C) have a less flexible structure of the binding pocket and often are encoded by a single gene copy on the chromosome. However, to increase β-lactam resistance, the production of non-ESBL enzymes can be induced by cell wall fragments which are increasingly released as a consequence of β-lactam action. In the absence of β-lactams, these fragments are imported into the cytoplasm for recycling. However, under β-lactam action, the recycling machinery is overloaded. As a consequence, the concentration of intermediates of the recycling reaction is increased resulting in the overexpression of a chromosomally encoded β-lactamase gene (*ampC*) as a stress response. In *Enterobacter* and *Citrobacter*

functional class	molecular class	enzymatic activity	genetic localisation distribution	examples β-lactamases
1	C	Cephalosporinase	Chromosom, Gram-negative	AmpC
2a	A,D	Penicillinase	*S.aureus, P.aeruginosa*	BlaZ
2b	A	"broad spectrum" TEM-1,-2; SHV-1	Plasmid, Gram-negative	TEM-1 SHV-1
2be	A	"extended spectrum"	Plasmid, Gram-negative Chromosom, K.oxytoca	TEM-3 bis SHV-2 bis 12 CTX-M1 bis
2br	A	"inhibitor resistant"	Plasmid, Gram-negative	TEM-30 bis 41 SHV-10
2c	A	Carbenicillinase PSE; CARB	Plasmid, Gram-negative	CARB-1
2d	D	Cloxacillinase	Plasmid, Gram-negative	OXA-1 bis
2e	A	Cephalosporinase	Chromosom, P.vulgaris	CepA
2f	A	Carbapenemase, ceph-, pen-ase	Chromosom, E.cloacae S.marcescens	SME-1, MNC-A, KPC-1
3	B	Metalloenzym	Chromosom, B.cereus	L1, CfiA, IMP-1, VIM-1
4	?	Penicillinase	Chromosom, P.cepacia	

Bacterial Resistance to Antibiotics, Fig. 10 Classification and characteristics of serine-type and metalloenzyme-type β-lactamases

species, this is a class C β-lactamase which has a low affinity for modern cephalosporins but can mediate resistance due to its extremely high concentration. Mutations inactivating the AmpD repressor of *ampC* constitutively overexpress AmpC β-lactamase (Lister et al. 2009).

(2) In Gram-negative bacteria, the outer membrane is an effective barrier for hydrophilic molecules. However, to ensure sufficient uptake of small hydrophilic molecules, such as nutrients, pore proteins (porins, see above) can be inserted into the outer membrane allowing passive diffusion through small water channels. β-Lactams can also diffuse into the periplasmic space through porins. To protect itself from small toxic molecules, a bacterial cell can downregulate the expression of porins in order to exclude, e.g., antibiotics. In *Pseudomonas aeruginosa*, mutational loss of a specific porin, the OprD protein, which is an effective entry for β-lactams, results in significantly increased resistance (Fig. 7). Resistance is synergistically increased in strains overexpressing the chromosomally encoded β-lactamase.

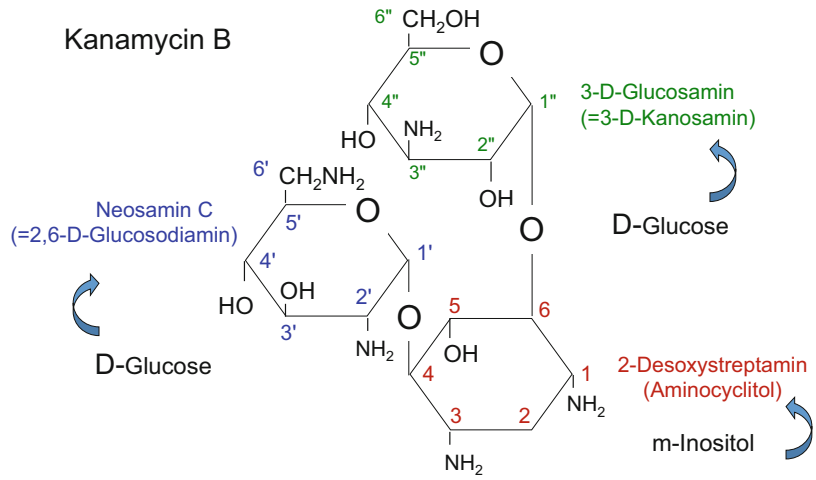
Resistance by Enzymatic Modification of Aminoglycoside-Aminocyclitol Antibiotics Streptomycin, the first aminoglycosid antibiotic which was isolated by Waksman et al. in 1943 from soil bacteria of the genus *Streptomyces*, was introduced into clinical use within 1 year due to its potent inhibitory activity against *Mycobacterium tuberculosis*. The isolation of several other aminoglycosides from species of the genera *Streptomyces* (e.g., kanamycin, neomycin, spectinomycin, tobramycin) and *Micromonospora* (e.g., gentamicin, isepamicin, sisomicin) followed. Except for streptomycin and apramycin, the core structural elements of clinically relevant antibiotics are the aminocyclitol 2-deoxystreptamine). It is linked by glycosidic bonds via the C4 and either the C5 or C6 position to two amino sugars. Consequently, they are named aminoglycoside-aminocyclitol antibiotics. Beside *M. tuberculosis*, their broad spectrum of activity includes many Gram-negative and several

Gram-positive pathogens. These highly hydrophilic compounds require an intravenous route of application.

Aminoglycosides act by specifically binding to the 30S subunit of the bacterial ribosome by interacting with the positively charged amino groups with negatively charged groups (e.g., phosphate) of ribosomal RNA regions in the aminoacyl-(A) site. The consequence is a deformation of the mRNA-tRNA binding site resulting in a less specific codon-anticodon interaction. This affects the discrimination between cognate and noncognate tRNA anticodons leading to an increase of misincorporated amino acids in growing peptide chains. Finally, such defect proteins are rapidly degraded or are inserted into membranes as misfolded membrane components which cause membrane disruption favoring an increased uptake of aminoglycosides and finally cell death.

Resistance to aminoglycoside-aminocyclitol antibiotics is a vital feature of bacterial producers of these antibiotics. The most effective strategy with a low impact of the producers' metabolism is the synthesis of an inactive precursor, which is activated after its export to the environment. This self-protecting strategy has been detected in the streptomycin producer *Streptomyces griseus*: it secretes the inactive phosphorylated streptomycin derivative together with a phosphatase, which activates the antibiotic outside the cell (Trieu-Cuot and Courvalin 1986).

Presumably, the gene for this streptomycin-modifying enzyme has been transferred to other bacteria which then have become resistant. Beside such aminoglycoside-O-phosphorylating (APH) enzymes which use ATP as cofactor, two other classes of aminoglycoside-modifying enzymes (AME) are known: aminoglycoside-O-nucleotidylating enzymes (ANT) and aminoglycoside-N-acetylating enzymes (AAC) using ATP and acetyl-CoA as cofactor, respectively. As a result, positively charged amino groups are masked, or hydroxy groups become negatively charged, both modifications interfering with the binding of the drug to its ribosomal target. Depending upon the pattern of substituents of the specific aminoglycoside, AMEs with different specificities are required to modify relevant functional groups. Thus, a broad variety of different AMEs have been identified in different pathogens. But, as a consequence, the presence of one specific AME does not necessarily mediate resistance to all aminoglycoside antibiotics (lack of cross-resistance). Thus, enzymes of one class are further subdivided according to their substrate specificity which is indicated as the position of the functional group modified. For example, among the AAC enzyme class, the AAC(6′) enzyme transfers an acetyl moiety to the 6′-amino group of, e.g., the amino sugar 2,6-D-glucosamine attached with the C4-OH via an O-glycosidic bond to the central aminocyclitol 2-deoxystreptamine (Fig. 11). The different AME subclasses are further divided into



Bacterial Resistance to Antibiotics, Fig. 11 Structural formula of kanamycin B and positions of enzymatic modification

molecular classes according to the degree of DNA homology of the respective gene, e.g., AAC(6′)-I and AAC(6′)-II, etc. Due to the restricted spectrum of activity of one subclass of AME, full resistance to all aminoglycosides is not found. However, since the genes encoding AMEs are located on transferable extrachromosomal elements, such as plasmids, combinations of AME genes may mediate a broad-spectrum resistance (Shaw et al. 1993; Vakulenko and Mobashery 2003).

Another mechanism of action to aminoglycosides is a mutational alteration of key residues associated with the ribosomal binding site. This mechanism is found for streptomycin in *M. tuberculosis* as a mutation altering the *rpsL* gene product which is part of the small ribosomal subunit.

Natural, i.e., primary resistance to all aminoglycoside antibiotics, is found in all anaerobic pathogens due to the fact that the uptake of the drugs into the cell requires a high membrane potential across the cytoplasmic membrane, only found under aerobic conditions.

Conclusion

During the period of antibiotic use, the introduction of a new antibiotic, either of natural origin or by pure chemical synthesis, was rapidly followed by the emergence of resistant bacteria.

The previous human counterstrategies, such as chemical modification of antibiotics, expanding the target preference (dual or multiple targeting strategy), combination of antibiotics showing different modes of action, and addition of inhibitors of enzymatic resistance mechanisms, have all proved to be ineffective after a short period of successful clinical use.

Thus, continuing to develop novel antibiotics combined with strategies, such as antibiotic stewardship programs, rapid diagnosis of present and developing resistance mechanisms as early as possible and antibiotic cycling will help to slower the development of resistance. However, considering the more than 2 billion-year-old presence of bacteria – and their resistance – on earth, these measures will not continuously abolish development of resistance and continue to provide a great challenge for successful anti-infective therapy.

B

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Bacterial Toxins

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Uptake

Protein toxins acting intracellularly are often composed of two subunits (A/B model). One subunit is catalytic (A-subunit) and the other is responsible for binding and cell entry (B-subunit). Following binding to an extracellular membrane receptor, the toxins are endocytosed. From the endosomes, the A-subunit is directly (pH dependent) transferred into the cytosol (e.g., diphtheria toxin and anthrax toxin) or the toxin is transported in a retrograde manner via the Golgi to the ER (e.g., cholera toxin), where translocation into the cytosol occurs (Schiavo and van der Goot 2001).

Mechanism of Action

Toxins Modifying Target Proteins

Protein toxins of this type are generally very potent and efficient, because they act catalytically on specific substrates. The toxins usually activate or inactivate key eukaryotic proteins involved in essential cellular functions by covalent modification. One subfamily catalyzes the ADP-ribosylation of target proteins. For unknown reasons, many toxins of this subfamily modify eukaryotic GTPases. Examples are the diphtheria toxin from toxigenic *Corynebacterium diphtheriae* and the *Pseudomonas aeruginosa* exotoxin A, which ADP-ribosylate elongation factor 2 at diphthamide to cause inhibition of protein synthesis (Table 1). Pertussis toxin from *Bordetella pertussis* and cholera toxin from *Vibrio cholerae* act on heterotrimeric G proteins. Pertussis toxin consists of the catalytic subunit S1 and five binding subunits (S2, S3, 2 × S4, and S5) with masses of ~11–26 kDa. Cholera toxin consists of a ~28 kDa A-subunit and five B-subunits (~12 kDa). Although pertussis toxin ADP-ribosylates the α-subunits of the G_i subfamily of G proteins (exception G_z) at a cysteine residue, cholera toxin and the related *E. coli* heat labile toxins ADP-ribosylate α-subunits of the G_s subfamily at an arginine residue. Pertussis toxin–induced ADP-ribosylation blocks the interaction of the G protein with heptahelical receptors (GPCR). The ADP-ribosylation of G_s inhibits intrinsic GTPase activity and persistently activates the G protein. Increase in cellular cAMP, activation of protein kinase A, and subsequent disturbance of cellular electrolyte secretion is suggested to be the cause of cholera toxin–induced diarrhea. A recently characterized class of toxins (toxin complexes, TC) is produced by the entomopathogenic bacterium *Photorhabdusluminescens*. It resembles a megadalton protein machinery able to mechanically inject a toxic enzyme into target cells, which can be insect cells as well as mammalian cells. Some enzymes are identified as ADP-ribosyltransferases which modify actin (leads to actin clustering) and Rho GTPases (leads to constitutive activation), respectively (Schiavo and van der Goot 2001).

Small GTPases of the Rho family are ADP-ribosylated (e.g., at Asn41 of RhoA) and inactivated by C3-like toxins from *Clostridium botulinum*, *Clostridium limosum*, and *Staphylococcus aureus*. These proteins have a molecular mass of 23–30 kDa and consist of the enzyme domain exclusively. Specific inhibition of Rho functions (Rho but not Rac or Cdc42 are targets) is the reason why C3 is widely used as a pharmacological tool (Aktories 2011).

Another subfamily of ADP-ribosylating toxins modifies G-actin (at Arg177), thereby inhibiting actin polymerization. Members of this family are, for example, *Clostridium botulinum* C2 toxin, *Clostridium perfringens* iota toxin, and *Clostridium* (recently renamed *Clostridioides*) *difficile* transferase CDT. These toxins are binary in structure. They consist of an enzyme component and a separate binding component, which is structurally related to the binding component of anthrax toxin (Aktories et al. 2011).

Bacterial Toxins, Table 1 Intracellularly acting exotoxins

Toxin	Protein substrate	Activity	Functional consequences
Diphtheria toxin, *Pseudomonas* exotoxin A	Elongation factor 2	ADP-ribosylation	Inhibition of protein synthesis (diphtheria, *Pseudomonas* infection)
Cholera toxin, heat labile *E. coli* toxins	G_s proteins	ADP-ribosylation	Activation of adenylate cyclase (cholera, "traveler"-diarrhea)
Pertussis toxin	$G_{i,o}$ proteins	ADP-ribosylation	Inhibition of G protein signaling (whooping cough)
C. botulinum C2-toxin and related toxins	Actin	ADP-ribosylation	Inhibition of actin polymerization
C. botulinum C3-toxin and related toxins	Rho proteins	ADP-ribosylation	Inhibition of RhoA, B,C Destruction of the cytoskeleton
E. coli CNF1, 2 and 3, *Yersinia* CNFy	Rho proteins	Deamidation	Activation of RhoA, (Rac, Cdc42)
Bordetella DNT	Rho proteins	Transglutamination	Activation of RhoA, Rac, Cdc42
Pasteurella multocida toxin PMT	$G_{q/11}$, $G_{i,o}$ $G_{12/13}$ proteins	Deamidation	Activation of the G proteins
C. difficile toxin A and B	Rho proteins	Glucosylation	Inactivation of Rho proteins Destruction of the cytoskeleton
Photorhabdus luminescens Toxin complex *PTC3* *PTC5*	(3) Actin (5) Rho proteins	ADP-ribosylation	(3) Forced, unregulated actin clustering (5) Activation of RhoA, Rac, Cdc42
Botulinum neurotoxins (A–G), tetanus toxin	Synaptic peptides: (a) Synaptobrevin (b) Syntaxin (c) SNAP25	Zinc-dependent endoprotease	Cleavage of synaptic peptides Inhibition of transmitter release (tetanus, botulism)
Shiga toxins and related toxins from *E. coli*	No proteins (!) 28S rRNA	*N*-glycosidase	Cleavage of 28S rRNA Inhibition of protein synthesis
Anthrax toxin Lethal factor	MEKs	Endoprotease Increase in intracellular cAMP	Inhibition of MAP-kinase pathways Calmodulin-dependent adenylylcyclase

The above-mentioned Rho GTPases are glucosylated by the family of clostridial glucosylating cytotoxins. Important members of this toxin family are *Clostridium* (*Clostridioides*) *difficile* toxins A and B, which are implicated in antibiotics-associated diarrhea and pseudomembranous colitis. The large clostridial cytotoxins are single-chain toxins with molecular masses of 250–308 kDa. The enzyme domain is located at the N terminus. The toxins are taken up from an acidic endosomal compartment. They glucosylate RhoA at Thr37; also, Rac and Cdc42 are substrates. Other members of this toxin family such as *Clostridium sordellii* lethal toxin possess a different substrate specificity and modify Rac but not Rho. In addition, Ras subfamily proteins (e.g., Ras, Ral, and Rap) are modified. As for C3, they are widely used as tools to study Rho functions (Aktories 2011; Aktories et al. 2017).

Rho GTPases are activated by cytotoxic necrotizing factors 1, 2, and 3 (CNF1, 2, 3) from *Escherichia coli*, CNFy from *Yersinia pseudotuberculosis*, and by the dermonecrotic toxin (DNT) from *Bordetella*. CNF1 to 3 are more than 90% identical, whereas the more distantly related CNFy has ~60% sequence identity with CNF1. All four toxins are single-chain proteins with molecular masses of about 115 kDa. Their cell-binding domains are located at the N terminus and the catalytic domain at the C terminus of the

toxins. DNT is a protein of ~160 kDa that shares significant homology with CNFs in the catalytic domain. All these toxins activate the small GTP-binding proteins of the Rho family by deamidation (CNFs) and transglutamination (DNT) of a specific glutamine residue (Gln63 in case of RhoA), which is necessary for GTP hydrolysis. Moreover, the inactivation of Rho GTPases by GTPase-activating protein (GAP) is blocked by CNFs and DNT. According to the functions of Rho GTPases, the toxins cause formation of stress fibers, filopodia, and membrane ruffles, and induce cell flattening and multinucleation. The role of CNFs in the pathogenesis of *E. coli* infections is still unclear. CNFs and DNT induce dermonecrosis after intradermal application (Hoffmann and Schmidt 2004). The ~146 kDa *Pasteurella multocida* toxin (PMT) shares the deamidation reaction with CNFs. Similarly, it deamidates a crucial glutamine residue involved in GTP hydrolysis but targets are heterotrimeric G_q, G_i, and $G_{12/13}$ proteins, which are activated by deamidation. PMT activates osteoclasts and inhibits osteoblasts and thereby is responsible for bone loss in atrophic rhinitis of pigs (Aktories 2011).

The anthrax toxin is a tripartite toxin and consists of the binding component protective antigen (PA), the lethal factor (LF), which is a metalloprotease, and the edema factor (EF), which is a calmodulin-dependent adenylylcyclase. Both enzyme components are translocated via PA into target cells. PA is activated by furin-induced cleavage and forms heptamers, which are similar to the binding components of C2 toxin and iota toxin. In the low pH compartment of endosomes, the heptamers form pores to allow translocation of LF and EF. LF cleaves six of the seven MEKs (MAPK-kinases), thereby inhibiting these enzymes. The functional consequence is the blockade of the MAPK pathways that control cell proliferation, differentiation, inflammation, stress response, and survival. Whether this is the reason for the LT-induced cell death of macrophages is not clear (Schiavo and van der Goot 2001).

Shiga toxin is produced (i) by *Shigella dysenterica*, the cause of bacillary dysentery, (ii) by certain *E. coli* strains (EHEC, enterohaemorrhagic *E. coli*; cause of the hemolytic uremic syndrome, HUS), and (iii) by various enterobacteriaceae (e.g., *Enterobacter cloacae*). The toxin consists of an A-subunit (~32 kDa) and a pentameric B-subunit (7.7 kDa each). The toxin enters cells after retrograde transport to the Golgi. In the cytosol, the A-subunit acts as an *N*-glycosidase to remove one adenine residue in position 4324 of the 28S rRNA at the ribosome and blocks protein biosynthesis (Schiavo and van der Goot 2001).

Clostridial neurotoxins are mainly two-chain toxins having a ~50 kDa enzyme component and a ~100 kDa binding/translocation subunit. They are the cause of botulism, a generalized flaccid paralysis of skeletal muscles, mainly acquired by foodborne poisoning, and tetanus, which occurs subsequent to wound infection. Botulism is induced by *C. botulinum* neurotoxins types A, B, C1, D, E, F, and G. Tetanus is induced by tetanus toxin from *Clostridium tetani*. The toxins belong to the most potent agents known. About 1 ng of botulinum neurotoxin per kg body mass may be lethal for man or animal. The toxins are zinc metalloproteases and cleave synaptic peptides involved in transmitter release. Botulinum neurotoxins B, D, F, and G and tetanus toxin cleave synaptobrevin; neurotoxins A and E cleave SNAP25, and neurotoxin C cleaves syntaxin. The botulinum neurotoxins induce flaccid muscle paralysis (botulism) because they act presynaptically at the peripheral neuromuscular junction to block acetylcholine release. Tetanus toxin is taken up at the neuromuscular junction but is then transported in a retrograde manner to the spinal cord. Within the spinal cord, tetanus toxin migrates to interneurons and blocks the release of inhibitory transmitters to cause spastic paralysis (Schiavo and van der Goot 2001).

Bacterial Phospholipases

The α-toxin from *C. perfringens* is involved in the pathogenesis of gas gangrene and the sudden death syndrome of young animals. This toxin is a zinc metalloenzyme with phospholipase activity. Other phospholipase C-toxins are from *Listeria monocytogenes* and *Mycobacterium tuberculosis*. The phospholipase C-toxin from

Bacillus cereus is specific for phosphatidylinositol. It cleaves phosphatidylinositol and its glucosyl derivatives. In cell biology, this toxin can be used as a tool to study whether a protein is anchored to GPI. A second phospholipase C-toxin produced by *B. cereus* is specific for sphingomyelin. Cleavage of sphingomyelin generates ceramide, a second messenger involved in processes like apoptosis and differentiation.

Pore-Forming Toxins

Pore-forming toxins act by punching holes into mammalian cell membranes. Many different types are known. They can be divided into small and large pore-forming toxins. Pore-forming toxins oligomerize in the plasma membrane of the mammalian cell to build circular structures. These ring-like structures can be composed of a few molecules, generating small pores that allow the exchange of ions and nucleotides (*Aeromonas* aerolysin, *S. aureus* α-toxin). Large pores, which allow the passage of peptides or proteins are formed by toxins which insert up to 50 molecules into the plasma membrane generating a pore with up to 35 nm diameter. Examples for such toxins are *Streptococcus pyrogenes* streptolysin O or *C. tetani* tetanolysin.

Injected toxins (often called "effectors") are directly delivered into the cytosol of eukaryotic target cells mostly involving the bacterial type III or IV secretion systems. The pathogens (e.g., *P. aeruginosa*, *Yersinia*, and *Salmonella*) produce a set of proteins that are delivered into mammalian cells by complex secretion machines depending on the direct contact between bacterium and host cells. Some of these injected toxins (e.g., Yops in the case of *Yersinia*) do not covalently modify mammalian targets but act as modulators on important signal transduction pathways; they act as molecular mimics of cellular proteins. For example, they regulate the activity of small GTP-binding proteins as exchange factors to activate the small G proteins or as activators of GTP-hydrolysis to inhibit them. Notably, *Salmonella* produces two contrary acting molecular mimics. They inject an activator of Rho GTPases (SopE) to induce ruffling and the uptake of the bacteria into the mammalian cell, and they inject an inactivator of Rho GTPases (SptP) probably to switch off the induced cytoskeletal rearrangements.

Superantigens

Like physiological ligands, bacterial toxins can influence cells by binding to cell surface molecules. Best known are the superantigens produced by *Staphylococcus* strains. Superantigens are bivalent molecules that bind to the major histocompatibility complex (MHC) class II and to the variable regions of the T-cell receptor. This bridging leads to the activation of the T-cell receptor in the absence of an antigenic peptide. This unspecific activation of T-cells is followed by a massive release of cytokines, which is thought to play a role in diseases like toxic shock syndrome and some exanthemas.

Clinical Uses

Botulinum neurotoxins are widely used as therapeutic agents to cause reduction or paralysis of skeletal muscle contraction. They are used to treat cervical dystonia, which causes regional involuntary muscle spasms often associated with pain. Moreover, they are used in strabism, blepharospasm, hemifacial spasm, and achalasia. Meanwhile, a number of studies indicate efficacy for botulinum toxin for the treatment of tension headache, but further studies are necessary to demonstrate its short-term and long-term efficacy. Botulinum toxin is also used as a cosmetic agent and the effects occur a few days after injection into the muscle and last for several months. The treatment can be repeated several times without major development of antineurotoxin antibodies. In the case of antineurotoxin antibody production, the treatment is continued with a different botulinum neurotoxin.

Some toxins (e.g., the diphtheria toxin) fused to antibodies are used as immunotoxins against cell surface molecules, for example, to deplete T-cells as targeted therapy for cutaneous T-cell lymphoma. The use of bacteria with the type III secretion system (e.g., *Salmonella*) producing only an injected toxin-antigen fusion protein as

live vaccines is under current investigation. The aim is to directly deliver protein fragments into antigen-presenting cells in order to improve immunization.

Cross-References

► Small GTPases

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Barbiturates

► GABAergic System

Basal Ganglia

► Dopamine System

Base-Excision Repair

► DNA Damage Response

B-Cell Stimulating Factor 2

► Interleukin-6

Bempedoic Acid

► Lipid-Lowering Drugs

Benralizumab

► Interleukin-5

Benzodiazepine

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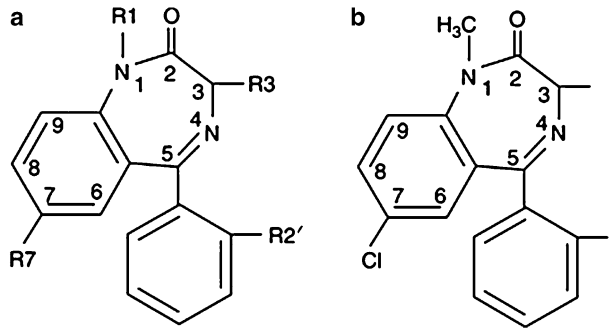
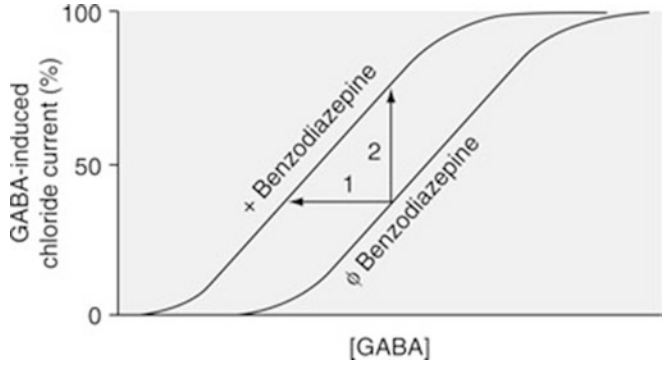
Definition

The term benzodiazepine refers to a chemical structure, consisting of a heterocyclic ring system in which the two N atoms are mostly located in positions 1 and 4 (1,4-benzodiazepines), e.g., in diazepam (Fig. 1b). Benzodiazepines have found wide therapeutic applications as anxiolytics, sedatives, hypnotics, anticonvulsants, and central muscle relaxants (Balon et al. 2018; Engin et al. 2018; Mohler et al. 2002; Rudolph and Knoflach 2011; Shader and Greenblatt 1993).

Mechanism of Action

GABA is the major inhibitory neurotransmitter in the mammalian central nervous system. Its fast synaptic actions are mediated by $GABA_A$ receptors, which are located on postsynaptic membranes. $GABA_A$ receptors have a central pore, with selectivity for chloride ions. Upon binding of GABA to $GABA_A$ receptors, negatively charged chloride ions flow into the postsynaptic





Benzodiazepine, Fig. 1 (**a**) Chemical structure of classical [1,4] benzodiazepines. R1, R2, R2', R3, and R7 denote variable substituents. (**b**) Chemical structure of the prototypical benzodiazepine diazepam

Benzodiazepine, Fig. 2 Schematic representation of the potentiation of the GABA-induced chloride current by benzodiazepines. The GABA dose–response curve is shifted to the left in the presence of benzodiazepines (arrow 1). The chloride current which is induced by a submaximal concentration of GABA is increased (arrow 2). Benzodiazepines are effective only in the presence of GABA (use-dependence) and cannot increase the inhibition by high concentrations of GABA (self-limiting action)

neuron, leading – in most cases – to hyperpolarization of the postsynaptic membrane and thus functional inhibition. In addition to a binding site for the physiological neurotransmitter GABA, most $GABA_A$ receptors contain binding sites for allosteric modulators, e.g., benzodiazepines, barbiturates, and neurosteroids. Benzodiazepines bind to a common modulatory site that is called benzodiazepine site. However, the ligands of the benzodiazepine site are not limited to ligands with a benzodiazepine structure. For example, the imidazopyridine zolpidem, a widely used hypnotic, also binds to the benzodiazepine site. The basic mechanism of action of benzodiazepines and nonbenzodiazepines acting via the benzodiazepine site appears to be the same. The binding of a benzodiazepine to the benzodiazepine site of the $GABA_A$ receptor enhances GABAergic inhibition by increasing the opening frequency of the GABA-gated ion channel. This leads to a shift of the GABA dose–response curve to the left, so that at any given concentration of GABA, the response is increased (Fig. 2). This can also be viewed as an increase in the affinity of GABA for the receptor. The action of benzodiazepines is use-dependent and self-limiting. Use-dependence indicates that benzodiazepines are only active in the presence of GABA. In the absence of GABA, benzodiazepines do not have an effect on their own, i.e., their action is dependent on the precondition that GABA is present and the respective synapse thus in use. Furthermore, benzodiazepines are not able to increase the response to GABA beyond its physiological maximum at high GABA concentrations, which is referred to as the self-limiting nature of their

action. The magnitude of the effect of benzodiazepines depends on the amount of GABA present in the synapse or in the extrasynaptic space and, hence, synaptic activity or activity mediated by extrasynaptic receptors. The self-limiting feature may help explain why the enhancement of GABA transmission by benzodiazepines is typically safe even at high doses, whereas overdoses with drugs that do not display this self-limiting feature, e.g., barbiturates, are life-threatening.

Subtype Specificity of BZ Actions

$GABA_A$ receptors that contain the α1, α2, α3, and α5 subunits in combination with β and γ subunits can bind classical benzodiazepines, e.g., diazepam, whereas $GABA_A$ receptors that contain the α4 and α6 subunits do not bind classical benzodiazepines. Essentially, all benzodiazepines that are currently in clinical use bind indiscriminately to $GABA_A$ receptors that contain the α1, α2, α3, and α5 subunits. The only clinically used drug that displays a significant subtype selectivity is the imidazopyridine hypnotic, zolpidem. Zolpidem has a high affinity at $GABA_A$ receptors containing the α1 subunit, an intermediate affinity at $GABA_A$ receptors containing the α2 or α3 subunits, and no affinity at $GABA_A$ receptors containing the α5 subunit. The $GABA_A$ receptor subtype specificity of benzodiazepine actions was assessed in genetically engineered mice. Whereas the diazepam-sensitive α1, α2, α3, and α5 subunits have a histidine residue in a conserved position in the N-terminal extracellular domain (H101 in α1, H101 in α2, H126 in α3, H105 in α5), the diazepam-insensitive α4 and α6 subunits have an arginine residue at the homologous position. By mutating the conserved histidine residue in the α1, α2, α3, and α5 subunits to arginine residues, the $GABA_A$ receptors containing the respective subunits were rendered diazepam-insensitive. Using this approach, it was discovered that the sedative and anterograde amnesic action of diazepam, and in part also the anticonvulsant action of diazepam, is mediated by $GABA_A$ receptors containing the α1 subunits, while the anxiolytic action of diazepam is mediated by $GABA_A$ receptors containing the α2 subunit. The central muscle relaxant action of diazepam is mediated by $GABA_A$ receptors containing the α2 or α3 subunits (Fig. 3) (Balon et al. 2018; Engin et al. 2018; Mohler et al. 2002; Rudolph and Knoflach 2011; Shader and Greenblatt 1993). The anxiolytic-like action of diazepam is observed at much lower doses than the muscle relaxant action. Interestingly, $GABA_A$ receptors containing the α3 subunit were not involved in mediating the anxiolytic-like action of diazepam in ethological tests of anxiety, indicating that this response is not dependent on neurons in the reticular activating system, where the α3 subunit is expressed. These findings demonstrate that subtype-selective drugs are likely to be

α1	α2	α3	α5
Sedation	Anxiolysis	Sensorimotor gating	Sensorimotor gating
Amnesia	Fear reduction	Myorelaxation	Cognitive impairment
Anticonvulsant	Myorelaxation	Antihyperalgesia	(low interference tasks)
Dependence liability	Antihyperalgesia	Itch suppression	Cognitive improvement
	Itch suppression		(high interference tasks)
			Anxiolysis
			Antidepressant

Benzodiazepine, Fig. 3 Benzodiazepine actions mediated by $GABA_A$ receptor subtypes. α1, α2, α3, or α5 indicates α1-, α2-, α3- or α5-containing $GABA_A$ receptors

of benefit, e.g., as anxiolytics without sedative side effects.

Interestingly, while the sedative action of diazepam is mediated by $GABA_A$ receptors containing the α1 subunit, its REM sleep inhibiting action, its enhancement of sleep continuity, and its effect on the EEG spectra in sleep and waking are mediated by $GABA_A$ receptors that do not contain the α1 subunit, indicating that the hypnotic effect of diazepam and its EEG fingerprint can be dissociated from its sedative action, defined as reduction of motor activity.

Several compounds have been developed which have activity at α2-containing (and also other) $GABA_A$ receptors but not at α1-containing $GABA_A$ receptors, e.g., L-838,417, which is a partial agonist at $GABA_A$ receptors containing the α2, α3, and α5 subunits, but has no activity at $GABA_A$ receptors containing the α1 subunit. This compound is active as an anxiolytic and anticonvulsant, but apparently does not impair motor performance (Rudolph and Knoflach 2011). The compound TPA023B has been tested in Phase 1 and Phase 2 studies, which were stopped prematurely as high doses of the related compound TPA023 were found to lead to cataracts and which were compatible with an anxioselective profile. These compounds have also been found to have antihyperalgesic actions in various rodent pain models, e.g., of inflammatory pain and of neuropathic pain, which are mediated by α2-, α3- and α5-containing $GABA_A$ receptors. TPA023B also reduces itching in mice and in dogs.

Positive and Negative Allosteric Modulators

Drugs that bind to the benzodiazepine site of the $GABA_A$ receptor and enhance GABA responses are termed positive allosteric modulators (or agonists). Essentially all ligands at the benzodiazepine site that are in clinical use are positive allosteric modulators. In contrast, negative allosteric modulators (or inverse agonists) diminish GABA responses. They are not in clinical use and have effects opposite to those of the positive allosteric modulators, e.g., they are convulsant and anxiogenic.

The Antagonist Flumazenil

Although flumazenil binds with high affinity to the benzodiazepine site of $GABA_A$ receptors, it has practically no action when given alone. However, flumazenil competitively blocks the action of benzodiazepine site positive allosteric modulators. Flumazenil can be used to (temporarily) terminate the action of benzodiazepines, e.g., after a benzodiazepine overdose. It may also serve as a diagnostic tool in this regard.

Pharmacokinetic Considerations

The benzodiazepines currently on the market differ in their pharmacokinetic properties, in particular, the duration of action, which guides the use of the drug to be used. The half-life is largely determined by the rate of metabolic degradation of the parent drug. In addition, long-acting metabolites (e.g., desmethyldiazepam) are generated that may contribute to the duration of action. Short-acting drugs might be used for patients with difficulties to fall asleep, with the expectation that there is no hangover effect on the next day. Long-acting drugs may be used if reawakening during the entire night is to be prevented. Short-acting benzodiazepines may have a half-life in the range of 2–6 h, e.g., midazolam, triazolam, and oxazepam; medium-acting benzodiazepines a half-life in the range of 10–12 h, e.g., lorazepam and lormetazepam; and long-acting benzodiazepines a half-life in the range of 20–50 h, e.g., diazepam and clobazam.

Clinical Use

Benzodiazepines are among the most frequently prescribed drugs; they have well-established uses in the treatment of anxiety disorders (anxiolytics) and insomnia, preanesthetic sedation, sedation and amnesia during medical procedures, suppression of seizures, and muscle relaxation.

Benzodiazepines such as alprazolam are used as tranquilizers to relieve anxiety states, e.g., in generalized anxiety disorder and panic attacks. The anxiolytic effects are observed at low doses, suggesting that only a small number of $GABA_A$

receptors need to be modulated to obtain the anxiolytic effect. As outlined previously, this action is, most likely, mediated by α2-containing $GABA_A$ receptors. In contrast, higher doses of benzodiazepines and thus a higher receptor occupancy are needed for the sedative action of diazepam, which is mediated by α1-containing $GABA_A$ receptors. When diazepam is used as an anxiolytic, sedative side effects are frequently troublesome. The reduction of the reactivity to external stimuli is the basis for the use of benzodiazepines as hypnotics in the treatment of sleep disorders. The anticonvulsant activity of diazepam can be explained by the GABAergic inhibition of neuronal responsiveness to excitatory inputs. Benzodiazepines (lorazepam and diazepam) are among the drugs of choice in the treatment of status epilepticus. Their use in the chronic treatment of epilepsy (e.g., clonazepam) is limited by the development of tolerance.

The definition of desired therapeutic and side effects in the case of the benzodiazepines very much depends on the clinical problem in question. The sedative and hypnotic actions are desired effects in the treatment of insomnia, but undesired effects in the treatment of anxiety disorders. Effects that are usually undesired include daytime drowsiness and potentiation of the sedative effects of ethanol. Anterograde amnesia is a desired when benzodiazepines are used during medical procedures. The known effects of benzodiazepines are mediated via the benzodiazepine site of $GABA_A$ receptors, since they can be antagonized with flumazenil. Repeated administration may lead to the development of tolerance to certain benzodiazepine effects; in particular, to the sedative, anticonvulsant, and muscle relaxant effects; and to the development of physical dependence, which can include withdrawal anxiety, insomnia, convulsions, and sensory hyperactivity and thus be similar to the symptoms that lead to the treatment. To avoid withdrawal symptoms, chronic treatment is discontinued by gradually tapering out the dose over a long period of time. The neurobiological nature of the adaptive changes, which occur after long-term treatment or withdrawal from long-term treatment, is poorly understood. Because of the adaptive changes that occur under chronic treatment, the long-term use of benzodiazepines is generally not recommended. For treatment of insomnia, benzodiazepines should not be given for more than, e.g., 4 weeks. For the treatment of anxiety disorders, benzodiazepines should not be used for more than, e.g., 6 months. Because of their potentiation of the sedative action of ethanol, benzodiazepines should not be used in patients with alcohol abuse. Likewise, the potential non-therapeutic use of benzodiazepines, for the purpose of euphoria, has to be kept in mind, and particular care should be taken while treating patients with a history of drug abuse. Benzodiazepines are frequently co-abused together with opioids, increasing the mortality of opioid abuse, potentially at least in part due to their respiratory depressant actions.

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Benzodiazepines

▶ GABAergic System

Benzoylcholinesterase

▶ Cholinesterases

Beta-Lactam Antibiotics

▶ Cephalosporins

Biocomputing

▶ Molecular Modelling

Biologics

▶ Rheumatoid Arthritis

Biomarkers

▶ Ca^{2+}-Binding Proteins
▶ S100 Proteins

Bio-originators

▶ Rheumatoid Arthritis

Biosimilars

▶ Rheumatoid Arthritis

Blood Glucose-Lowering Drugs

▶ Glucose-Lowering Drugs Other than Insulin

Blood Poisoning

▶ Sepsis

Blood Pressure Control

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Synonyms

Control of hypertension; Physiology of blood pressure

Definition

The blood pressure represents the tension or pressure of the blood within the arteries that is exerted against the arterial wall in vivo. Blood pressure is a quantitative trait that is highly variable. In population studies, blood pressure has a normal distribution that is slightly skewed to the right. The regulation of blood pressure within the intravascular system is a complex interaction of a number of systems and mechanisms. Raised blood pressure or hypertension is a substantial global health problem and continues to be the leading contributor to global burden of disease and mortality affecting over 1 billion adults worldwide (Global, regional, and national comparative risk assessment of 84 behavioural, environmental and occupational, and metabolic risks or clusters of risks for 195 countries and territories, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017 2018).

Despite important advances in our understanding of the pathophysiology of hypertension and the availability of effective treatment strategies, it still remains the leading modifiable risk factor for cardiovascular and renal disease. There is no specific level of blood pressure where clinical complications start to occur; thus the definition of hypertension is arbitrary but needed in clinical practice for patient assessment and treatment.

The relevance of clinical conditions with chronic low blood pressure or hypotension has been questioned, with the exception of a few rare clinical syndromes. Temporary increases or decreases of blood pressure are often seen in clinical medicine in the context of acute illnesses or interventions.

Basic Mechanisms

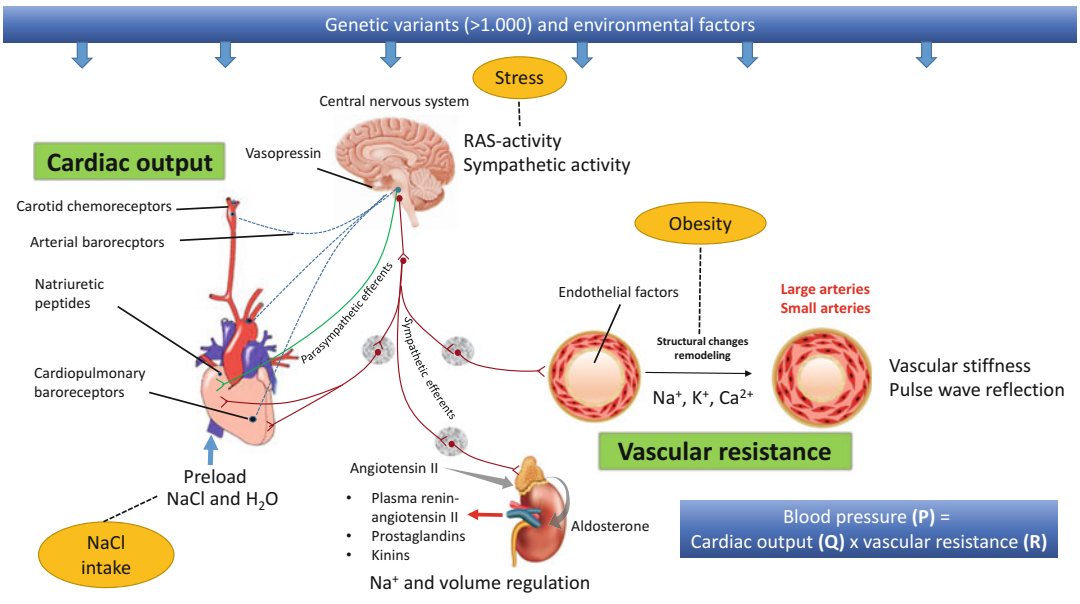
The circulation is divided into several compartments: the high-pressure arterial circuit, which contains 13% of the blood volume, the capillary bed containing 7% of the blood volume, and the low-pressure venous bed, which contains 64% of the blood volume. The pulmonary circulation contains 9% and the heart 7% of the blood volume. Although the venous system stores and propels large volumes of blood and regulates cardiac output by venous return to the heart, in considering blood pressure control, attention is focused on the high-pressure arteries. The basic function of the circulation is to provide nutrients to peripheral tissues. Blood vessels in local tissue beds regulate blood flow in relation to local needs. Blood flow (Q) is defined by Ohm's law and varies directly with the change in pressure (P) across a blood vessel and inversely with the resistance R ($Q = P/R$). It can be seen that pressure varies directly with blood flow and resistance ($P = QR$). Blood pressure is produced by the contraction of the left ventricle (producing blood flow) and by the resistance of the arteries and arterioles. Systolic pressure, or maximum blood pressure, occurs during left ventricular systole. Diastolic pressure, or minimum blood pressure, occurs during ventricular diastole. The difference between systolic and diastolic pressure is the pulse pressure (Systolic and Diastolic Blood Pressure and Pulse Pressure).

Although blood pressure control follows Ohm's law and seems to be simple, it underlies a complex circuit of interrelated systems. Hence, numerous physiologic systems that have pleiotropic effects and interact in complex fashion have been found to modulate blood pressure. Because of their number and complexity, it is beyond the scope of the current account to cover all mechanisms and feedback circuits involved in blood pressure control. Rather, an overview of the clinically most relevant ones is presented. These systems include the heart, the blood vessels, the extracellular volume, the kidneys, the nervous system, a variety of humoral factors, and molecular events at the cellular level. They are intertwined to maintain adequate tissue perfusion and nutrition. Normal blood pressure control can be related to cardiac output and the total peripheral resistance. The stroke volume and the heart rate determine cardiac output. Each cycle of cardiac contraction propels a bolus of about 70 ml blood into the systemic arterial system. As one example of the interaction of these multiple systems, the stroke volume is dependent in part on intravascular volume regulated by the kidneys as well as on myocardial contractility. The latter is, in turn, a complex function involving sympathetic and parasympathetic control of heart rate; intrinsic activity of the cardiac conduction system; complex membrane transport and cellular events requiring influx of calcium, which lead to myocardial fiber shortening and relaxation.

The ejection of blood into the aorta during systole acutely dilates the aortic wall and generates a pressure wave through the arterial tree, the velocity of which is a measure of arterial compliance. Aortic stiffening is associated with increased pulse wave velocity (PWV) as well as increased pulse wave reflection back to the heart at the end of the systole increasing the afterload and systolic blood pressure (Safar et al. 2018). On the other hand, a difference in PWV between distal and proximal arteries (impedance mismatch) protects small arteries and microcirculation from excessive pulsatility, a protective mechanism that is lost in hypertension. Aortic wall stiffening is a major contributor to the development of isolated systolic hypertension with age, an important risk factor for progression of hypertension and incident cardiovascular disease. Elevated PWV together with increased pulsatile diameter are standard features of hypertension

(Safar et al. 2018). The regulation of the total peripheral resistance also involves the complex interactions of several mechanisms. These include baroreflexes and sympathetic nervous system activity; response to neurohumoral substances and endothelial factors; myogenic adjustments at the cellular level, some mediated by ion channels and events at the cellular membrane; and intercellular events mediated by receptors and mechanisms for signal transduction. As examples of some of these mechanisms, there are two major neural reflex arcs (Fig. 1). Baroreflexes are derived from high-pressure baroreceptors in the aortic arch and carotid sinus and low-pressure cardiopulmonary baroreceptors in ventricles and atria. These receptors respond to stretch (high pressure) or filling pressures (low pressure) and send tonic inhibitory signals to the brainstem. If blood pressure increases and tonic inhibition increases, inhibition of sympathetic efferent outflow occurs and decreases vascular resistance and heart rate. If blood pressure decreases, however, less tonic inhibition ensues from the baroreflexes, and both heart rate and peripheral vascular resistance increase, thereby increasing blood pressure. In addition, the neural control of renal function produces alterations in renal blood flow; glomerular filtration rate (GFR); excretion of sodium, other ions, and water; and release of renin and other vasoactive substances. These, in turn, have effects on the regulation of intravascular volume, vascular resistance, and blood pressure. Activation of carotid chemoreceptors is also transmitted to the vasomotor center and responds not only to arterial pressure but also to oxygen tension and carbon dioxide tension (in opposite directions). A drop in blood pressure, a drop in oxygen tension, or a rise in dioxide tension results in increased sympathetic outflow to the adrenal medulla, heart, and resistance vessels.

In addition to the numerous changes, molecular changes at the tissue and cellular level that have been associated with blood pressure changes. Some important mechanisms that are linked to blood pressure regulation involve membrane sodium transport mechanisms, Na^+/K^+ ATPase, Na^+/Li^+ countertransport, Na^+-H^+ exchange, Na^+-Ca^{2+} exchange, Na^+-K^+ 2Cl



Blood Pressure Control, Fig. 1 Basic mechanisms involved in blood pressure control. The most important organs and mechanisms involved in blood pressure control are shown (for explanations see text). RAS, renin–angiotensin–aldosterone system

transport, passive Na^+ transport, calcium channels, and potassium channels.

Numerous vasoactive substances have major effects on blood vessels, the heart, the kidneys, and the central nervous system (CNS) and often serve to counterbalance one another. Nitric oxide is an important endothelium-derived vasoactive factor released in response to shear stress and activation of a variety of receptors (Hermann et al. 2006). Nitric oxide exerts a multitude of effects ranging from vasodilatation and antiproliferative effects on vascular smooth muscles to the inhibition of thrombocyte aggregation and leukocyte adhesion, hence contributing to blood pressure regulation but also additional important cardiovascular effect including hemostasis and inflammation (Hermann et al. 2006). Endothelial dysfunction that implies a risk for hypertension and cardiovascular diseases is characterized by impaired bioavailability of nitric oxide. The bioavailability of nitric oxide is reduced in hypertension due to increased generation of reactive oxygen species that scavenge nitric oxide (Brandes Ralf 2014). Another example of physiologic actions, norepinephrine (noradrenaline), via α-adrenergic mechanisms, is a potent vasoconstrictor, while epinephrine (adrenaline), via α- and β-adrenoceptors, increases primarily heart rate, stroke volume, systolic blood pressure, and pulse pressure. The ▶ Renin–Angiotensin–Aldosterone System generates angiotensin II (Ang II). Ang II, in turn, constricts vascular smooth muscle; stimulates aldosterone secretion; potentiates sympathetic nervous system activity; leads to salt and water reabsorption in the proximal tubule; stimulates prostaglandin, nitric oxide, and endothelin release; increases thirst; and is a growth factor. Aldosterone activates the epithelial sodium channel (ENaC) in the cortical collecting duct in the kidney, leading to sodium reabsorption and potassium excretion. Prostaglandin E_2 and prostacyclin (PGI_2) act to counterbalance vasoconstriction by Ang II and norepinephrine.

Vasopressin (antidiuretic hormone [ADH]) secretion increases in response to decreased blood volume and/or reductions in effective blood volume via a decrease in inhibitory tone from both low-pressure and high-pressure baroreceptors to the hypothalamus. The neuronal pathways that mediate hemodynamic regulation of vasopressin release are completely different from those involved in osmoregulation, and unlike the latter, small decreases in blood pressure or blood volume have little effect on vasopressin secretion. A rise in blood pressure causes a decrease in secretion of vasopressin related to increased baroreceptor activity, which inhibits hypothalamic vasopressin-releasing hormones. Vasopressin works by causing water conservation at the distal collecting duct of nephron. This alone, however, is a relatively inefficient mechanism of increasing intravascular volume because conserved water is distributed among total body water and only a small portion is intravascular. In addition, vasopressin is a potent vasoconstrictor, and the greater vasopressin secretion observed in response to more severe hypovolemia or hypotension serves as a mechanism to stave off cardiovascular collapse during periods of large reductions of blood pressure or blood volume. Two important endothelial-derived factors have opposite effects on the blood vessels: nitric oxide is a vasodilator acting via activation of soluble guanylyl cyclase in vascular smooth muscle cells, whereas the endothelins, particularly endothelin-1, are vasoconstrictors coupled to G-protein signaling. The kallikrein–kinin system produces vasodilator kinins, which in turn may stimulate prostaglandins and nitric oxide. Natriuretic peptides induce vasodilation, induce natriuresis, and inhibit other vasoconstrictors (renin–angiotensin, sympathetic nervous system, and endothelin).

When the temporal sequence of adjustments of blood pressure is analyzed, it seems that CNS mechanisms (e.g., baroreflexes) will provide regulation of the circulation within seconds to minutes. Other mechanisms, such as the renin–angiotensin–aldosterone system and fluid shifts, occur over minutes to hours. Only the kidneys seem to have the ability for long-term adjustment in blood pressure, predominantly through regulation of extracellular volume. This theoretical concept has been – although indirectly – confirmed by genetic approaches applied to the analysis of rare

familial syndromes of high blood pressure, i.e., hypertension, or low blood pressure, i.e., hypotension. In those studies carried out in families with monogenetic forms of the disease, several molecular pathways have been successfully delineated. All defects identified so far raise or lower blood pressure through a common pathway by increasing or decreasing salt and water reabsorption by the nephron. Thus, these studies point to the kidney as a pivotal organ for chronic determination of blood pressure.

Pharmacological Intervention

The clinical consequences of high blood pressure have been documented by epidemiologic studies, which demonstrate a strong positive and continuous correlation between blood pressure and the risk of cardiovascular disease (stroke, myocardial infarction, and heart failure), renal disease, and mortality. This correlation is more robust with systolic than with diastolic blood pressure. While hypertension was once thought to be "essential" for perfusion of tissues through sclerotic and narrowed blood vessels and to maintain a normal sodium balance, the pathologic nature of elevated blood pressure has become clear. Primary (essential) or idiopathic hypertension is defined as high blood pressure in which causes such as renovascular disease, renal failure, aldosteronism, pheochromocytoma, or other causes of secondary hypertension or monogenic (mendelian) forms are not present. Primary hypertension accounts for the vast majority of all cases of hypertension. This condition is a heterogeneous disorder, with different patients having different causal factors that lead to high blood pressure. Primary hypertension is also influenced by a large number of genetic variants that interact with environmental factors such as salt intake, alcohol consumption, obesity, physical activity, and stress predisposing an individual to hypertension. Recently, more than 900 independent blood pressure-associated loci with more than 1000 genetic variants across the genome have been identified (Evangelou et al. 2018). There are no established clinical or laboratory tests to identify the factors that are responsible for the blood pressure elevation in an individual patient. Consequently, pharmacologic treatment of primary hypertension is largely empiric. Clinical treatment algorithms try to account for comorbidities and expected or observed side effects of drugs in individual patients. Pharmacologic treatment should always be implemented "on top" of non-pharmacologic interventions such as control of body weight, intake of salt and alcohol, regular physical activity, smoking cessation, and other modifications of lifestyle as necessary in the individual patient.

Randomized trials of pharmacologic treatment of hypertension have documented that blood pressure reduction lowers morbidity and mortality, with profound reduction in stroke and smaller reductions in cardiac and renal disease (for details see ▶ "Antihypertensive Drugs").

As a result of such studies, hypertension has been operationally defined as the blood pressure level above which therapeutic intervention has clinical benefit. As intensified treatment has continued to demonstrate benefits, this level has gradually reduced over time and is currently defined in Europe as systolic blood pressure $\geq$140 mmHg and/or diastolic blood pressure $\geq$90 mmHg (Table 1A). Isolated systolic hypertension is the predominant form of hypertension in older adults and defined as systolic blood pressure $\geq$140 mmHg and diastolic blood pressure $<$90 mmHg. In 2017 the definition of hypertension was changed in the USA by proposing even lower cutoff values of $\geq$130 mmHg for systolic and $\geq$90 mmHg for diastolic blood pressure (Table 1B) due to different interpretations of available research data, which is, however, a matter of ongoing controversy (Tsioufis et al. 2018). Despite some apparent differences between international guidelines, they share several important commonalities by shifting toward lower blood pressure thresholds for initiating antihypertensive drug therapy, and lower blood pressure targets during treatment, which collectively should lead to lower blood pressure-related complications and mortality (Whelton and Williams 2018).

Blood Pressure Control, Table 1 Definitions and classifications of blood pressure levels

Category	Systolic [mmHg]		Diastolic [mmHg]
A. **According to European guidelines** (Williams et al. 2018)			
Optimal	<120	and	<80
Normal	120–129	and/or	80–84
High normal	130–139	and/or	85–89
Grade 1 hypertension	140–159	and/or	90–99
Grade 2 hypertension	160–179	and/or	100–109
Grade 3 hypertension	≥180	and/or	≥110
Isolated systolic hypertension	≥140	and	<90
B. **According to US guidelines** (Whelton et al. 2018)			
Normal	<120	and	<80
Elevated	120–129	and	<80
Hypertension			
Grade 1 hypertension	130–139	or	100–109
Grade 2 hypertension	≥140	or	≥90

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Blood Pressure Lowering Drugs

► Antihypertensive Drugs

Blood Thinners

► Anticoagulants

Blood-Brain Barrier

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Synonyms

Blood-brain interface; Brain capillary endothelial cells; Cerebral microvessels

Definition

Homeostasis of the extracellular milieu in the brain and its protection against neurotoxic compounds, infections, and continuously changing composition of the blood are important for normal function of the neurons. It is warranted by a structure formed between blood and brain which is, therefore, called the blood-brain barrier (BBB). The blood-brain barrier is an anatomical formation, and on the other hand, it has also a molecular background associated with the endothelial cell membrane of cerebral microvessels.

Basic Mechanisms

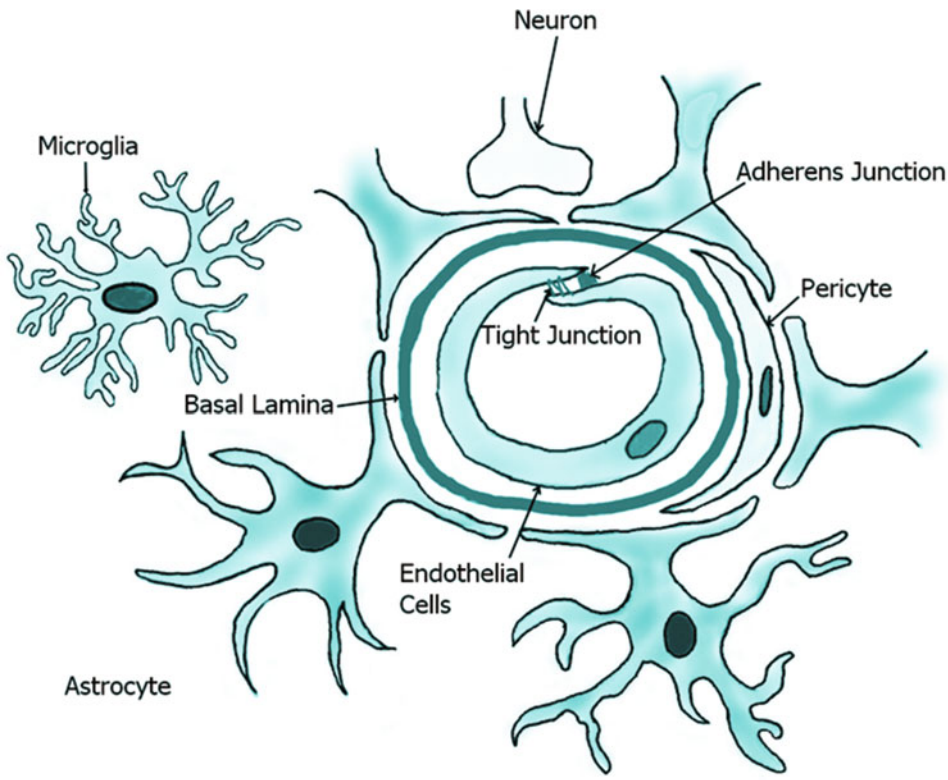
Cellular Background

The most important cell types forming the BBB are the capillary endothelial cells surrounded with pericytes and astrocyte end feet. There are some supporting cell types which are not directly connected to the BBB but also contributing the barrier function: these are microglia and neuronal cells. In the next points, these cell types and their roles in the BBB are presented (Fig. 1).

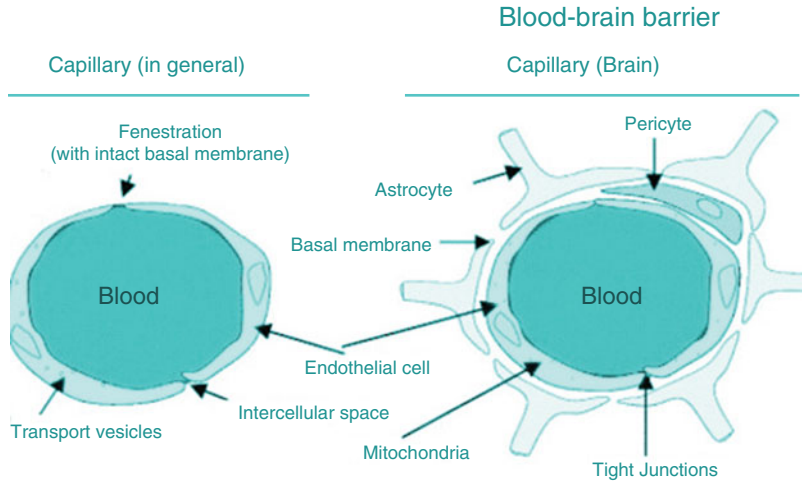
Endothelial Cells (ECs)

Compared to peripheral capillaries, BBB ECs are characterized by increased mitochondrial content, exhibit minimal pinocytotic activity, and lack of fenestrations (Fig. 2). Increased mitochondrial content is essential for these cells to maintain active transport mechanisms to transport ions, nutrients, and waste products into and out of brain parenchyma, thus contributing to precise regulation of the central nervous system (CNS) microenvironment and ensuring proper neuronal function (Oldendorf et al. 1977). The high concentration of mitochondria in cerebrovascular ECs might account for the sensitivity of the BBB to oxidant stressors. The physiology and pathophysiology of ECs are closely linked to their mitochondria, and mitochondrial dysfunction is an important mediator of disease pathology in the brain (Grammas et al. 2011).

The brain endothelium is highly reactive because it serves as both a source of, and a target for, inflammatory proteins and reactive oxygen species. Neuroinflammation and oxidative stress lead to BBB breakdown, which are implicated in the pathogenesis of CNS diseases. Cell polarity of ECs means different functional expression of transporter proteins and metabolic enzymes (Betz et al. 1980) on the luminal (or apical) and abluminal (or basolateral) membranes, which contribute to the high selectivity of the BBB. Of the many transporters expressed at the BBB endothelium membrane, several have been implicated in influx and/or efflux of drugs into/out of the CNS. Decreased expression and/or defective function of transporters leads to increased BBB permeability, toxic adverse effects of drugs, and can be an early event in the pathology of several disorders (Obermeier et al. 2013). Factors that can disrupt the BBB can be secreted elements, immune cells, and pathogens. Impaired BBB integrity manifests mainly as increased barrier permeability. In addition to direct effects on ECs, other members of the neurovascular unit can also be affected, like pericytes, astrocytes, and basement membrane, which in turn contribute impairment of BBB functions. Consequences can be dysregulated molecular and ionic flux across the BBB or the initiation of a central inflammatory response. Despite different causes, characteristics, and consequences, BBB breakdown generally culminates in neuronal dysfunction, neuroinflammation, and neurodegeneration. Pathological outcomes and potential for recovery are diverse (Erdő et al. 2017).



Blood-Brain Barrier, Fig. 1 The cellular structure of blood-brain barrier (BBB). The brain capillary endothelial cells connected to each other by tight junctions and adherens junctions. These cells are surrounded with pericytes and astrocyte end feet. Neurons and microglia cells are also contributing to the function of the BBB. (Modified from Erdő et al. 2017)



Blood-Brain Barrier, Fig. 2 Comparison of a peripheral (left) and a central (right) capillaries. While in the periphery the capillary endothelial cells are not connected tightly, there are fenestrations and intercellular space between them; in the brain there are no fenestrations but tight junction proteins between the endothelial cells. Endothelial layer and pericytes are embedded into basal membrane in the brain, and astrocytes are tightly connected to the parenchymal surface of blood microvessels (Modified from Neearti et al. 2012)

Astrocytes

Astrocytes are the most abundant cell type in the brain and display a fibroblast-like morphology within gray matter (Raff et al. 1983); however, this morphology can be influenced by their CNS location and associations with other cell types. Astrocyte end feet cover over 99% of cerebral capillaries (Abbott et al. 2006), leading to critical cell-cell interactions that directly modulate and regulate BBB characteristics. Several studies have demonstrated that astrocytes play a vital role in maintenance, and perhaps induction, of BBB characteristics (Ronaldson and Davis 2012).

Astrocytes are fundamental for homeostasis, defense, and regeneration of the CNS. Loss of astroglial function and astroglial reactivity contributes to the aging of the brain and to neurodegenerative diseases. Changes in astroglia in aging and neurodegeneration are highly heterogeneous and region-specific.

Several inducing factors secreted by astrocytes have been identified, including tumor growth factor-beta (TGF-β), glial cell-derived neurotrophic factor (GDNF), and basic fibroblast growth factor (BFGF), which are involved in induction and regulation of the BBB phenotype. Additionally, astrocytes can regulate brain microvascular permeability via Ca^{2+} signaling involving astrocyte-endothelial gap junctions and purinergic transmission. Astrocytes play a critical role in preventing excitotoxicity induced by acute elevations of glutamate in the brain. This is mediated via expression of astrocyte glutamate transporters excitatory amino acid transporters (EAAT1 and EAAT2) that are responsible for glutamate uptake into the astrocyte cell, thus reducing glutamate levels in the parenchyma.

Lipoproteins in plasma transport lipids between tissues; however, only high-density lipoproteins (HDL) appear to traverse the BBB; thus, lipoproteins found in the brain must be produced within the central nervous system. Apolipoprotein E (ApoE) and ApoJ are the most abundant apolipoproteins in the brain, which are mostly synthesized by astrocytes. ApoE has an important role in the pathology and BBB injury during the development of Alzheimer's disease.

There is also evidence that astrocytes accumulate in the normal aging brain, increasing in both number and size. These astrocyte changes in normal brain aging may contribute to the exacerbated injury response and poorer outcomes observed in older traumatic brain injury patients. Astrocyte morphology changes, and glial fibrillar acidic protein (GFAP) expression increases progressively during aging in humans and rodents.

Pericytes

Pericytes are multifunctional mural cells of the microcirculation that wrap around the endothelial cells that line the capillaries and venules throughout the body. Pericytes are embedded in basement membrane, where they communicate with endothelial cells of the body's smallest blood vessels by means of both direct physical contact and paracrine signaling. Pericytes are BBB associated cells. During aging, pericytes show ultrastructural changes such as vesicular and lipofuscin-like inclusions: an increased size of mitochondria and a foamy transformation. In the aging human brain degeneration and loss of pericytes were shown. Pericytes in aging brain begin to lose their contact to the EC and begin to migrate. This behavior seems to be a response of the pericytes to a yet unidentified age-related effect. Platelet-derived growth factor (PDGF) released by ECs is known to bind to the PDGF receptor expressed by pericytes and to attract pericytes to ECs (Erdő et al. 2017). If this ligand-receptor system is disturbed, the functional integrity of the vessel is impaired. With age, this system could deteriorate as well, but data are not available as yet.

Pericyte injury and possibly early degeneration correlate with increased BBB permeability within the hippocampus, a region known to be affected by pericyte loss and BBB breakdown on postmortem tissue analysis in Alzheimer's disease.

Microglia

Microglia, the primary immune cells of the brain, are ubiquitously distributed in the CNS and are activated in response to systemic inflammation, trauma, and several CNS pathophysiologies. This cell type is not the basic element of the BBB, but it has an impact on the BBB function and integrity. Microglia present with a ramified morphology that is characterized by a small soma and fine cellular processes during their "resting state."

Microglial activation in response to pathophysiological stressors can trigger changes in cell morphology, which include reduced complexity of cellular processes and transition from a ramified morphology to an amoeboid appearance. Activated microglia produce high levels of neurotoxic and proinflammatory mediators such as nitric oxide, peroxide, tumor necrosis factor alpha (TNF-α), and proteases, all of which result in cell injury and neuronal death. As immune cells microglia scavenge apoptotic cells, tissue debris after trauma, or microbes. They can also act as scavengers of extracellular molecules such as amyloid-β. Activation of microglia is associated with altered TJ protein expression and increased BBB permeability.

Briefly the major physiological protective effector functions of microglia: (1) proliferation, (2) morphological transformation, (3) motility and migration, (4) intercellular communication, (5) phagocytosis, and (6) proteostasis. With aging, microglia shift their morphology and may display diminished capacity for normal functions related to migration, clearance, and the ability to shift from a proinflammatory to an anti-inflammatory state to regulate injury and repair (Erdő et al. 2017).

Neurons

There is considerable evidence for direct innervation of both brain microvessel ECs and associated astrocyte processes via distinct connections with noradrenergic, serotonergic, cholinergic, and GABAergic neurons. Therefore, neurons are in connection with the BBB and have influence on the behavior of capillary endothelial barrier. For example, studies have shown that loss of direct noradrenergic input from the locus coeruleus results in increased BBB reactivity to effects of acute hypertension, resulting in significantly increased permeability to 125-I labeled albumin.

With aging, cell senescence can contribute to BBB disintegration. The damaged BBB allows an influx of inflammatory cytokines to enter the brain. These cytokines lead to neuronal and glial failure. Several factors contribute to the deterioration of synaptic plasticity with age, and one of these factors appears to be a heightened level of activation of microglia, which may reflect impairment in the homeostatic ability of these cells with age, or an increase in responsiveness to modulatory molecules. The age-related decrease in tissue perfusion, together with the increase in BBB permeability, may alter the microenvironment in the brain; this, combined with the age-related compromised homeostatic capability of microglia, may be a significant factor in the neuroinflammatory changes which were described in the aged brain and which had an impact on synaptic function. These combined changes may contribute to the deficit in long-term potentiation (LTP) in synapses of aged animals. Ultimately, the functional neuronal changes within the brain can cause age-related diseases.

Molecular Background

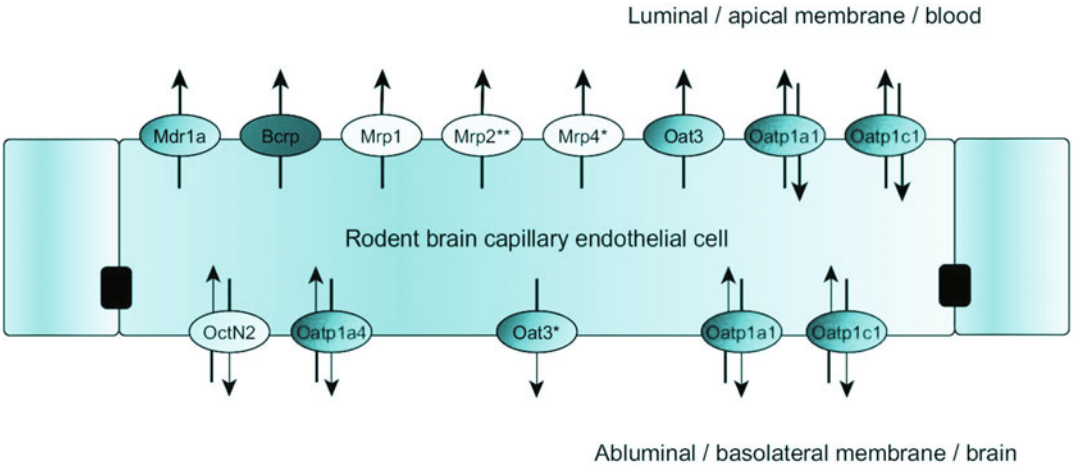
Membrane Transporters

Efflux transporters include P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidrug resistance proteins (MRPs) in the brain. Transporters that facilitate drug entry into the brain (uptake or influx transporters) include organic anion transporting polypeptides (OATPs), organic anion transporters (OATs), organic cation transporters (OCTs), nucleoside transporters, monocarboxylate transporters (MCTs), and putative transport systems for peptide transport (Sanchez-Covarrubias et al. 2014). The localization of the main membrane transporter proteins in the apical and basolateral surface of rodent ECs is presented in Fig. 3.

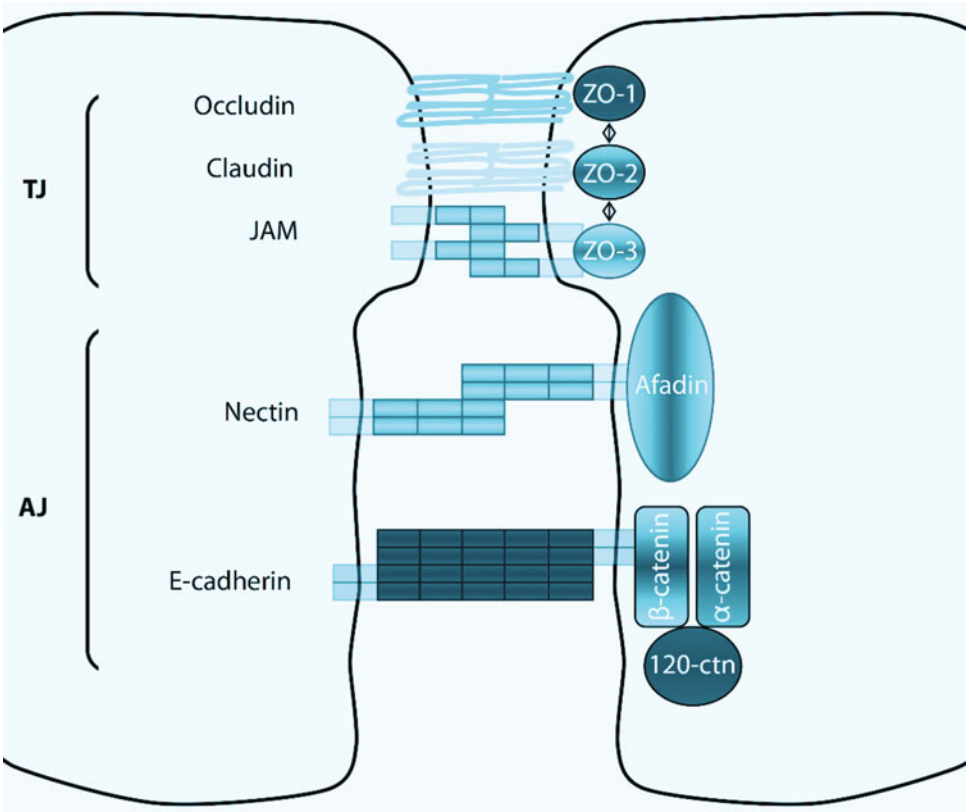
The restricted paracellular permeability of capillary EC layer is warranted by two intercellular molecular binding systems: the adherens junctions (AJ) and the tight junctions (TJ) (Fig. 4**)**.

Adherens Junctions

Adherens junctions (AJs) are found throughout the CNS microvasculature and are responsible for intercellular adherence between adjacent ECs (Vorbrodt and Dobrogowska 2003). AJs are composed of multiple protein components including vascular endothelium (VE) cadherin, actinin, and catenin. Cell-cell adhesion is mediated by homophilic interactions of VE-cadherin expressed on



Blood-Brain Barrier, Fig. 3 Schematic representation of the main efflux and uptake transporters on apical and basolateral surface of rodent brain capillary endothelial cells. (Modified from http://www.solvobiotech.com/)



Blood-Brain Barrier, Fig. 4 Schematic structure of tight junction (TJ) proteins and adherens junction (AJ) proteins making connection between the endothelial cells in the brain capillaries. (Modified from Erdő et al. 2017)

adjacent ECs. Such interactions mediate calcium-dependent cell adhesion by binding to the actin cytoskeleton. Cytoskeletal binding occurs via catenin accessory proteins. Specifically, β-catenin links VE-cadherin to α-catenin, an interaction that induces the direct binding to actin (Fig. 4).

Tight Junctions

Although disruption of AJs can result in increased BBB permeability, TJs are primarily responsible for restricting paracellular permeability at the BBB (Vorbrodt and Dobrogowska 2003). TJs form the primary physical barrier component of the BBB and function to greatly restrict paracellular entry of various endogenous and exogenous substances that can potentially be neurotoxic. Such TJs impart a high transendothelial electrical resistance (TEER) across the BBB (1500–2000 Ω cm2) that restricts free flow of ions and solutes. TJs are dynamic complexes of multiple protein constituents including junctional adhesion molecules (JAMs), occludin, claudins (i.e., claudin-1, −3, and − 5), and membrane-associated guanylate kinase (MAGUK)-like proteins (i.e., ZO-1, −2, and − 3) (Fig. 4).

Basement Membrane

Basement membranes (BMs) (it is also called as basal lamina) are considered to be uniform, thin extracellular matrix sheets that serve as a substrate for ECs. To find out whether BMs maintain their ultrastructure, protein composition and biophysical properties throughout life, the natural aging history of the human inner limiting membrane (ILM) was investigated by Candiello and coworkers (Candiello et al. 2010). Transmission electron microscopy showed that the ILM steadily increases in thickness from 70 nm at fetal stages to several microns at age 90. Furthermore, the relative concentrations of collagen IV and argin increase, and the concentration of laminin decreases with age. Force-indentation measurements by atomic force microscopy also showed that ILMs become increasingly stiffer with advancing age.

Extracellular Matrix

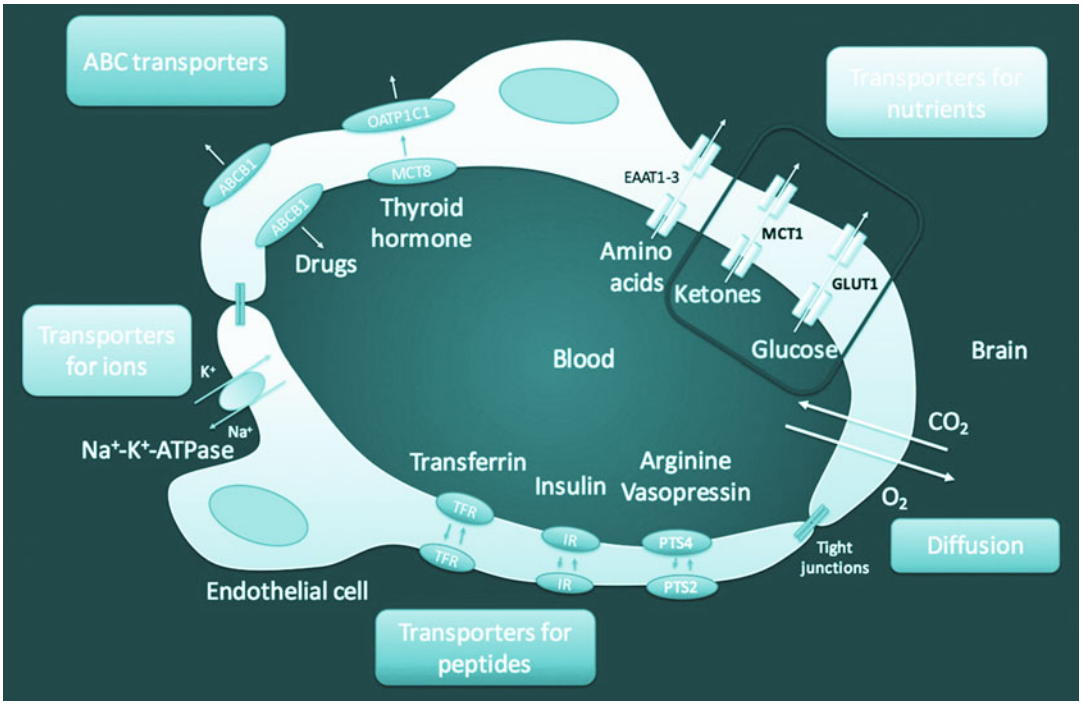
The extracellular matrix (ECM) of the basal lamina serves as an anchor for the cerebral microvascular endothelium. The anchoring function of the extracellular matrix is mediated via interactions between endothelial integrin receptors, laminin, and other matrix proteins. Disruption of extracellular matrix is associated with loss of barrier function, resulting in increased permeability. Additionally, matrix proteins have been shown to influence the expression of TJ proteins, such as occludin, suggesting that the extracellular matrix plays a role in maintaining TJ protein integrity.

Pharmacological Interventions

The number of molecules that can freely cross the BBB is very limited (O_2, CO_2, etc.). However, a huge amount of the oral or systemic drugs should reach the brain and their central target to develop their CNS effect. For this, they need to travel through the BBB (Takakura et al. 1991). To assist achieving the therapeutic goals, there are different technological, mechanistic, and physical approaches available to overcome the BBB and enhance the delivery of the compounds into the target brain regions (Bors and Erdő 2019) (Fig. 5).

How to Overcome the BBB?

Nanocarriers, nanoparticles (lipid, polymeric, magnetic, gold, and carbon-based nanoparticles, dendrimers, etc.), viral and peptide vectors and shuttles, sonoporation, and microbubbles all are able to improve the central drug delivery. The modulation of receptors and efflux transporters in the endothelial cell membrane can also be an effective approach to enhance brain exposure to therapeutic compounds. Intranasal administration is a noninvasive delivery route to bypass the blood-brain barrier because it has a direct connection to the brain through olfactory and trigeminal nerves (Erdő et al. 2018). Direct brain administration is an invasive mode to target the brain regions with therapeutic drug to reach proper concentrations locally. Nowadays, both technological and mechanistic tools are available to assist in overcoming the blood-brain barrier (Kacem et al. 2014). With these techniques more effective and even safer drugs can be developed for the treatment of devastating brain disorders.



Blood-Brain Barrier, Fig. 5 Schematic representation of different transport systems expressed on brain capillary endothelial cells. ABC transporters (ATP-binding cassette transporters) are responsible for drug transport through the BBB. MCT8 and OATP1C1 are uptake transporters of thyroid hormone. Nutrient transporters are involved in the transport of amino acids, ketones, and glucose. Transferrin receptor (TFR), insulin receptor (IR), and arginine-vasopressin receptor (PTS4) are transporters for peptides. Na^+-K^+-ATPase is responsible for ion transport. The gas molecules (O_2, CO_2) can freely diffuse across the BBB (Modified from Helbig 2015)

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Blood-Brain Interface

▶ Blood-Brain Barrier

Blood-Testis Barrier

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Synonyms

Blood-tissue barrier in testes; Seminiferous epithelial barrier; Sertoli cell barrier

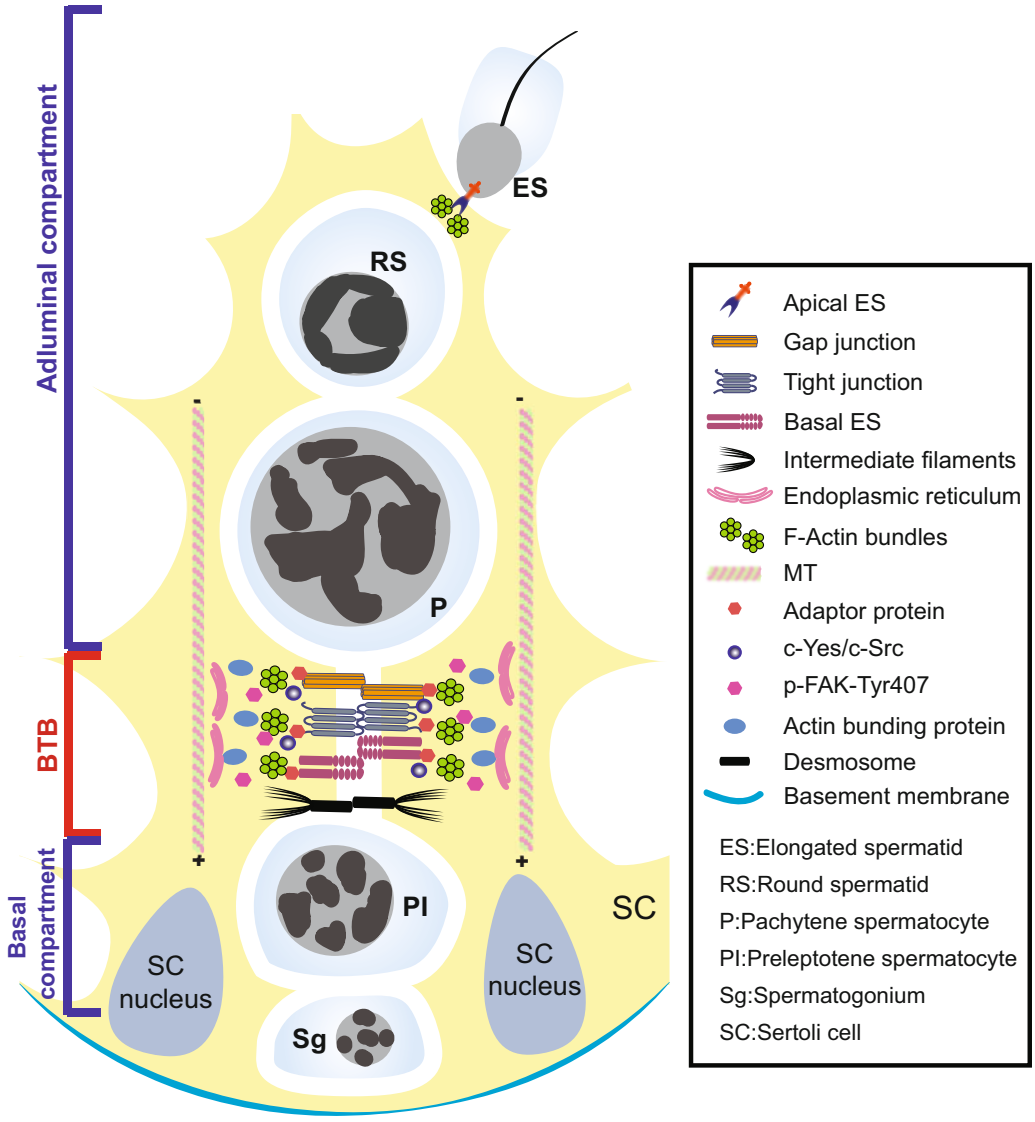
Definition

The blood-testis barrier (BTB) is a blood-tissue barrier found in the testis, located near the base of the seminiferous epithelium (Fig. 1) as noted in the cross section of seminiferous tubules which are the functional unit of the mammalian testis that produce millions of sperm daily in the male of any adult rodent or human. The BTB is created by specialized junctions between adjacent Sertoli cells near the base of the epithelium, which are composed of the actin-based tight junction (TJ), basal ectoplasmic specialization (basal ES), and gap junction, but also intermediate filament-based desmosome (Fig. 1). Thus, endothelial TJ-barrier in the microvessels located at the interstitial space between seminiferous tubules contributes virtually no barrier function to the BTB. The BTB limits paracellular (i.e., between cells) and transcellular (i.e., across cells) transport of water, electrolytes, biomolecules, and other substances across the barrier to protect postmeiotic spermatid development in the adluminal compartment of the seminiferous epithelium.

Basic Mechanisms

Background

The concept of the blood-testis barrier (BTB) in the testis originated from studies in the early 1900s when dyes injected into laboratory animals, they stained tissues in virtually all organs with the notable exceptions of the brain and the seminiferous tubules of the testis (Cheng and Mruk 2012). Since then, the concept and functions of the BTB have been considerably expanded and better understood (Cheng and Mruk 2012). In brief, the BTB restricts the paracellular and transcellular transport of biomolecules, ions, electrolytes, water, and other substances across the Sertoli cell barrier, providing the "gate" and the "fence" function. On the other hand, the BTB also divides the seminiferous epithelium into the basal and adluminal (apical) compartments (Fig. 1). To date, it is well established that the BTB is conferred almost exclusively by specialized junctions between adjacent Sertoli cells near the base of the seminiferous epithelium (Fig. 1). Interestingly, the TJs between adjacent Sertoli cells near the base of the seminiferous epithelium are supported by the testis-specific actin-rich ectoplasmic specialization (ES) designated basal ES, which together with the gap junction and the intermediate filament-based desmosome constitute the BTB (Fig. 1). The BTB is an important ultrastructure



Blood-Testis Barrier, Fig. 1 Schematic illustration of the cross section of a seminiferous tubule in the mammalian testis to show the relative location of the blood-testis barrier (BTB). The BTB divides the seminiferous epithelium into the basal and adluminal (apical) compartments. The BTB is comprised of actin-based basal ectoplasmic specialization (ES), tight junction (TJ), and gap junction (GJ), but also intermediate filament-based desmosome. Preleptotene spermatocytes transformed from type B spermatogonia at stages VII-VIII of the epithelial cycle are being transported across the BTB, while differentiating into leptotene spermatocytes, and then zygotene and pachytene spermatocytes. Following meiosis, haploid spermatids undergo spermiogenesis to produce elongated spermatids via 19 different steps in the rat testis. Once step 8 haploid spermatids arise in stage VIII tubule, they anchor onto the Sertoli cell via the apical ES which replaces gap junction and desmosome as the only anchoring device. The testis-specific apical ES adhesion protein complex α6ß1-integrin/laminin-α3/ß3/γ3 can be broken down by MMP-2 (matrix metalloprotease-2) to generate the F5-peptide, specifically released from laminin-γ3 chains. Seminiferous epithelium is in close contact with the basement membrane, wherein the structural proteins collagen α3 (IV) chains and laminin-α2 chains can also be proteolytically cleaved to generate the NC1- and LG3/4/5-peptides, respectively. These bioactive peptides, namely F5-, NC1- and LG3/4/5-peptides, in turn, regulate basal ES/BTB and apical ES remodeling to support spermatogenesis (see text for details)

in the testis to support spermatogenesis since the onset of meiosis that produces haploid spermatids in rodents and humans tightly correlates with the establishment of a functional immunological barrier.

Ultrastructural Features of the Blood-Testis Barrier (BTB)

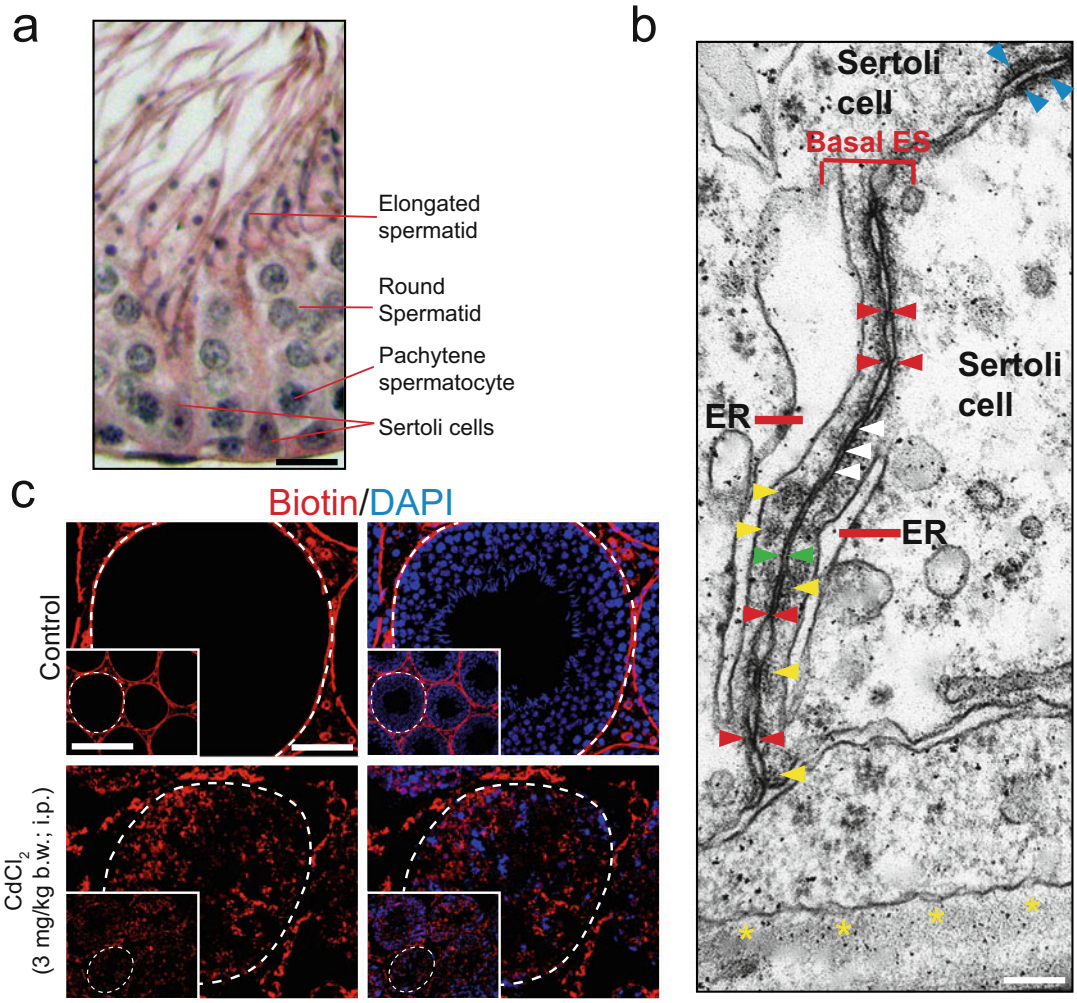
The most notable feature of the BTB under electron microscopy is the extensive network of actin filament bundles that lay across the Sertoli cell cytosol that close to the apposing plasma membranes, creating a unique ultrastructure in the testis designated the apical ES (Fig. 1). These bundled actin filament networks thus reinforce the TJs, making the BTB one of the tightest blood-tissue barriers. On the other hand, besides this network of F-actin at the basal ES/TJ to constitute the BTB, another network of microtubule (MT)-based cytoskeleton is located nearby. Interestingly, while the BTB provides the "gate" and the "fence" function to limit paracellular and transcellular transport across the barrier, it is not a static ultrastructure. The BTB undergoes rapid molecular remodeling in response to the stages of the seminiferous epithelial cycle, which is mediated by rapid internalization of BTB-associated proteins at the site, including TJ (e.g., claudins) and basal ES (e.g., N-cadherin) proteins, at stages VII–early IX of the epithelial cycle. These changes thus facilitate the transport of preleptotene spermatocytes across the immunological barrier once they transformed from type B spermatogonia. These observations also prompt investigators to examine if local regulating bioactive molecules are produced locally to modulate this specific cellular event.

Pharmacological Intervention

Regulatory Bioactive Peptides of the BTB

Studies in the past decade have shown that the testes generate several biologically active peptides at multiple sites across the seminiferous epithelium during the epithelial cycle, which exert their biological effects through several downstream signaling proteins. These biologically active peptides and their downstream signaling proteins have been shown to modulate the BTB function, either promoting BTB remodeling (i.e., to make the barrier "leaky") or BTB integrity (i.e., to make the barrier "tighter"), and other specific cellular events during spermatogenesis such as potentiating the degeneration of apical ES to facilitate the release of sperm at spermiation. At least three bioactive peptides and their unique downstream signaling proteins/pathways have been identified to date.

The F5-peptide. Studies have shown that the F5-peptide is a bioactive peptide capable of inducing Sertoli cell BTB remodeling and also apical ES degeneration, thereby promoting the transport of preleptotene spermatocytes across the BTB and the release of sperm at spermiation, respectively, based on studies in vitro and/or in vivo through the use of its recombinant protein or its overexpression following cloning of the F5-peptide cDNA into the mammalian expression vector pCI-neo (Yan et al. 2008; Su et al. 2012; Gao et al. 2016). These findings are important because they demonstrate that the F5-peptide is being used to coordinate two distinctive cellular events that take place concurrently but at the opposite ends of the epithelium. This includes the release of sperm at spermiation and BTB remodeling that take place at the luminal edge and the base of the epithelium, respectively (Fig. 2). Studies have shown that the F5-peptide is derived from domain IV of the laminin-γ3 chain (Yan et al. 2008; Su et al. 2012), an integrated component of the apical ES expressed specifically by elongated spermatids in stage VII–VIII tubules, via the action of MMP-2 (matrix metalloproteinase-2) through proteolytic cleavage (Siu and Cheng 2004). In brief, the testis is generating a bioactive peptide endogenously when the apical ES is undergoing degeneration to prepare for the sperm release at spermiation (Fig. 2), which in turn is used to potentiate further breakdown of the apical ES and to induce BTB remodeling (Su et al. 2012; Gao et al. 2016). More importantly, studies have shown that the F5-peptide exerts its regulating effects through integrin-based receptors (Su et al. 2012) via "inside-out" and "outside-in" signaling



Blood-Testis Barrier, Fig. 2 Ultrastructural features and function of the BTB. (**a**) Cross section of a stage VIII tubule, illustrating the two Sertoli cell nuclei near the basement membrane that create the BTB as ultrastructurally noted in (**b**). BTB is created by coexisting actin-based (i) tight junction (TJ) as noted by the "kisses" (annotated by the opposing red arrowheads), (ii) basal ES as shown herein is typified by the presence of actin filament bundles (yellow arrowheads) that are sandwiched in-between the endoplasmic reticulum (ER) and the adjacent Sertoli cell plasma membranes (see apposing green arrowheads), and (iii) gap junction (see white arrowheads); as well as intermediate filament-based desmosome (see blue arrowheads) typified by the presence of electron dense substances on both sides of the Sertoli cell plasma membranes. The seminiferous epithelium is in direct contact with the basement membrane (annotated by yellow asterisks). (**c**) The functional BTB is noted herein by its ability to block the diffusion of small biotin (visualized by red fluorescence) across the base of the seminiferous tubule at the site of the BTB (annotated by the dashed white line). However, in tubules from rats treated with a toxicant, cadmium chloride (at 3 mg/kg) via intraperitoneal administration which is known to damage the BTB function, biotin was able to permeate the BTB to enter the adluminal compartment. DAPI (4′,6-diamidino-2-phenylindole, blue fluorescence) stained cell nuclei in the seminiferous epithelium. Scale, 40 μm in (**a**), 0.2 μm in (**b**), and 200 μm (2 mm, inset) in (**c**)

cascade by inducing disruptive distribution and downregulating the expression of p-FAK-Y407 (Su et al. 2012; Gao et al. 2016). It is noted that p-FAK-Y407 is a signaling protein known to promote BTB integrity (Lie et al. 2012). Furthermore, the F5-peptide/p-FAK-Y407 cascade exerts its effects at the apical ES and the basal ES/BTB through disruptive cytoskeletal organization of

the actin and MT networks (Su et al. 2012; Gao et al. 2016).

The NC1-peptide. In the testis, the basement membrane is a modified form of extracellular matrix (ECM). It is universally found and located basolaterally to all epithelium and endothelium in the mammalian body, by providing structural and functional support to tissues by modulating epithelial and endothelial cell function. One of the major constituent proteins of the basement membrane in the testis is collagen α3 (IV) chains. Studies have shown that NC1 domain released from the C-terminal region of collagen α3 (IV) is a potent biologically active peptide designated NC1-peptide, capable of inducing reversible BTB disruption in vitro and/or in vivo through the use of its purified recombinant protein (Wong and Cheng 2013) or via its overexpressed following cloning of the NC1-peptide cDNA into the mammalian expression vector pCI-neo (Chen et al. 2017). Similar to the F5-peptide, besides promoting BTB remodeling, NC1-peptide is also able to induce apical ES degeneration, thereby affecting spermatid adhesion in the epithelium, which in turn leads to germ cell exfoliation (Chen et al. 2017). In the testis, NC1-peptide has been shown to exert its effects through an "outside-in" and "inside-out" signaling since the presence or absence of the signal peptide cloned into the NC1-peptide neither potentiates nor diminishes the biological effects of NC1-peptide that perturbs the Sertoli cell TJ-barrier function (Chen et al. 2017). A recent study has shown that NC1-peptide exerts its biological effects on Sertoli cell BTB function through an activation of Cdc42, but not Rho A, also involving an activation of the mTORC1/rpS6 downstream, which in turn modulates the cytoskeletal organization of F-actin and MTs in Sertoli cells (Su and Cheng 2019). On the other hand, NC1-peptide also induces an increase in endocytic vesicle-mediated protein trafficking by enhancing the endocytosis of BTB-associated protein CAR (Chen et al. 2017). These findings thus illustrate that while the F5- and NC1-peptides have similar effects on the Sertoli cell BTB function since their overexpression or the use of their recombinant proteins can both promote BTB remodeling by making the barrier "leaky," they exert their effects via distinctive signaling pathways downstream.

The LG3/4/5-peptide. Besides collagen α3 (IV) chains, the other major constituent protein of the basement membrane in the mammalian testis is the laminin-α2 chain. Using RNAi to knockdown laminin-α2 by using shRNA that targeted laminin-α2 in Sertoli cell epithelium, it was noted that this led to a transient disruption of the Sertoli cell TJ-barrier function, concomitant with disruptive organization of F-actin and MTs across the Sertoli cell cytosol (i.e., these cytoskeletons were extensively truncated across the Sertoli cell cytosol), thereby causing disruptive distribution of TJ (e.g., CAR, ZO-1) and basal ES (N-cadherin, ß-catenin) proteins at the Sertoli cell-cell interface since these proteins utilize actin for their attachments and supported by the MTs (Gao et al. 2017a). These findings also illustrate that the physiological function of laminin-α2 chain is to support BTB and spermatogenic function. More interestingly, laminin-α2 chain localized at the basement membrane could be cleaved (possibly via the action of MMP-9 at the site as earlier reported (Siu et al. 2003)) to generate an 80 kDa fragment containing LG3/4/5 domains as demonstrated by immunoblot analysis and immunofluorescence analysis, which was transported across the seminiferous epithelium via a microtubule (MT)-dependent mechanism since treatment of the testis with Taxol was shown to perturb the transport of this laminin-3/4/5 fragment from reaching the apical ES (Gao et al. 2017a). These observations thus support the notion that the testis is generating an 80 kDa LG3/4/5-peptide from the basement membrane to exert is regulatory effects at the apical ES. Subsequent study has shown that LG3/4/5-peptide exerts its effects to perturb Sertoli cell TJ-barrier function, via the use of shRNA to knockdown laminin-α2 in Sertoli cell epithelium by as much as 75%, through an activation of mTORC1/rpS6 signaling complex, including a surge in the expression of p-rpS6-S235/S236 and p-rpS6-S240/S244, and a concomitant downregulation of p-Akt1/2 (Gao et al. 2017b). Furthermore, the use of a specific p-Akt1/2 activator

SC79 is able to block the laminin-α2 knockdown-mediated cytoskeletal disorganization of F-actin and MTs (Gao et al. 2017b). This observation thus illustrates that the mTORC1/rpS6/Akt1/2 is the downstream signaling cascade used by LG3/4/5-peptide to support BTB and spermatogenic function in the testis. In brief, the NC- and LG3/4/5-peptides are utilizing the mTORC1/rpS6 signaling cascade downstream to mediate their corresponding effects to promote Sertoli cell TJ-barrier disruption (i.e., making the barrier "leaky" and integrity (i.e., making the barrier "tighter"), respectively, via an upregulation (activation) or downregulation (inactivation) of the mTORC1/rpS6 signaling complex. This thus provides a novel mechanism to induce BTB remodeling and also apical ES degeneration to support the transport of preleptotene spermatocytes across the BTB and the release of sperm at spermiation during the epithelial cycle of spermatogenesis.

Summary

The BTB is regulated by endogenous regulatory peptides produced locally in the testis during the epithelial cycle of spermatogenesis, which are being used to induce BTB remodeling to facilitate the transport of preleptotene spermatocytes across the barrier (such as the F5- and NC1-peptides), or to maintain its integrity (such as the LG3/4/5-peptide), to support spermatogenesis. These peptides are also used to modulate spermatid transport and adhesion. The bioactive peptides exert their effects through changes in the cytoskeletal organization of actin and microtubule cytoskeletons with distinctive downstream signaling pathways.

Cross-References

► A-Kinase Anchoring Proteins (AKAPs)
► Blood-Brain Barrier

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Blood-Tissue Barrier in Testes

▶ Blood-Testis Barrier

BMH1

▶ 14-3-3 Proteins

BMH2

▶ 14-3-3 Proteins

Bone Metabolism

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Synonyms

Bone Remodeling; Bone Turnover; Skeletal Metabolism; Skeletal Turnover

Definition

Bone metabolism involves the processes of ***bone formation*** and ***bone resorption***, the key actions by which skeletal mass, structure, and quality are accrued and maintained throughout life. In the mature skeleton, anabolic and catabolic actions are balanced through tight regulation of the activity of bone forming cells (***osteoblasts***) and bone resorbing cells (***osteoclasts***). This process is called ***coupling*** and mediated through the action of circulating ***osteotropic hormones,*** locally active factors such as cytokines, growth factors, and small glycoproteins, some of which are secreted by **osteocytes.**

Basic Mechanisms

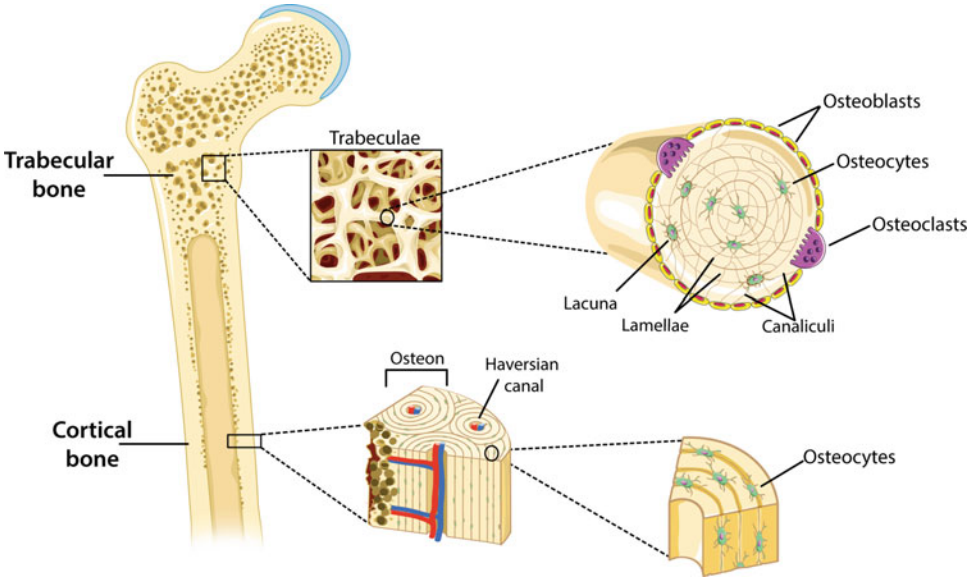
Biochemical Composition of Bone

Bone is composed of approximately 50% to 90% mineral (mainly calcium phosphate in the form of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] crystals), 5% to 40% organic matter (i.e., cells, collagen, and noncollagenous proteins) and water. The mineral component of bone provides mechanical strength and rigidity to the skeleton. The highest mineral content is found in the small bones of the middle ear, which need to be stiff and unyielding in order to transmit sound. Most other bones have a much lower mineral content, which gives them elasticity and the capacity to shock absorb. In these bones, the mineral is also sufficiently labile to be easily removed, enabling it to act as a store for supporting systemic calcium and phosphorus homeostasis.

The organic extracellular matrix of bone, termed "osteoid," is predominantly composed of 90% type I collagen, which assembles into fibrils that are covalently cross-linked to generate tensile strength. Of the noncollagenous proteins, osteocalcin, bone sialoprotein (BSP), and osteopontin are the most abundant. Most of the noncollagenous proteins play important roles in the organization of the extracellular matrix (cell-cell and cell-matrix attachment, cell migration, growth, development, fibril formation, and mineralization). Besides these components, several specific enzymes and small peptides are an integral part of the organic matrix and play an important role in skeletal metabolism (see below). The calcium-collagen-cell composite ensures the two main functions of bone: providing a structural framework and a reservoir for mineral ions.

Bone Structure

Most bones of the human skeleton are composed of two structurally distinct types of tissue: cortical (dense, compact) and trabecular (cancellous,



Bone Metabolism, Fig. 1 Structure of bone tissue

spongy) bone (Fig. 1). Both types contain the same elements: cells (osteocytes) embedded in a mineralized matrix and connected by small canals ("canaliculi"). In cortical bone, which makes up 85% of the skeleton, these components form elongated cylinders of concentric lamellae surrounding a central blood vessel (called osteon or Haversian system). Cortical bone constitutes the cortex of many bones, shows a high degree of mineralization, a comparatively low number of cells, and provides the framework for the interior cancellous parts of bone. In contrast, trabecular (or cancellous) bone is organized in a sponge-like network of thin, interconnected spicules (trabeculae) with a larger number of cells (**osteoblasts** and **osteoclasts**) distributed over a huge surface. Consequently, the metabolic rate of trabecular bone is much higher than that of cortical bone.

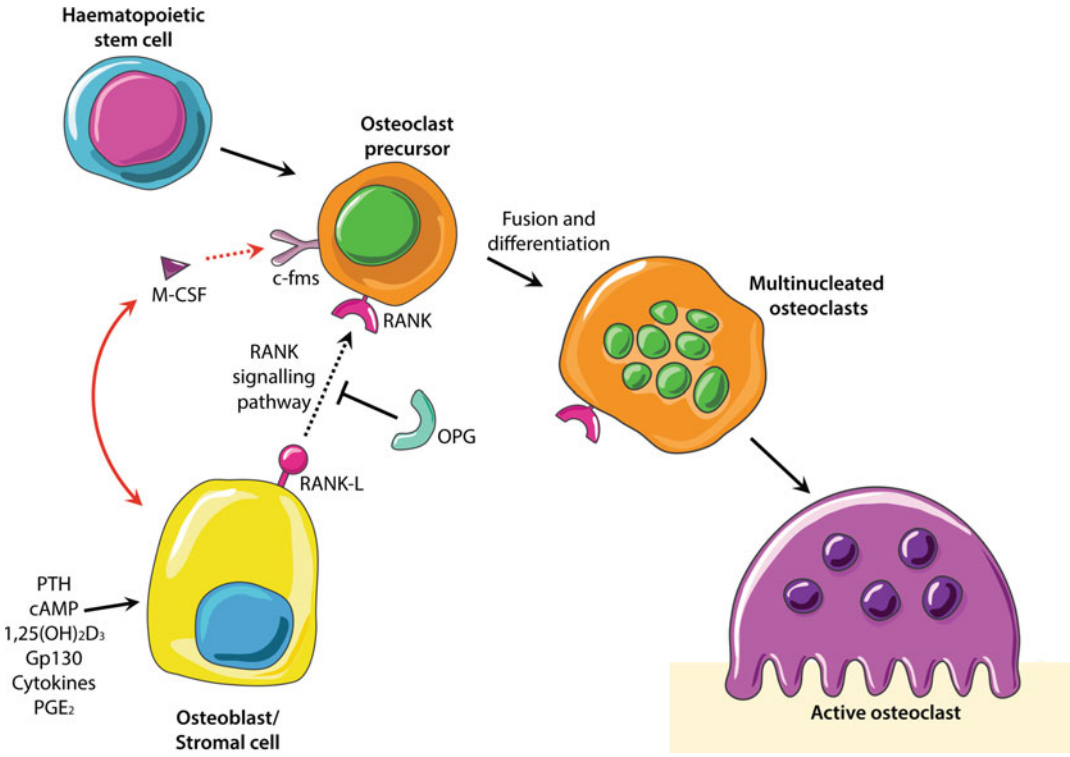
Mechanisms of Bone Remodeling

Bone is a metabolically active tissue that undergoes constant *remodeling*. The term "*modeling*" is restricted to the period of skeletal growth, when the size and shape of the bone is determined. *Bone remodeling*, on the other hand, continues throughout adult life and results in renewal of the entire skeleton every 7–10 years. Healthy remodeling requires that bone formation and resorption are in balance with each other, that is, osteoblasts replace the same amount of bone previously removed by the osteoclasts. Normal bone remodeling is necessary for repair and maintenance of normal bone structure in response to continuous stress and strain, as well as for mineral homeostasis (e.g., release of calcium in times of dietary calcium deficiency) and systemic fuel metabolism. While bone formation is achieved through cells of the osteoblast lineage (osteoblasts, bone lining cells, and osteocytes), osteoclasts are responsible for bone resorption. The coordinated actions of these cells together form the bone remodeling unit (BRU, also known as basic multicellular unit, BMU).

Osteoblast lineage cells – Osteoblasts are derived from mesenchymal stem cells (MSCs) that first differentiate into preosteoblasts and then into mature, bone matrix producing osteoblasts. The key molecular switch in the commitment of MSCs to the osteoblast lineage is "runt-related transcription factor 2" (*RUNX2*), which has multiple upstream regulators such as the Notch and Wnt signaling pathways. Canonical Wnt signaling regulates most aspects of osteoblast

physiology including cell commitment, differentiation, maturation, bone matrix formation, mineralization, and apoptosis. As preosteoblasts transition to mature osteoblasts, they increase the expression of osterix and secrete bone matrix proteins including osteocalcin, bone sialoprotein (BSP) I/II, and collagen type I. Resting or quiescent osteoblasts become "bone lining cells," covering the bone surfaces where neither bone resorption nor formation occurs. These cells serve as a reservoir for bone forming cells and reacquire their secretory activity during the next bone remodeling cycle. Osteoblasts trapped and embedded in the mineralized matrix are called osteocytes and are important for many properties of living bone (Fig. 1).

Osteoclasts – Osteoclasts are large, terminally differentiated multinucleated cells that originate from cells of the hematopoietic stem cell lineage (macrophages). Osteoclast formation is controlled by circulating hormones such as parathyroid hormone (PTH), 1,25-dihydroxyvitamin D_3, and estrogens, as well as by local growth factors (e.g., transforming growth factor (TGF) - β) and cytokines generated in the bone and bone marrow microenvironment. This control is mostly indirect as the receptors for majority of these factors are located on the osteoblast surface. Osteoblastic cells therefore control osteoclast formation through the expression of various stimulatory and inhibitory factors. For example, osteoblasts and stromal cells secrete macrophage colony-stimulating factor (M-CSF), which binds to the colony-stimulating factor-1 receptor (c-fms) on preosteoclasts, resulting in their differentiation to mature osteoclasts. Osteoblasts and osteocytes also secrete a cytokine known as receptor activator of nuclear factor kappa-B (RANK) ligand (RANKL), which acts by binding to its receptor, RANK, on osteoclast precursors to induce these cells to fuse and differentiate into mature osteoclasts. RANKL also stimulates existing osteoclasts to increase their bone resorbing activity (Fig. 2). The activity of RANKL is



Bone Metabolism, Fig. 2 Mechanisms of osteoclast activation

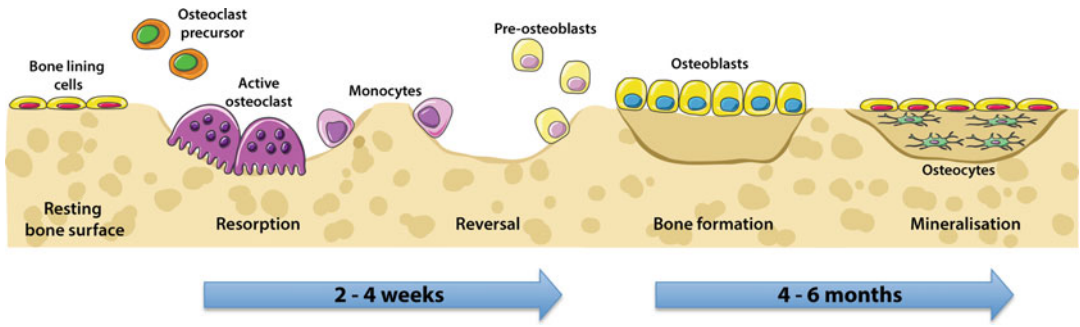
regulated by another osteoblast-derived protein, osteoprotegerin (OPG), which is a decoy receptor for RANKL, preventing its binding to RANK.

The bone remodeling cycle – Initiation of bone remodeling is the first important step that ensures remodeling only takes place when required. In *targeted remodeling*, the initiating signal comes from osteocytes that use their network of dendritic processes to receive and signal to other cells. For example, micro-damage to the matrix disrupts the osteocyte canaliculi, which in turn induces apoptosis of the osteocyte at that site. This leads to the release of paracrine factors that increase local angiogenesis and recruitment of osteoclast and osteoblast precursors. In contrast, *nontargeted remodeling* is not directed towards a specific site and refers to remodeling in response to systemic changes in hormones such as PTH, allowing access to bone calcium stores.

Once initiated, the remodeling cycle occurs in a highly regulated manner involving activation, resorption, reversal, formation, and termination, which occur over the course of 120–200 days (Fig. 3). Firstly, osteoclast precursor cells are recruited from the circulation and ***activated*** as described above. Next, bone lining cells separate from the underlying bone by forming a raised canopy over the site to be resorbed. Activated osteoclasts then attach to the exposed bone surface to form a sealed cavity, known as the resorption pit. This "construction site" is therefore well isolated from the surrounding bone.

The actual process of bone ***resorption*** involves rearrangement of the osteoclast cytoskeleton, resulting in the development of a specialized membranous structure referred to as the "ruffled border." It is through this structure that acids (H+) and proteases (cathepsin K and matrix metalloproteases) are secreted, dissolving both the mineral and matrix components of bone and ultimately creating a resorption pit, also known as Howship's lacuna. The minerals and protein fragments released during this process are absorbed into the osteoclast by endocytosis and are partly processed and later secreted from the distal side of the osteoclast, from where they eventually reach the circulation. The resorption phase is terminated by programmed cell death, ensuring that excess resorption does not occur.

The ***reversal*** phase couples bone resorption to bone formation at the remodeling site. Mononuclear cells, including monocytes and preosteoblasts, occupy the resorption lacuna. Identifying the coupling signals and mechanisms coordinating osteoclast and osteoblast activities has been the subject of ongoing research (see Sims and Martin (2020) for review). Several osteogenic molecules, likely released by osteoclasts, have been identified. These include factors released from the bone matrix during bone resorption (e.g., TGF-β, insulin-like growth factor 1 (IGF-1), platelet-derived growth factors) that stimulate the expansion and differentiation of osteoprogenitor cells, as well as coupling factors secreted by mature osteoclasts that are independent of its resorptive activity (e.g., cardiotrophin-1, BMP6, Wnt10b, sphingosine-1-phosphate). Factors expressed on the osteoclast cell membrane (e.g., EphrinB2 and Semaphorin



Bone Metabolism, Fig. 3 The bone remodeling cycle

D) that require cell-cell contact with osteoblast lineage cells have also been identified.

The ***formation*** stage lasts approximately 3–4 months. New bone formation can be divided into two parts. Once osteoblasts are fully matured, they first synthesize and secrete collagen (mainly type I collagen) and noncollagen proteins such as osteocalcin, osteonectin, BSP, osteopontin, and proteoglycans, which together form the organic bone matrix or "osteoid." Osteoblasts then co-regulate osteoid mineralization, which occurs in two steps: the relatively rapid primary mineralization, and the much slower process of secondary mineralization. The final phase of the formation process is cessation of osteoblast activity. It is not known how this level of spatiotemporal regulation is achieved. One possibility is that factors produced during osteoblast differentiation decrease osteoblast activity. One such factor could be TGF-β since active TGF-β decreases differentiated function in osteoblasts.

The remodeling cycle reaches the ***termination*** stage once mineralization is complete. At this stage, the mature osteoblasts can undergo apoptosis, change into bone lining cells, or become embedded within the bone matrix and terminally differentiate into the master of the bone universe, the osteocyte. Osteocytes play a key role in signaling the end of the remodeling process via secretion of antagonists of osteoblastogenesis, specifically antagonists of the Wnt signaling pathway such as sclerostin.

Hormonal Regulation of Mineral Homeostasis and Bone Remodeling

As bone remodeling is part of the body's mineral (calcium/phosphate) homeostasis as well as a mechanism for repair and adaptation, all cellular events are regulated by systemic and local factors. The latter include autocrine and paracrine signals produced by the bone cells (e.g., growth factors, prostaglandins, and cytokines) but also molecules released from the bone matrix during bone resorption (e.g., glycoproteins, collagen fragments, osteocalcin, and certain growth factors laid down in the osteoid during bone formation). Systemic (hormonal) modulators include parathyroid hormone, 1,25-dihydroxyvitamin D, calcitonin, glucocorticoids, androgens and estrogens, insulin-like growth factors, thyroid hormone, etc.

Parathyroid hormone (PTH) plays a central role in the regulation of bone remodeling and mineral homeostasis. PTH is an 84-amino acid polypeptide secreted by the parathyroid glands in response to reductions in blood levels of ionized calcium. The primary physiological effect of PTH is to increase serum calcium concentrations. It does so by increasing renal calcium reabsorption (while increasing phosphate excretion), and stimulating conversion of the inactive metabolite of vitamin D, 25-hydroxy-vitamin D, to its biologically active metabolite 1,25-dihydroxyvitamin D. PTH acts on bone to acutely increase bone resorption and thus release skeletal calcium from the skeleton into the circulation.

The steroid hormone **1,25-dihydroxyvitamin D** increases both intestinal calcium absorption and bone resorption, but has also been found to stimulate bone formation. In contrast, **calcitonin** rapidly inhibits osteoclast activity and thus decreases serum calcium levels. Calcitonin is synthesized in the parafollicular C-cells of the thyroid and acts on osteoclasts by increasing the intracellular cyclic AMP content of the cell. This then causes contraction of the ruffled border.

Estrogen binds directly to estrogen receptors (ER) α and β on bone cells. It plays a crucial role in bone homeostasis and is a central regulator of osteoclast activity and life span. The decline in estrogen levels postmenopause is considered the main cause of bone loss and osteoporosis in older women. At physiological (premenopausal) levels, estrogens reduce bone loss by inhibiting the generation of new osteoclasts, reducing the activation frequency and promoting apoptosis of mature osteoclasts. Some of the effects of estrogens seem to be mediated via the modulation of growth factors and cytokines, while others are associated with binding to at least two different estrogen receptors (ERα, ERβ).

Androgens, like estrogen, are essential for skeletal development and maintenance of bone mass and can act directly and indirectly through both the androgen receptor and ERα on bone cells. Testosterone's actions are mostly "anabolic," that is, supporting bone formation. However, as

testosterone is metabolized via the cytochrome P450 aromatase enzyme complex into 17-β-estradiol, it can also have antiresorptive effects.

The hypothalamic-pituitary-thyroid axis plays a major role in bone mineral homeostasis, skeletal development and peak bone mass achievement. **Thyroid hormones** increase the metabolic rates of both osteoclasts and osteoblasts and thus, increase the rates of both bone resorption and formation. With excessive amounts of thyroid hormone, there is increased bone surface engaged in bone remodeling, which in turn can result in low bone mineral density.

At physiological (endogenous) levels, **glucocorticoids** direct mesenchymal stem cells to differentiate towards osteoblasts, and thus increase bone formation via upregulation of the Wnt/β-catenin pathway in mature osteoblasts (Zhou et al. 2009). However, at supraphysiological concentrations (glucocorticoid therapy, endogenous hypersecretion), glucocorticoids inhibit bone formation. The main targets of glucocorticoids in bone are the cells of the osteoblast lineage: thus, high-dose glucocorticoids suppress osteoblast activity within hours of administration, and induce osteoblast and osteocyte apoptosis (Rauch et al. 2010; Weinstein et al. 1998). Excess glucocorticoids may also indirectly promote bone resorption via changing RANKL/OPG balance, sclerostin action, and decreasing renal calcium reabsorption and intestinal calcium absorption. From a pathophysiological point of view, glucocorticoid-induced osteoporosis (GIO) is a disease entity completely distinct from postmenopausal osteoporosis.

Pharmacological Intervention

Since the 1980s, numerous effective pharmacological strategies have been developed to modulate bone turnover, cell differentiation, or activity to prevent or treat bone loss/osteoporosis or metabolic bone diseases such as Paget's disease of bone (Table 1). Agents that inhibit osteoclast formation and/or activity are usually classified as antiresorptive or anticatabolic drugs, while stimulators of osteoblast formation and/or activity are

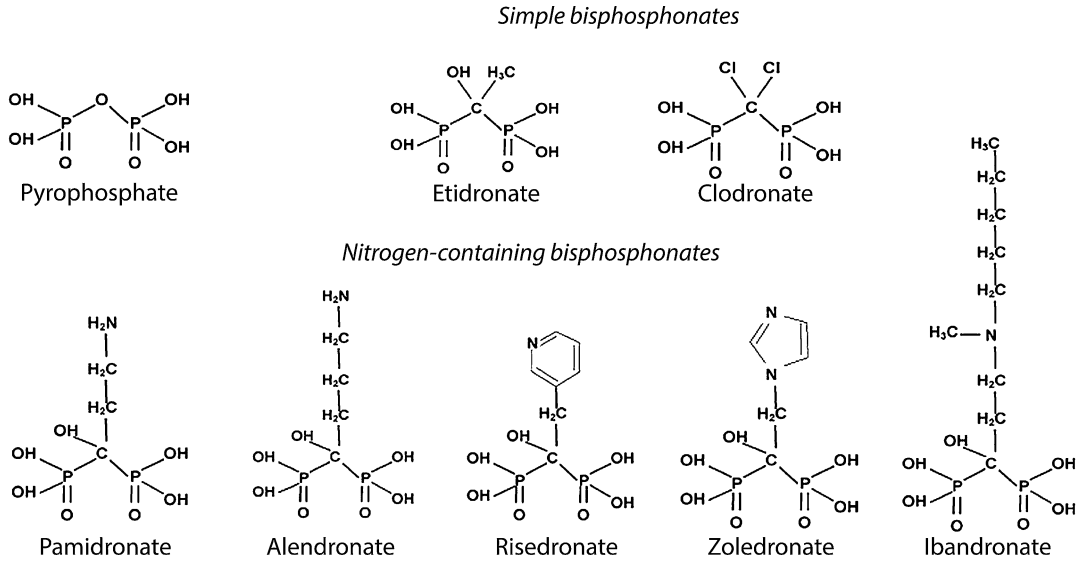
Bone Metabolism, Table 1 Current and future agents to modulate bone metabolism

Inhibitors of osteoclast activity	Stimulators of osteoblast activity
Agents currently approved in multiple countries for pharmacotherapy	
Bisphosphonates (e.g., alendronate, risedronate, zoledronic acid, ibandronate, etc.)	Parathyroid hormone and parathyroid hormone analogs (e.g., teriparatide)
RANKL antibody (denosumab)	Sclerostin antibody (romososumab)
Selective estrogen receptor modulators (SERMs) (e.g., raloxifene)	Calcitrol (1,25dihydroxy vitamin D) and analogs (e.g., 1-alpha-hydroxy vitamin D; very limited use; not for osteoporosis)
Strontium salts (e.g., Sr Ranelate; very limited use)	Androgens (limited use)
Estrogens	High dose estrogens (limited use)
Calcitonin and synthetic analogs (very limited use)	PTH-rP and PTHrP analogs (currently limited use)
	Fluorides (very limited use)
Agents currently under investigation	
Enzyme inhibitors (e.g., MMPs, cathepsin K)	Growth factors (e.g., GH, IGFs FGFs)
Calcium receptor modulators	Prostaglandins (PGs) and PG mimetics
Osteoprotegerin	Endothelins and analogs
Anticytokines (e.g., IL-1, TNFs, IL-1 Rc constructs)	Amylin and analogs
Protein pump inhibitors	Mechanoreceptor modulators (e.g., glutamate)
Nitric oxide modulators	Purinergic modulators
Adhesion molecule inhibitors	Proteosome inhibitors
	Intracellular signaling targets (e.g., SMADs)

called anabolic agents. Some compounds, such as bioactive vitamin D or estrogens, seem to affect both bone formation and bone resorption. Over the past 10 years, more and more specific agents have been developed that target a single molecule key to either of the two processes. However, due to the physiological coupling of bone formation and resorption, it has been difficult to reach the holy grail of osteoporosis therapy, that is, bone anabolic activity without an increase in bone resorption, or reduction in bone resorption without a synchronous decrease in bone formation activity.

Bisphosphonates are counted amongst first line treatments of benign and malignant bone diseases. Bisphosphonates are analogs of pyrophosphate (Fig. 4), an effective endogenous inhibitor of ectopic mineralization. All bisphosphonates have the phosphate-carbon-phosphate structure, with side chains bonded to the carbon. These side chains confer different properties, varying in their affinity to bind to hydroxyapatite and enzymes within the osteoclast, and thus their ability to resorb bone. Bisphosphonates can be classified into at least two groups with different mechanisms of action. The simpler nonaminobisphosphonates such as etidronate and clodronate can be metabolically incorporated into intracellular analogs of ATP and cause cell death. The newer and more potent nitrogen-containing bisphosphonates (e.g., alendronate, risedronate, ibandronate, and zoledronate) have a different mechanism of action. After entering the cell by fluid-phase endocytosis, they inhibit farnesyl pyrophosphate synthase, an enzyme in the mevalonate pathway that is necessary for osteoclast ruffled border formation. Bisphosphonates have been shown to be highly effective in the treatment of osteoporosis, cancer bone metastasis, multiple myeloma, and Paget's disease of bone. While generally very well tolerated, very rare adverse effects are osteonecrosis of the jaw (ONJ) and atypical femur fractures (AFF). ONJ is mostly seen in patients with multiple myeloma or bone metastases who receive high-dose intravenous bisphosphonate treatment in conjunction with other cell-toxic agents (chemotherapy, glucocorticoids) (Bamias et al. 2005). Atypical femur fractures are more often seen in osteoporotic patients on longer-term oral or intravenous bisphosphonate therapy.

The anti-RANKL antibody, denosumab, prevents RANKL from binding to its receptor RANK



Bone Metabolism, Fig. 4 Chemical structure of pyrophosphate, simple bisphosphonates, and nitrogen-containing bisphosphonates

on osteoclast precursors, thus inhibiting osteoclastogenesis, activation, and survival and thereby increasing bone mass and strength. Denosumab is monoclonal antibody administered by subcutaneous injection and has been demonstrated in numerous studies to reduce fracture risk in patients with osteoporosis, as well skeletal related events in patients with cancers metastasis to bone (Cummings et al. 2009; Gul et al. 2016).

Estrogens (usually in the form of hormone replacement therapy; HRT) have been shown to reduce the risk of osteoporotic fracture (and of colon cancer) but carry a small risk of breast cancer and cardiovascular events after prolonged use. While effective in controlling menopausal symptoms, the long-term use of estrogens to prevent bone loss or treat osteoporosis is not being recommended, particularly in older women. **Selective estrogen receptor modulators (SERMs)** have been designed to selectively activate estrogen receptors (ERs) in bone. Tamoxifen, a triphenylethylene compound used in the treatment of breast cancer, has long been known to moderately inhibit bone loss, while at the same time stimulating endometrial proliferation. In contrast, raloxifene reduces vertebral but not non-vertebral fracture risk in postmenopausal women without the unwanted endometrial effects (Ettinger et al. 1999). In fact, raloxifene has been shown to significantly reduce the risk of breast cancer recurrence. Estrogens and SERMs are not potent enough to inhibit the grossly exaggerated osteoclast activity in malignant or Paget's disease of bone. Combined SERM and estrogen drugs are also being developed (also known as **tissue-selective estrogen receptor complexes; TSECs**). The molecular mechanism is difficult to delineate as both components of the TSEC compete for the same ligand-binding pocket on the ER.

The antisclerostin antibody, romosozumab, is a potent anabolic agent targeting the Wnt signaling pathway. Romosozumab works by preventing the inhibitory actions of osteocyte-derived sclerostin on osteoblast activity. Unlike other Wnt antagonists such as dickkopf-related protein-1 (Dkk-1) or secreted frizzled-related protein-1 (sFRP-1) that are widely expressed, sclerostin is predominantly expressed in the bone. Twelve months of treatment with romosozumab, an injectable humanized monoclonal antibody, has been shown to increase bone formation without significant changes in bone resorption and results in rapid gains in bone mass (McClung et al. 2014; Chavassieux et al. 2019). It has been approved in several countries for the prevention and treatment of postmenopausal osteoporosis.

Cathepsin K inhibitors target osteoclasts to impair bone resorption by inhibiting the major protease response for type I collagen degradation. Several cathepsin K inhibitors have been developed and clinically evaluated; however, they have not been pursued due to safety concerns. For example, odanacatib was a promising agent to treat osteoporosis and bone metastasis as it substantially reduced risk of clinical vertebral fractures; however, its multibillion dollar development program was terminated in 2016 due to an increased risk of stroke found in clinical safety analyses (Mullard, 2016). MIV-711 is another cathepsin K inhibitor, which is currently still under development for the treatment of osteoarthritis.

Due to its strong and acute antiresorptive effect, **calcitonin** has been used in the past to treat a variety of metabolic bone diseases, including osteoporosis, hypercalcaemia of malignancy, and Paget's disease of bone, However, calcitonin has all but disappeared from clinical use as more potent and better tolerated drugs have become available.

PTH and PTH analogs have a dual effect on bone cells depending on the temporal mode of administration. Given intermittently, PTH and the PTH analog teriparatide stimulate osteoblast activity and lead to substantial increases in bone density. In contrast, when given (or secreted) continuously, PTH stimulates osteoclast mediated bone resorption and suppresses osteoblast activity. The anabolic effects of PTH on osteoblasts are probably both direct and indirect via growth factors such as IGF-1 and TGF-β. The multiple signal

transduction pathways mediating the effects of PTH on bone cells include activation of cyclic AMP, intracellular protein phosphorylation, activation of phospholipase C, protein kinase C, tyrosine kinase c-src, and the generation of inositol 1,4,5-triphosphate (IP3).

Teriparatide is a fragment of full length PTH (1-34 PTH) and has been shown to increase bone mass and reduce the risk of fractures in men and women with osteoporosis (Neer et al. 2001). The drug is administered via daily subcutaneous injection over 24 months; however, its anabolic effect diminishes with time, along with an increase in bone resorption. Abaloparatide is a highly selective and high affinity PTH-related protein (PTHrP) analog, which was approved by the US FDA in 2017 to treat postmenopausal osteoporosis.

Vitamin D and calcium supplements are often used as an adjuvant in the treatment of osteoporosis. In patients with clear calcium deficiency, oral calcium at doses of 1000–1200 mg per day corrects a negative calcium balance and suppresses PTH secretion. Sufficient calcium intake is important for the accrual of peak bone mass in the young. Its role in the prevention and treatment of osteoporosis (i.e., the effect of calcium supplements on actual fracture risk) is controversial.

Cholecalciferol (vitamin D_3) is synthesized in the skin through UV radiation (sun exposure) and is the main source of vitamin D in humans. Ergocalciferol (vitamin D_2) is derived from the diet and plays a minor role in human vitamin D supplies. Both forms are metabolized in the liver to 25-hydroxyvitamin D (calcidiol). The latter is the storage form of vitamin D and is believed to have little biologic activity. The active form of vitamin D is generated in the kidney through the hydroxylation of C1, leading to 1,25-dihydroxyvitamin D. This last step is tightly controlled by a number of regulators such as PTH. 1,25-dihydroxyvitamin D (calcitriol) is a potent steroid hormone with almost countless effects throughout the body. In bone, calcitriol has differentiation inducing as well as activating effects on both osteoblasts and osteoclasts. It also increases the absorption of calcium from the gut.

Data from clinical trials show that the daily supplementation with 500–1200 mg of calcium and 700–1000 IU of oral Vitamin D reduce the rate of bone loss in postmenopausal women (Tang et al. 2007; Boonen et al. 2007, DIPART (vitamin D Individual Patient Analysis of Randomized Trials) Group, 2010). Whether this treatment regimen also reduces the risk of fragility fractures in community-living younger men and women is rather controversial. If any, the effect in this population is minor. However, in the elderly, supplementation with oral calcium and vitamin D reduces the risk of hip and nonvertebral fracture by 30–40%. Warning: So-called "active" vitamin D-metabolites (i.e., calcitriol or 1-alpha calcidol) have no place in osteoporosis therapy and are outright dangerous drugs due to their very narrow therapeutic window (hypercalcaemia, renal calculi).

Strontium salts have long been under investigation as anabolic agents for bone. Strontium is naturally present in bone in trace amounts, with around 100 μg in every gram of bone. Given therapeutically, strontium substitutes for calcium in hydroxylapatite crystals, which is the major reason for the clinically observed, apparent increase in bone density. In humans, studies in high-risk patients have shown borderline reductions in vertebral fracture risk (Reginster et al. 2012). The mechanism of action is largely unknown and may involve modulation of the calcium receptor or calcium channels. Strontium ranelate has been discontinued in many countries due to associated life-threatening allergic reactions and increased risk of cardiac events.

Fluoride ions stimulate bone formation by a direct mitogenic effect on osteoblasts mediated via protein kinase activation and other pathways. Further to these cellular effects, fluorides alter hydroxyapatite crystals in the bone matrix. In low doses, fluorides induce lamellar bone, while at higher doses abnormal woven bone with inferior quality is formed. The effect of fluorides on normal and abnormal (e.g., osteoporotic) bone

therefore depends on the dose administered. Fluoride salts for the treatment of osteoporosis have been popular in some countries, but like calcitonin and strontium have virtually disappeared from clinical use.

The evolution of our understanding of bone metabolism has simultaneously driven considerable progress in the development of effective therapeutics to treat bone disease. Although several antiresorptive and anabolic drugs are now available, their use (or prolonged use) is hindered by side effects and long-term efficacy, thus the need for continuous drug discovery and development. Several new agents that target bone cell signaling/activity and local and systemic regulators of bone remodeling are currently under investigation (see Table 1).

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Bone Morphogenetic Proteins

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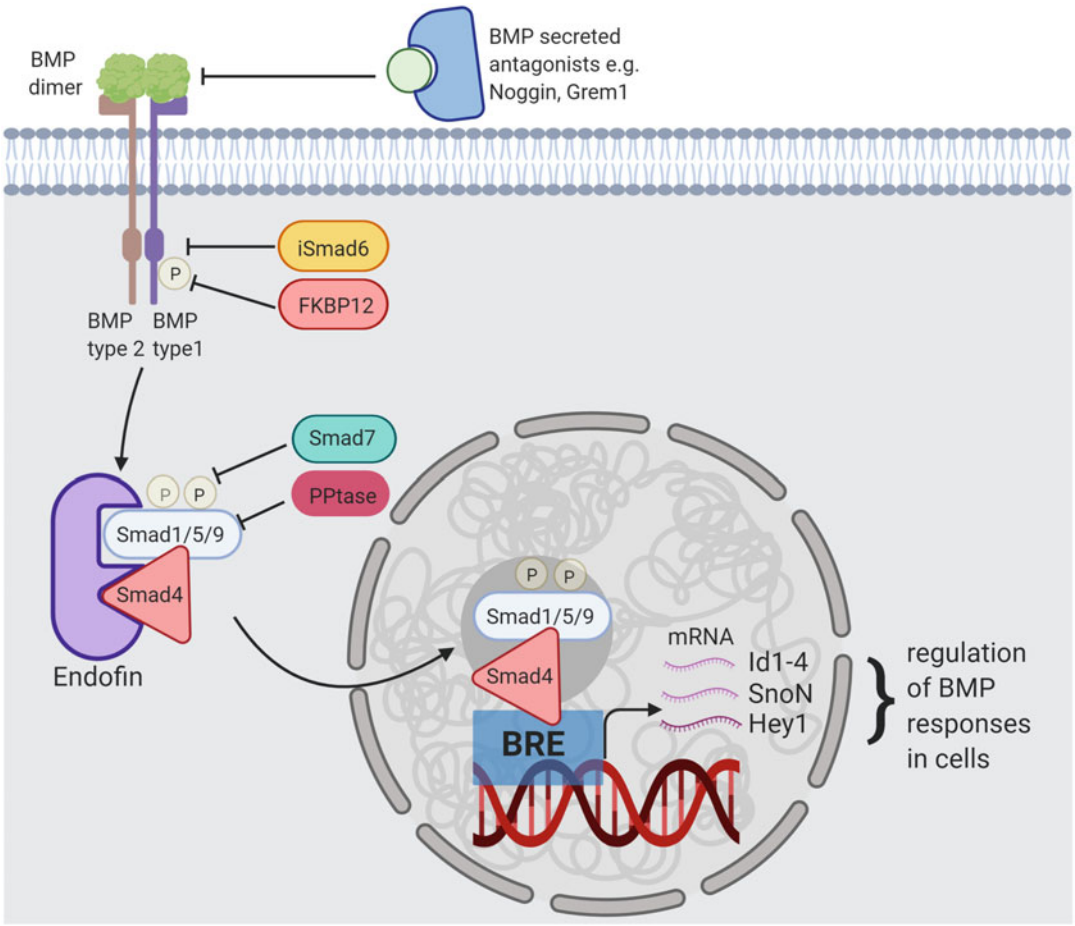
Introduction

Bone morphogenetic proteins (BMPs) are secreted glycoprotein members of the transforming growth factor-beta (TGFβ) superfamily. BMP signalling plays pivotal roles in developmental processes such as bone and cartilage, teeth, and kidney formation. There are about 22 members of the BMP family in mammalian cells, and all engage specific plasma membrane receptors to activate Smad signalling pathways that transmit BMP signals to the nucleus where a specific subset of genes are regulated. BMP signalling pathways are tightly controlled at multiple nodes inside and outside the cell. Significantly, a family of secreted BMP antagonists exist in the extracellular matrix that bind to and inhibit BMP signalling. This rigorous control of BMP signalling is critical for normal developmental processes, and disruption of this process leads to developmental disorders such as defective bone/cartilage formation, altered kidney development, and pulmonary artery hypertension (PAH). Aberrant BMP signalling is implicated in many human diseases including fibrosis of the kidney and lung, iron metabolism, and a range of cancers. Several pharmacological strategies based on increasing or decreasing BMP signalling have been developed as potential novel therapies for kidney and lung fibrosis, cancer, and vascular disease. This essay will provide an overview of BMP biology, summarize recent updates in BMP signalling, and provide sample vignettes of therapeutic targeting of BMPs and related proteins.

Basic Characteristics

Several excellent comprehensive reviews have been published in recent years that provide in-depth detail of BMP family biology and signalling (Miyazono et al. 2010; Gomez-Puerto et al. 2019; Walsh et al. 2010; Wang et al. 2014; Canalis et al. 2003; Massagué 2012; Brazil et al. 2015). The first BMP was discovered by Marshall Urist in 1965 as a factor that induced bone or cartilage formation when added to mesenchymal stem cells. At a very basic level, the basic model of BMP signalling is similar to many growth factors and cytokines. BMP dimers engage two distinct plasma membrane tyrosine kinase receptors (type 1 receptors, e.g., BMPR1A/1B or Alk1/2, and type 2 receptors, e.g., BMPRII or ActRIIA/B (Yadin et al. 2016)) that then dimerize and lead to receptor kinase activation and receptor autophosphorylation. This in turn leads to phosphorylation of intracellular mediators called receptor Smad proteins (R-Smad1/5/8) which then bind to Smad4 causing nuclear translocation of the Smad protein complex. The assembly of this complex of proteins is mediated by a scaffold protein called Endofin. Once in the nucleus, R-Smad1/5/8 localize to chromatin complexes centered around BMP response elements (BREs) in DNA to regulate the transcription of BMP target genes such as inhibitor of differentiation (Id1-4), SnoN, and Hey1 transcription factors in osteoblasts (Miyazono et al. 2005). Integrated within this somewhat simplified description of BMP signalling are multiple additional regulatory processes that add a high level of complexity to BMP signalling (summarized in Fig. 1). For example, proteins such as FKBP12 and Smad6 (an inhibitory Smad, iSmad) bind to BMPRI in the absence of BMP ligand and inhibit receptor activation. Binding of BMP proteins to their receptors leads to FKBP12 and iSmad6 dissociation from BMPRI, facilitating BMP receptor activation and signalling. The mechanism of iSmad6 dissociation involves methylation of Smad6 by PRMT1 on Arg74 and 81, allowing R-Smad1/5/8 phosphorylation and BMP signalling. The requirement for this methylation event to occur prior to transduction of BMP signalling may explain why the kinetics of BMP signalling is slower (~10 min) compared to other growth factors such as insulin or EGF (~1–2 min). A similar mechanism exists to control TGFβ1 signalling (see below).



Bone Morphogenetic Proteins, Fig. 1 Schematic summary of canonical BMP signalling pathway. BMP dimers bind to a heterotetrameric complex of two BMP type 1/type 2 receptors, which dimerize and lead to phosphorylation of the type 1 receptor by the type 2 receptor on serine/threonine residues. R-Smads 1/5/8 are then phosphorylated by the activated BMP receptor complex, which leads to their binding to Smad4, facilitated by the Endofin anchor protein. The R-Smad/Smad4 complex translocates to the nucleus where it binds to specific BMP response elements (BREs) on a subset of target genes such as Id1-4, SnoN, and Smad6. The protein products of these genes mediate the complex cellular responses to BMP proteins. Regulation of the pathway is achieved at multiple levels including secreted BMP antagonist binding to BMP dimers in the extracellular matrix, iSmad6 and FKBP12 preventing BMP receptor activation, and Smad7/protein phosphatase inhibition of R-Smad phosphorylation and nuclear translocation

A family of BMP antagonists that include Noggin, Gremlin (Grem1), Chordin, USAG-1, and crossveinless (CV2) control BMP action outside the cell. These secreted antagonists bind to BMPs in the extracellular milieu and prevent binding to their cognate receptors, attenuating signal transduction. Each of these BMP antagonists binds to specific target BMPs to regulate specific developmental processes. Perturbations in the balance of BMP v BMP antagonist levels can lead to defective mammalian development, as well as human disease (see below). Additional regulatory processes such as epigenetic modification of BMP response gene promoters/enhancers, miRNAs, ubiquitin ligases such as Smurf1 that target Smad proteins, and pseudo-receptors such as BAMBI create a hugely complex BMP signalling network in cells.

Despite the similarities in their signal transduction mechanisms, each BMP has distinct roles in mammalian development and physiology, linked to the tissue-specific temporospatial expression

and their interaction with secreted antagonists such as Noggin, Grem1, and USAG-1. Much of our knowledge of the biological roles of BMPs come from the phenotypes of BMP knockout mice. These data are beautifully summarized in a table from a review article from the Shi laboratory in Chicago (Wang et al. 2014). Perhaps not surprisingly, many BMP knockout mice display a broad range of defects in bone formation, increased fractures, defective fracture repair, and altered tooth formation. For example, mice lacking BMP2 are non-viable and die in utero due to defects in amnion/chorion and cardiac development. BMP4 knockout mice are also embryonic lethal due to a lack of mesoderm formation. Targeted disruption of BMP6 in mice causes a rapid and massive accumulation of iron in the liver, pancreas, heart, and kidney. BMP7 null mice survive gestation, but die shortly after birth, likely as a result of defective kidney formation and skeletal patterning. BMP9 knockout mice display defects in tooth development, lymphatic vessel formation, and retinal vascular remodelling. GDF5/6/7 (BMP14/13/12) have been shown to regulate tendon formation and fracture healing (Wang et al. 2014). BMP-Smad signalling plays a pivotal role in iron homeostasis via crosstalk with the hemojuvelin (HJV) receptor, which is a member of the repulsive guidance molecule (RGM) family. HJV acts as a co-receptor for BMP ligands and potentiates their signalling to Smad proteins. This leads to increased hepcidin transcription and iron uptake into liver cells and macrophages. Mutations in the BMP response element of the hepcidin gene are associated with iron overload in patients with hemochromatosis. Similarly, a range of mutations in HJV, the BMP ligand co-receptor, have been identified in patients with juvenile hemochromatosis.

Other genetic data from patients highlights the consequences of overactive BMP signalling in development and health. R206H mutations in ALK2 (the BMP type 1 receptor) lead to hyperactive BMP receptor signalling, likely due to a reduced receptor affinity for the inhibitory FKBP1A protein, leading to enhanced Smad phosphorylation. These patients develop a debilitating condition called fibrodysplasia ossificans progressive (FOP), which is characterized by heterotopic ossification, where bone forms in muscle and other soft tissue in the body (Song et al. 2010). Pulmonary artery hypertension (PAH) is another life-threatening disease where patients present with increased pulmonary artery resistance which can lead to right heart failure (Gomez-Puerto et al. 2019). Approximately 70% of familial PAH patients are heterozygous for inactivating mutations along the BMPR2 gene that lead to reduced BMP signalling and the PAH phenotype.

Myostatin/GDF8 signals via Alk4/5/ActRIIB receptors→Smad2/3 to inhibit myogenesis. Mutations in myostatin decrease myostatin signalling and lead to a "double-muscled" phenotype as seen in Belgian Blue cattle and very rare human patients. Mutations in Smad4 cause Myhre syndrome, characterized by muscle hypertrophy. The effect of these Smad4 mutations is linked to inhibition of myostatin signalling, leading to increased muscle mass similar to the examples above.

Overexpression of BMP2 has been identified in osteoarthritis and has been linked to cartilage degradation. Increased serum levels of BMP2 and BMP4 have been identified in osteoarthritis and have been suggested as markers of disease severity. Levels of the BMP antagonist Gremlin1 have also been identified in the synovial fluid of patients with rheumatoid arthritis. Amplified Gremlin1 expression may contribute to cartilage degeneration in arthritis by excessive mechanical loading and over-activation of the NFκB pathway. BMP7 has been explored as a potential treatment for osteoarthritis, given its anti-fibrotic potential. Downstream targets such as NFκB may be mediating the inflammatory effects of BMPs in these cells (see below).

BMP4 has been identified as a key regulator of both white and beige adipogenesis, and defective BMP signalling has been implicated in human hypertrophic obesity (Gustafson et al. 2015; Hammarstedt et al. 2018; Hedjazifar et al. 2019). Reduced BMP signalling in hypertrophic obesity

may occur due to increased Grem1 levels, which binds to BMP-4 and antagonises its receptor activation. A recent report identified that levels of Gremlin1 are upregulated in obesity and type 2 diabetes, leading to antagonism of insulin action in metabolic tissues. BMP9 regulates energy balance via inhibition of liver SREBP1 expression. BMP9 acts to reduce triglyceride accumulation and reduce fat accumulation in liver. Levels of BMP9 are reduced in insulin resistance and diabetes, suggesting a previously unappreciated role for BMP signalling in glucose metabolism, diabetes, and obesity.

Upregulated expression of the BMP antagonist Gremlin1 is also a feature of diabetic nephropathy (Walsh et al. 2008, 2010; Lappin 2002), pulmonary hypertension, and multiple human cancers, including colorectal, glioma, and breast cancer. A rare genetic form of colorectal cancer called hereditary mixed polyposis syndrome (HMPS) is caused by a 40-kb duplication event on chromosome 15 leading to ~2500-fold increase in Gremlin1 mRNA levels in colonic epithelial cells. This increase in Gremlin1 expression disrupts the finely tuned balance between BMP-4, Wnt Sonic Hedgehog (SHH), and Grem1 in the colonic villi, leading to increased epithelial cell proliferation at the tip of the villi and polyp/tumor formation. Other reports have corroborated these findings, showing that Grem1 levels are associated with metastasis and poor prognosis in ER-negative breast cancer. The source of Grem1 in these tumors is likely to be cancer-associated fibroblasts.

Apart from Grem1, other BMP antagonists have been implicated in human disease. USAG-1 is the most abundant BMP antagonist in the human kidney and acts to inhibit the renoprotective effects of BMP7 in this organ. Twsg1 is the main BMP antagonist secreted by glomerular podocytes. Expression of USAG1 and Twsg1 expression (as well as Grem1 discussed above) are increased in diabetic nephropathy, as well as other models of glomerular and tubular kidney injury. In liver disorders such as chronic hepatitis, cirrhosis, and hepatitis C, levels of BMP antagonists Grem1 and Follistatin have been shown to be amplified.

There are numerous papers describing roles for BMPs and their antagonists in cancer (Rider and Mulloy 2010). For example, Noggin has been implicated in melanoma, where it inhibits the anti-proliferative actions of BMP7, leading to tumor progression. Noggin has also been implicated in bone metastases of prostate cancer in men. The BMP antagonist Coco promotes the proliferation of breast cancer cells that have migrated to the lung, likely by overcoming the anti-proliferative drive mediated by locally produced BMPs. BMPs and their antagonists also play crucial roles in the regulation of cancer stem cell (CSC) phenotypes. For example, BMP2 produced by CSCs drives their differentiation and reduces CSC proliferative capacity. In extracts from glioblastomas from patients, expression of BMP2 was found to be high, and yet BMP2 activity was reduced in these cells. This anomaly was explained by high levels of Grem1 expressed by these tumors, leading to inhibition of BMP2 activity reducing CSC differentiation and attenuating the anti-proliferative effect of BMP2 (Seoane 2014). Crosstalk between BMP2 and TGFβ1 is critical in regulating the CSC phenotype, with TGFβ signalling implicated in maintaining CSC stemness and prevention of cell differentiation (see below).

BMP Signalling Crosstalk

There is a wealth of data in the literature describing crosstalk between BMP signalling and pathways critical in development and disease such as TGFβ, Wnt/β-catnenin, Notch, SHH, and NFκB. TGfβ1 ligand signalling (of which there are 32 members (Batlle and Massagué 2019)) occurs in a very similar manner to BMP signalling. TGFβ1 is produced in a pro-form that requires cleavage and activation by the extracellular endoprotease Furin. Active TGFβ then induces the dimerization of TGFβR1/TGFβR2 receptors triggering to the phosphorylation of R-Smad2/3, followed by Smad4 binding, nuclear

translocation, DNA binding, and gene regulation. In addition to canonical BMP→Smad1/5/8 and TGFβ→Smad2/3 pathways, crosstalk between these pathways has been described in a range of cellular contexts. In vascular endothelial cells, BMP and activin receptor membrane bound inhibitor (BAMBI) function as a competitive pseudo-receptor for the TGFβ family. Expression of BAMBI decreases TGFβ1-induced angiogenesis, by driving alternative TGFβ signalling via Smad1/5 and ERK1/2 phosphorylation. BAMBI expression and alternative TGFβ signalling have also been shown to play a role in the diabetic kidney disease, with evidence that targeting alternative TGFβ signalling may be a potential avenue for new treatments for diabetic nephropathy. TGFβ1 signalling via Smad1/5/8 pathways has been shown to regulate breast cancer epithelial cell migration and anchorage-independent epithelial cell growth.

Alternative BMP signalling to Smad2/3 has also been demonstrated during embryonic development and in invasive cancer of the breast and prostate. Both TGFβ and BMP alternative signalling pathways occur via the formation of non-canonical receptor complexes in specific cells that then facilitate the phosphorylation of specific Smad phosphorylation and alternative signalling. The balance between BMP and TGFβ1 signalling via canonical and alternative pathways is critical during development and disease. A protein called Kielin/Chordin-like protein-1 (KCP-1) acts as an inhibitor of TGFβ signalling by binding to TGFβ ligand, preventing receptor activation and reducing pro-fibrotic signalling (Vrijens et al. 2013). In contrast, KCP-1 binding to BMP7 enhances its receptor binding and increases anti-fibrotic BMP7 signalling. KCP-1 has been shown to attenuate high-fat diet-induced obesity and acute and chronic renal injury in mouse models. These reports demonstrate the potential of modulating the counterbalance between TGFβ and BMP signalling to improve outcomes in human disease.

BMPs and Wnt interact at several key signalling nodes during embryogenesis. Expression of BMP2 ligand is regulated by Wnt3a and β-catenin overexpression in osteoblasts during development. Smad4 has been identified in nuclear complexes with β-catenin, suggesting crosstalk at the DNA/transcriptional level. The interaction between BMP and Wnt signalling is exemplified in the intestine, where opposing gradients of BMP and Wnt expression exist. Levels of Wnt are highest at the villus base and lowest in the epithelial cells at the tip of the villus. In contrast, BMP expression is lowest at the villus base and increases as cells migrate up toward the tip of the villus. Dysregulation of this balance of BMP and Wnt has been identified in colon cancer. Loss of function of the APC tumor suppressor gene occurs in >90% of sporadic colon cancers. Loss of APC leads to enhanced Wnt signalling via stabilization of β-catenin, leading to increased β-catenin nuclear localization and amplified Wnt-mediated gene expression (Bertrand et al. 2012). This increased Wnt signalling leads to inappropriate epithelial cell proliferation, polyp formation, and increased cancer risk. A similar scenario exists when BMP signalling is perturbed in the intestine. Overexpression of Grem1, a BMP antagonist, leads to reduced anti-proliferative BMP signalling at the villus tip, leading to increased epithelial cell division, polyp formation, and cancer. BMP signalling also inhibits intestinal stem cell renewal via suppression of Wnt/β-catenin signalling. BMP-mediated suppression of Wnt/β-catenin-driven intestinal stem cell renewal has been shown to occur via PI3K/PTEN/Akt/14-3-3ζ signalling. Finally, Wnt/β-catenin signalling activates the transcription of the pseudo-receptor BAMBI, thus leading to suppression of TGFβ signalling as discussed in detail above.

The Notch pathway has been shown to act synergistically with BMP signalling in developing blood vessels and heart valves. Notch signalling is activated by Delta/Jagged ligand-mediated cleavage of the Notch transmembrane receptor, releasing the Notch intracellular domain (NICD) which translocates to the nucleus and forms a complex with the CBF-1 transcription factor to drive the expression of genes such as Hes1 and Hey1. BMP4 has also been shown to induce Hes1 and Hey1 in C2C12 mouse myoblasts, an event that is required for BMP-mediated myogenic differentiation. Physical complexes of Smad1 and NICD have been isolated and shown to

synergistically activate Hey1 expression to regulate fate determination in mouse neuroepithelial cells. In contrast, BMPs promote the migration of endothelial cells and the formation of stalk cells via induction of Id1 expression, a process that is inhibited by Notch-mediated Hey1 which represses Id1 expression. Others have shown that Notch signalling inhibits BMP9-induced osteogenic differentiation of mesenchymal stem cells (MSCs) by blocking JunB expression.

Sonic hedgehog (SHH) is a morphogen that regulates dorsoventral patterning during development (Zhu et al. 2019). SHH induces BMP4 expression in subepithelial fibroblasts in *Xenopus*, leaving to the development of cell-renewable epithelium. SHH is also a key morphogen in the colon and is primarily expressed at the tip of the villus in the differentiated zone. SHH has been shown to activate the expression of the BMP antagonist Grem1 in pancreatic cancer. Formation of limb buds in mice is regulated by a complex interplay of SHH, BMP/Grem1, and Jag1 acting on mesenchymal stem cells. A key element of this regulation is the repression of Grem1 expression via the action of Gli1, a transcriptional mediator of SHH signalling. Similar to Notch signalling, both SHH and Notch can both positively and negatively regulate BMP signalling, depending on the cellular context. Crosstalk between BMP signalling and the NFκB pathway has been identified by a number of groups. NFκB is the primary transcriptional mediator of pro-inflammatory cytokines such as TNF1 and IL-1β. NFκB activation has been shown to repress BMP2-mediated osteoblast activation and bone formation via interference with Smad proteins to BMP2 response genes in the nucleus. Signalling to NFkB may mediate many of the effects of BMPs and their antagonists on inflammatory diseases such as rheumatoid arthritis (see below).

The above examples highlight the complex nature of BMP signalling, with a range of regulatory loops, signalling nodes, and feedback circuits that can both amplify and dampen BMP signalling. These insights have given researchers useful leads on how best to intervene pharmacologically to manipulate BMP signalling in human disease. Some examples of these data are discussed below.

BMP Pharmacology

Pharmacological interventions aimed at both stimulating and inhibiting BMP action are in use and development in medicine. BMPs have been used as therapeutic agents for the treatment of bone fractures for a number of years. Recombinant human BMP2 (InFuse® from Medtronic) or rhBMP7 (OP-1 from Olympus) have been used as adjunct therapies for non-union fractures of long bones such as the femur (Gautschi et al. 2007). The osteoinductive properties of these BMPs are thought to accelerate bone healing in these patients, although reports questioning the efficacy and associated complications of BMP use exist in the literature. Given the proposed anti-fibrotic effects of BMP7 in kidney and other organs, its potential as a therapeutic anti-fibrotic agent has been investigated. Disappointingly, despite encouraging preliminary data, rhBMP7 did not display any clear benefit in treating fibrosis in the kidney, lung, and skin. Alternative approaches such as Thr123, a peptide mimetic of BMP7, and activators of BMP7 signalling such as tilerone and members of the flavonoid chalcone family have shown potential in mouse models of lung fibrosis. A novel peptide agonist of ALK2/BMPRI called P123 can block the growth of primary tumor cells and the progression of EMT in human breast cancer stem cells (CSCs). The BMP7 agonist THR-184 has been shown to have anti-inflammatory/anti-fibrotic actions in preclinical models of kidney injury. A recent study tested the ability of THR-184 to reduce acute kidney injury in patients post cardiac surgery in a placebo-controlled trial. No significant benefit was detected in THR-184 patients compared to placebo group. A recent report identified benzoxazole compounds as potent and efficacious agonists of BMP signalling in human renal cells. These compounds could be explored as potential anti-fibrotic agents based on their ability to activate BMP signalling.

Most of the focus in terms of BMP therapeutics centers on inhibiting overactive BMP signalling as the outcome of choice. Despite the low numbers of patients who develop FOP (97% of whom have the R206H mutation in the ALK2/BMPRI), significant efforts have focussed on developing

drugs to reduce excessive BMP signalling in these patients. The Bullock group in Oxford have identified a range of small molecule inhibitors of ALK2, and the crystal structure of ALK2 in complex with one of these (LDN-212854) has been solved and provides a useful template for developing new and improved treatments for FOP. Regeneron Pharmaceuticals has recently announced encouraging results from a Phase 2 trial to assess the efficacy of garetosmab (REGN2477), a drug designed to inhibit the R206H mutated form of ALK2/BMPRI in FOP patients (https://clinicaltrials.gov/ct2/show/study/NCT03188666). Other therapeutic strategies for FOP including saracatinib (a small molecule kinase inhibitor of ALK2/BMPR2) and rapamycin (an inhibitor of mTORC1 pathway) are also being explored for FOP. These and other strategies have been reviewed by Wentworth and colleagues (2019).

ALK2 inhibitors may also have potential as novel therapeutics for diffuse intrinsic pontine glioma (DIPG), where 25% of patients possess somatic mutations in ALK2. BMP9/10 have also been targeted in cancers, with Dalantercept, an ALK1-Fc fusion protein targeting the ALK1 receptor shown to reduce BMP9/10-mediated tumor angiogenesis. Dalantercept is now in Phase 2A clinical trials for advanced renal cell carcinoma. BMP9/10 are implicated in patients with hereditary hemorrhagic telangiectasia (HHT), a life-threatening vascular disorder with no current cure. Pulmonary artery hypertension (PAH) is characterized by vascular remodelling of the vasculature of the lung that can lead to death from heart failure within 3–5 years of diagnosis. The heritable form of PAH is caused by mutations in the BMPRII that reduce or inactivate receptor activity. Reversing BMPRII deficiency has therefore been explored as a novel treatment modality for PAH. One preclinical study has demonstrated that recombinant BMP9 enhanced BMPRII signalling and reversed lung damage in a genetic mouse model of PAH. A drug called sildenafil is used to treat PAH, as it enhances BMP4-mediated Smad1/5 phosphorylation and reduced smooth muscle cell proliferation in the lung. Sildenafil treatment has the potential to partially reverse the effects of reduced BMPR2 signalling in the lungs of PAH patients.

BMP9/10 have been shown to be crucial for postnatal retinal vascular remodelling in mice, with crosstalk to increased Notch signalling significant in this regard. Many patients present with mutations in the gene for the ALK1 receptor or Smad4. These mutations lead to defective BMP9/10 signalling and increased endothelial proliferation leading to inappropriate angiogenesis in these patients. Thus, BMP9/10 mimetic drugs have been sought to try to rectify the angiogenic imbalance in these patients. A drug-repurposing strategy screening the FDA-approved catalogue identified tacrolimus (FK506) as a potent activator of ALK1 in BMP9-challenged C2C12 cells. These authors also showed that tacrolimus could reduce the hypervascularization response in the retinas of BMP9/10-immunodepleted mice.

BMP2 and BMP4 have been identified as potential molecular targets in Barrett's esophagus, a chronic inflammatory condition associated with increased risk of esophageal adenocarcinoma. BMP4 has been identified as a driver of the altered columnar epithelial differentiation seen in Barrett's esophagus. Targeting the BMP4 pathway using the BMP antagonist Noggin has been explored as a novel therapy in a rat model of Barrett's esophagus. Targeting of the BMP antagonist Grem1 has also been suggested for a range of human diseases such as diabetic nephropathy, PAH, and colorectal cancer. Neutralizing antibodies against Grem1 have been developed by several pharma companies, and one report from Novartis demonstrated that inhibition of Grem1 action using a neutralizing antibody reduced the severity of lung damage in a mouse model of pulmonary artery hypertension. Small molecule inhibitors of Grem1 are also being explored as novel therapeutics for colorectal cancer. The predicted structures of Grem1 protein in complex with BMP2, as well as the crystal structure of Grem2 with GDF5, will be useful in pursuing a

rational drug design-based approach toward the development of novel Grem1 inhibitors.

Future Directions

As the above essay demonstrates, there are many exciting developments emerging in the field of BMP signalling. Insights into the molecular mechanisms of altered BMP signalling in diseases such as PAH and FOP have facilitated novel avenues of therapeutic exploration. Significant advances in our understanding of the role of BMPs and their antagonists such as Grem1 provide hope that targeting these pathways may enhance the effective treatment options for hard-to-treat diseases such as glioblastoma and colorectal cancer. Deeper dives into the cellular signalling pathways engaged by BMPs and their antagonists will shed new light on the pathogenic mechanisms of BMP and BMP antagonist signalling in human diseases. We look forward to new advances in our understanding of BMP signalling, which will likely extend our treatment options and improve patient outcomes for tissue fibrosis, iron metabolism, pulmonary hypertension, and cancer.

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Bone Remodeling

▶ Bone Metabolism

Bone Turnover

▶ Bone Metabolism

Bortezomib (Velcade)

▶ Proteasome Inhibitors

Botulinum Neurotoxins

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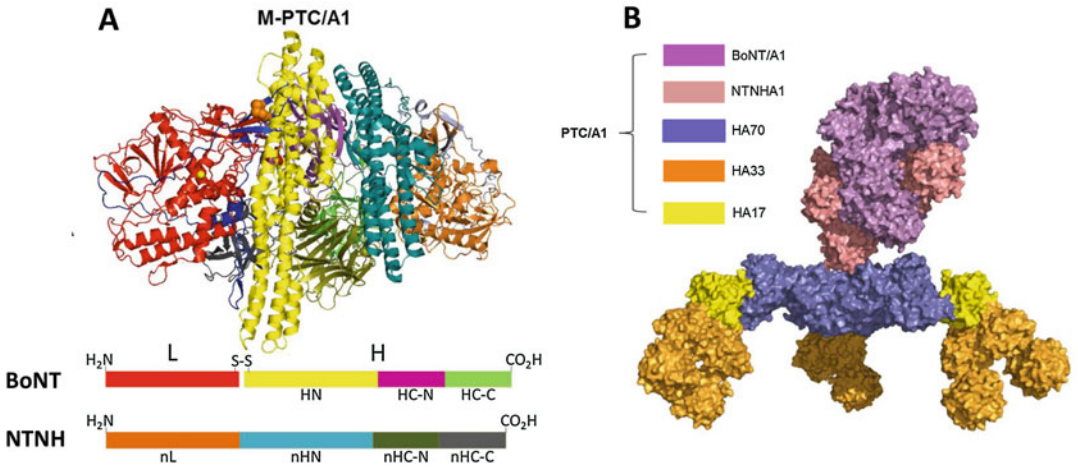
Definition, Serotypes, and Subtypes

The botulinum neurotoxins (BoNTs) form a family of protein neurotoxins endowed with a set of particular properties that have made them powerful therapeutics for a variety of human syndromes. Their development as medicines is still undergoing. They are synthesized by different *Clostridia* though bacteria of other phyla may harbor the gene encoding for BoNTs. They consist of two chains (L, 50 kDa and H, 100 kDa) linked by a single interchain disulfide bond (Fig. 1a). They are produced in eight different serotypes (indicated by letters BoNT/A, /B, /C, /D, /E, /F, /G, and /X) (Rossetto et al. 2014; Dong et al. 2019). In addition many subtypes of a serotype are known (dubbed with a suffix number: BoNT/A1, BoNT/A2, etc.) plus chimeric neurotoxins (BoNT/CD, BoNT/DC, BoNT/FA). Thus, the BoNT family comprises several dozens of proteins, and their number is growing as result of massive DNA sequencing. None the less all BoNTs share a four-domain structure, with a similar spatial folding of the polypeptide backbone, as depicted in Fig. 1a. The N-terminal domain is formed by the L chain, which is zinc endopeptidases (protease family M27) responsible for the intracellular biochemical lesion caused by the BoNTs. The L domain is followed by the HN domain (the first half of the H chain) which is responsible for the translocation of the L chain into the cytosol of nerve terminals. The third domain (HC-N, 25 kDa) likely contributes to neurospecific binding carried out by the C-terminus domain (HC-C, 25 kDa). HC-C has a conserved binding site for the oligosaccharide portion of polysialogangliosides, glycolipids highly enriched in the presynaptic membrane of neurons, and another site specific for the lumenal domain of the synaptic vesicle protein SV2 (BoNT/A and /E) or synaptotagmin (BoNT/B, /F, and /G). The neurospecific binding of BoNT/C and /D is not well characterized, but there is evidence that oligosaccharides of glycolipids or protein-linked N-glycans are involved together with an hydrophobic loop of the toxin that insert in the lipid bilayer (Rossetto et al. 2014; Dong et al. 2019).

The BoNT molecule is complexed with a BoNT-like molecule (NTNH: nontoxic non-hemagglutinin), devoid of protease activity, forming a hand-in-hand heterodimer creating a medium size protein complex (M-PTC) of 300 kDa that is more stable than BoNT to the acidic and proteolytic environment of the stomach (Fig. 1a). This heterodimer is then complexed with accessory nontoxic proteins (HA proteins), as shown in Fig. 1b, to form a so-called progenitor toxin complex (PTC ≈ 900 kDa in the case of BoNT/A) which is stable at acidic pH values, but dissociates in slightly alkaline physiologic solutions. The role of the HA proteins is believed to be that of assisting the translocation of the BoNT molecule from the intestinal lumen, across the mucus layer and the polarized epithelial monolayer, into the mucosa in the alimentary and infant forms of botulism. More detailed information are available in recent reviews (Rossetto et al. 2014; Dong et al. 2019).



Botulinum Neurotoxins, Fig. 1 (**a**) Crystal structure of BoNT/A1 in complex with the NTNH/A1 protein. NTNH/A1 exhibits the same domain organization as BoNT/A1, and the two proteins form a hand-in-hand complex, which suggests that NTNH/A1 protects BoNT/A1 from proteases and other damaging agents that the toxin meets in the stomach. The lower bar shows the schematic organization of the two proteins. (**b**) Structure of the precursor toxin complex of BoNT/A1 (PTC/A1) which consists of the NTNH/A1-BoNT/A1 heterodimer and the hemagglutinin proteins (HA). There are six HA33 proteins, three HA17 proteins, and one HA70 per one NTNHA1-BoNT/A1 complex. PTC/A1 forms three spiderlike legs that are suggested to have a role in binding to the intestinal epithelium to facilitate absorption of the toxin

Botulism and Botulinum Neurotoxins

Botulism was first described about 200 years ago following episodes of flaccid paralysis developed in people that had shared food. In 1897 van Ermengem demonstrated that the disease was due to a bacterium that produced a powerful poison of peripheral nerves causing the flaccid paralysis characteristic of botulism (Erbguth 2004). Botulism is rare but potentially fatal, and the death rate depends on the capability of identifying the symptoms at hospital admission (Fleck-Derderian et al. 2017). The first symptoms are a paralysis of cranial nerves with ocular and facial palsy, diplopia and ptosis, and dysphagia and dysarthria, followed by a descending flaccid paralysis that includes the respiratory muscles which is the most frequent cause of death. Additional symptoms are due to paralysis of the autonomic cholinergic nerves with abdominal pain, vomiting, nausea, dry mouth, and dizziness. The display of symptoms varies somewhat as well as the time intervening between intoxication and development of overt symptoms, and they mainly depend on the amount and type of BoNT and on the anatomical site of toxin assumption/injection (Rossetto et al. 2014; Fleck-Derderian et al. 2017).

The botulism patient remains conscious but cannot operate any muscle. If the disease is rapidly identified and the respiration is mechanically assisted, the patient survives and recovers almost completely after few to several months, depending on dose and type of BoNT: from the few weeks of BoNT/E to several months of BoNT/A. However, pathologies caused by prolonged permanence in the emergency room and by variable levels of permanent fatigue may follow.

All the manifestations of botulism are uniquely due to the actions of the BoNT, whose toxicity is due to their neurospecificity and to the neuroparalysis that results from the intraneural catalytic action of the metalloprotease L domain. BoNTs are, together with tetanus neurotoxin, the most poisonous substances known, and therefore they are included in the list A of substances with a possible bioterrorist use. At the same time, their neurospecific high affinity binding and the reversibility of the induced neuroparalysis are at the

basis of their therapeutic and cosmetic use. They are cell-specific biochemical scalpels of great therapeutical value in the treatment of a variety of human pathologies and conditions characterized by hyperfunction of peripheral cholinergic nerve terminals (Pirazzini et al. 2017).

Toxicity is generally measured as the mouse lethal dose 50% (MLD50), i.e., the dose that kills 50% of mice within 4 days, after a single intraperitoneal injection. The MLD50 values of the various BoNTs are comprised in the range 0.01–5 ng/Kg depending on the BoNT types and less on the mice strain. The human lethal dose can be extrapolated from data obtained with primates. For a 70 Kg man, the lethal doses are 90–150 ng when injected intravenously, 800–900 ng when inhaled, and 70 μg when introduced orally.

Basic Mechanism of Nerve Terminal Paralysis by the Botulinum Neurotoxins

Biological evolution of these neurotoxins has led to a structural organization designed to deliver the metalloprotease domain into the nerve terminal cytosol. This remarkable result has been attained by exploiting several physiological functions of nerve terminals. On the basis of the presently available experimental notions, the BoNT mechanism of nerve terminal paralysis consists of five major steps, as depicted in Fig. 2: (1) binding to cholinergic nerve terminals, (2) entry inside recycling synaptic vesicles (SV), (3) the L domain crossing of the vesicle membrane driven by low pH, (4) release of L in the cytosol by reduction of the interchain disulfide bond, and (5) cleavage with inactivation of one or more of the three proteins that form the core of the nanomachine that performs synaptic vesicle neurotransmitter release causing neuroparalysis (Pirazzini et al. 2017).

Neurospecific Binding

After entering the lymphatic and blood circulations, following intestinal absorption or inspiration or injection, the BoNTs rapidly gain access to the peri-neuronal fluid compartment, but do not cross the blood-brain barrier. The local intramuscular injection of very small doses (few MLD50s) of BoNT/A shows the remarkable and therapeutically very useful property of a very limited diffusion from the injection site (Pirazzini et al. 2017). The BoNTs bind with high affinity on the presynaptic plasma membrane of skeletal and autonomic cholinergic nerve terminals. The high affinity is due to a double receptor binding: (a) to the oligosaccharide portion of a polysiaganglioside and (b) to the intravesicular domain of synaptic vesicle proteins (SV2 for BoNT/A and /E or synaptotagmin for BoNT/B, /F, and /G); the binding of BoNT/C and /D is less defined (Rossetto et al. 2014; Pirazzini et al. 2017; Dong et al. 2019).

Internalization into Nerve Terminals

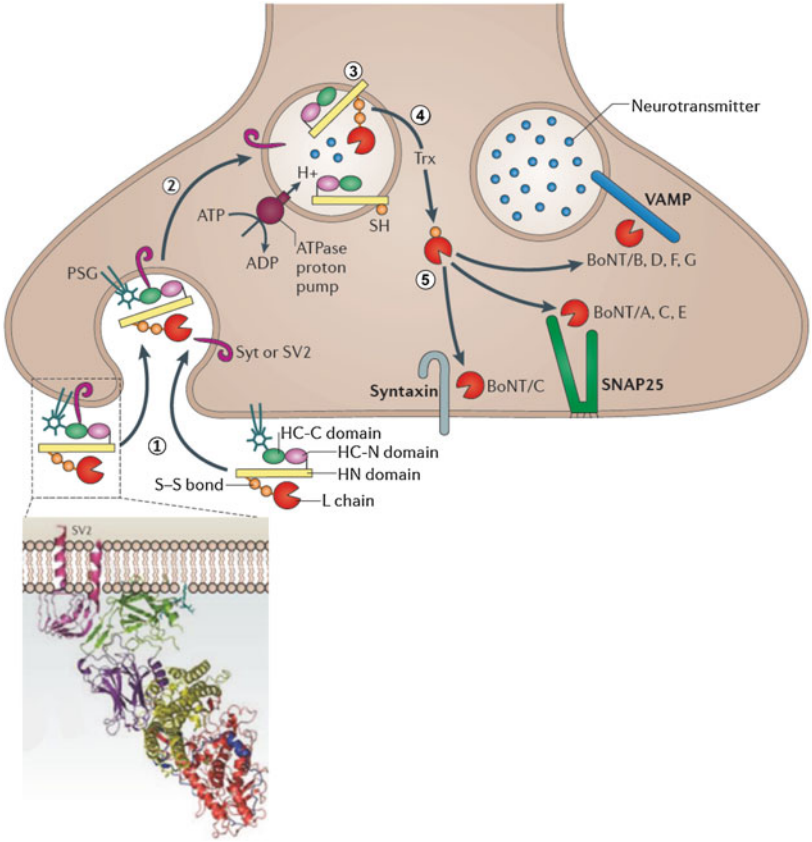
After intramuscular injection, one or two molecules of BoNT/A1 are rapidly taken up and found within the lumen of SV at the neuromuscular junction. The entry of other BoNTs is less defined. SV exocytosis is strictly coupled to their endocytosis, and this explains the fact that the time required for the development of paralysis after injection of BoNT/A decreases with increasing synaptic activity.

Membrane Translocation

After endocytosis, a proton pump present on the SV membrane drives protons inside generating a transmembrane pH gradient that drives the uptake of neurotransmitter from the cytosol to the lumen. The acidic pH induces a structural change of BoNT with translocation of the L domain across the SV membrane remaining attached via the SS bond to the HN domain.

Reduction of the Disulfide Interchain Bond

This bond is specifically reduced by the NADPH-thioredoxin reductase-thioredoxin redox system bound to the cytosolic surface of SV, thus releasing the metalloprotease L domain to display its catalytic activity on its very specific protein targets (Pirazzini et al. 2017). This notion has an important translational potential because inhibitors of this redox system do prevent the nerve terminal intoxication and paralysis and are strong candidates for the prevention of botulism in



Botulinum Neurotoxins, Fig. 2 The first step in intoxication involves the binding of the C-terminal part of the binding domain (HC-C in green) to a polysialoganglioside receptor present on the presynaptic membrane, followed by binding to a protein receptor (Syt or SV2) located inside the synaptic vesicle which becomes available upon vesicle exocytosis with neurotransmitter release (step 1). The crystallographic structures of BoNT/A1 bound to ganglioside and a luminal loop of SV2 are shown in the inset. The BoNT is then endocytosed inside synaptic vesicles (step 2), which are acidified by the vesicular ATPase proton pump to drive the reuptake of neurotransmitter. This physiological process is exploited by BoNTs, which become protonated, resulting in the translocation of the L chain of the toxin across the SV membrane (step 3) into the cytosol. The thioredoxin reductase-thioredoxin system (TrxR-Trx) breaks the interchain S-S bond (step 4) leading to the release of the L metalloprotease into the cytosol where it cleaves its specific SNARE target (step 5). (Adapted from Rossetto et al. 2014)

humans and for the treatment of those forms of botulism where there is a continuous production of novel toxin molecules.

SNARE Protein Cleavage

The vesicular neurotransmitter release is mediated by a nanomachine consisting of several proteins. Its core is composed by three proteins: the SV protein VAMP (vesicle-associated membrane protein) and the presynaptic membrane proteins SNAP-25 and syntaxin, which project into the cytosol. These three proteins are present in a number of isoforms and are collectively termed SNAREs. The SNARE proteins form a coiled coil heterotrimer termed SNARE complex, which contributes to bring the SV close to the presynaptic membrane, ready to fuse when the [Ca^{2+}] trigger is elicited by opening of the presynaptic membrane voltage-gated Ca^{2+} channels. BoNT/B, /D, /F, /G, and /X cleave VAMP at single sites within the coiling domain. BoNT/C cleaves both syntaxin and SNAP-25, while BoNT/A and /E cleave SNAP-25. These cleavages prevent the formation and/or function of the SNARE complex

and, consequently, of neurotransmitter release; for detailed information on the specificity of BoNTs for the various isoforms of the three SNARE proteins and the cleaved peptide bonds, see Rossetto et al. (2014) and Dong et al. (2019). Therefore, BoNT exploits three essential physiological functions of SV to paralyze the synapse: SV endocytosis, SV uptake of neurotransmitters, and SV surface redox activity.

Duration of the Peripheral Neuroparalysis Induced by Botulinum Neurotoxins

At variance from most bacterial and plant toxins, which kill cells, the BoNTs do not kill the intoxicated neurons; they merely paralyze it, and they are so toxic because respiration driven by the neuromuscular apparatus is essential for most vertebrates. All cytosolic proteins turn over, and the BoNT L chains are no exception. But they do so with different kinetics in such a way that the recovery of neurotransmission, which requires abolition of the L metalloprotease activity, takes place with different time courses for the different BoNTs: the L chain of BoNT/A (and paralysis) has the longest duration (3–4 months for human skeletal terminals, 9–12 months for autonomic cholinergic nerve terminals), while the L chain of BoNT/E is the shortest-acting one (paralysis of skeletal terminal lasting very few weeks). The duration of action of BoNT/A in humans is of major importance because it determines the therapeutic benefit length. Indeed long-lasting BoNTs require fewer injections and lower doses, limiting the possibility of immunization (see below).

Long-Distance Effects of Botulinum Neurotoxins

Peripheral neuroparalysis is the most evident symptom of botulism and is the one at the bases of the therapeutic use of BoNTs. However, indirect evidence that these neurotoxins could act at a distance from the injection site, i.e., within spinal cord and brain neuronal circuits, were reported long ago, and in some cases, it was shown to be due to retroaxonal transport of BoNTs similar to that occurring with TeNT (Mazzocchio and Caleo 2015). Compelling evidence of BoNT/A1 retrotransport to the central nervous system (CNS) was provided by tracing the cleavage of the SNARE within CNS neurons after peripheral injection of the toxin, using an antibody very specific for the novel epitope generated by the BoNT/A1 cleavage of SNAP-25. BoNT/A1 retrograde transport can occur also via sensory neurons, as the injection in the whisker pad induces the appearance of truncated SNAP-25 also in the trigeminal nucleus caudalis. These long-distance effects are mediated by an active retroaxonal transport of catalytically competent toxins inside motor axons or sensory neurons and not by passive spread of BoNT/A or to that of cleaved SNAP-25. Moreover, BoNT/A can undergo subsequent events of transcytosis and transport, remaining catalytically active (Mazzocchio and Caleo 2015).

Clinical Use

Many years before the discovery of *C. botulinum*, the clinical observation of the disease by Kerner inspired suggestions that the "sausage poison" causing botulism might have therapeutic potential. Based on observations and experiments described in his 1822 monograph, Kerner concluded that the toxin applied in minimal doses should reduce or block the hyperactivity and hyperexcitability of the motor and the autonomic nervous system (Erbguth 2004). After his farsighted vision, the therapeutic potential of botulinum toxin was tested and successfully illustrated 150 years later in the experiments of Dr Alan Scott, in monkeys first and then in human patients, showing that BoNT may be used as an alternative to strabismus surgery. Botulinum toxin was first approved by the US Food and Drug Administration (FDA) in 1989 for the treatment of strabismus, blepharospasm, and facial nerve disorders, including hemifacial spasm. Since that time the use of BoNT has continued to expand within and outside movement disorders and beyond

neurology, including aesthetic medicine and pain treatment (Jankovic 2018; Pirazzini et al. 2017). Today, BoNT is used in at least nine different medical specialties for more than 40 major indications (Pirazzini et al. 2017). Only a few of the indications are approved by regulatory agencies; most clinical uses are off label (Table 1).

Botulinum Neurotoxins, Table 1 Therapeutic uses for botulinum neurotoxin

Ophthalmology
Strabismus[a, b, c]
Nystagmus
Neurology
Focal dystonias
Blepharospasm [a, b, c]
Cervical dystonia [a, b, c] (torticollis, anterocollis, laterocollis)
Occupational dystonias (writer's cramp [b], musician's cramps)
Oromandibular dystonia
Lingual dystonia
Non-dystonic disorders
Hemifacial spasm [a, b, c]
Tremor (essential, parkinsonism)
Tics
Bruxism
Spasticity (poststroke, multiple sclerosis, brain or spinal cord injury)
Focal spasticity [a,b,c]: upper and lower limb spasticity
Non-focal: hemispasticity, paraspasticity, tetraspasticity
Hyperhidrosis[a,b,c]
Focal: axillary, palmar, plantar
Diffuse
Hypersalivation
Sialorrhea[c] (motor neuron diseases/amyotrophic lateral sclerosis)
Drooling[c] (Parkinsonian syndrome)
Pediatrics
Infantile cerebral palsy[b,c]
Otorhinolaryngology
Laryngeal dysphonia [c]
Crocodile's tears
Chronic rhinitis
Aesthetic medicine
Glabellar rhytides[a,b,c]
Pain
Muscular
Dystonia
Spasticity
Chronic myofascial pain
Temporomandibular disorders
Low-back pain
Nonmuscular
Migraine (chronic[a] and tension-type migraine)
Neuropathic pain
Trigeminal pain
Pelvic pain
Urology
Detrusor sphincter dyssynergia
Overactive bladder[a,b,c] (Idiopathic or neurogenic detrusor overactivity)
Urinary retention
Bladder pain syndrome
Pelvic floor spasms
Benign prostate hyperplasia
Gastroenterology
Achalasia
Chronic anal fissures
Psychiatry
Depression[c,d]

[a]US-approved indication
[b]EU-approved indication
[c]Evidence-based therapeutic indication
[d]To be evaluated

BoNT Formulations

Despite the variety of botulinum toxin types, therapeutic preparations are currently only available for BoNT types A1 and B1. The commercially available neurotoxins that have been approved by US FDA and are sold in the Western Hemisphere include three main brands of BoNT/A1, OnabotulinumtoxinA (Botox®), AbobotulinumtoxinA (Dysport®), and IncobotulinumtoxinA (Xeomin®), and a brand of BoNT type B RimabotulinumtoxinB (Myobloc® in the USA or NeuroBloc® in Europe) (Table 2). The major Asiatic BoNT type A manufactures are sited in Korea (Medytox with Neuronox®, Hugel with Botulax®, and Daewoong with Nabota®/DWP-450) and in China (Lanzhou Institute of Biological Products with BTXA™, distributed by local/regional companies under different trade names). Overall, the BoNT/A containing drugs are a

Botulinum Neurotoxins, Table 2 Physical and chemical characteristics of major formulations of botulinum neurotoxins A1 and B1 for therapeutical use in humans

Brand name	Botox®/Vistabel®[a]	Dysport®/Azzalure®[a]	Xeomin®/Bocouture®[a]	NeuroBloc®/ Myobloc®
Generic name	OnabotulinumtoxinA	AbobotulinumtoxinA	IncobotulinumtoxinA	RimabotulinumtoxinB
Manufacturer	Allergan (USA)	Ipsen Biopharm Ltd. (UK)	Merz Pharmaceuticals (Germany)	US WorldMeds (USA)
***C. botulinum* strain**	Hall A-hyper	Hall A	Hall A (ATCC 3502)	Bean
Toxin type	A1	A1	A1	B1
MW	900 kDa complex	500–900 kDa complex	150 kDa Purified toxin	700 kDa complex
Pharmaceutical form	Vacuum-dried powder for reconstitution	Freeze-dried powder for reconstitution	Freeze-dried powder for reconstitution	Ready-to-use solution
Shelf life	2–8 °C 36 months	2–8 °C 24 months	Room temperature 36 months	2–8 °C 24 months
pH (reconstituted)	7.4	7.4	7.4	5.6
Excipients	In 100 U vial: HSA 500 μg NaCl (900 μg/vial)	In 500 U vial: HSA 125 μg Lactose (2.5 mg/vial)	In 100 U vial: HSA 1000 μg Sucrose (4.7 mg/vial)	HSA 500 μg/ml Succinate 10 mM NaCl 100 mM
Unit∗/vial	100 U or 200 U Botox® 50 U Vistabel®	300 U or 500 U Dysport® 125 U Azzalure®	100 U or 200 U Xeomin® 50 U Bocouture®	5000 U/ml
Protein load/vial	5 ng/100 U	4.35 ng/500 U	0.44 ng/100U[a]	40–70 ng/5000 U
Clinical activity in relation to Botox®	1	1:2–1:3	1	1:40–1:50
Label indications	Cervical dystonia Primary axillary hyperhidrosis Blepharospasm and strabismus Spasticity Bladder dysfunction Chronic migraine Glabellar lines	Cervical dystonia Upper limb spasticity Lower limb spasticity in pediatric patients glabellar lines	Cervical dystonia Blepharospasm Chronic sialorrhea Upper limb spasticity Glabellar lines	Cervical dystonia

[a]Vistabel, Azzalure, and Bocouture are the corresponding formulations for cosmetic treatment

multibillion US dollars business per year, with millions of patients injected over the world.

Botox®, Dysport®, and Myobloc® consist of the PTC including the neurotoxin and the nontoxic accessory proteins NTNHA and the HAs (Fig. 1b), whereas Xeomin® contains only the purified BoNT/A1 (Fig. 1a). They are sold in glass vials as a dry powder to be reconstituted in physiological medium/saline before injection into the patient (for BoNTa/A-based drugs) or an injectable sterile solution (BoNT/B drug). All formulations contain excipients (i.e., human serum albumin) to stabilize toxicity, to improve toxin shelf life, and to minimize nonspecific adsorption of the BoNT molecules onto the walls of vials and syringes.

Although the BoNT/A containing products have similar efficacies, they are differently formulated and demonstrate unique characteristics. BoNTs preparations potency is expressed in units (U), where 1 U corresponds to the amount of product needed to achieve 1 LD_{50} in the mouse bioassay. Since the bioassays used to assess the commercial toxins are performed under slightly different conditions, the units of biological activity are different and manufacturer-specific. Consequently, these products are not interchangeable, and doses are specific to each preparation. Comparison of data in the clinical literature assesses a dose conversion ratio Dysport®/Botox® between 2 and 3, while Botox® and Xeomin® are reported as equivalent in potency in most cases.

Notwithstanding the different formulations, these three toxin products display the same limited diffusion from the site of injection.

Therapeutic Applications

Depending on the target tissue, BoNT can block the cholinergic neuromuscular transmission, but also the cholinergic autonomic innervation controlling sweat, tear, and salivary glands and smooth muscles. In addition, in experimental animals, it induces blockade of transmitters involved in pain perception, transmission, and processing. Dosages for BoNT/A drugs vary widely in different therapeutic applications with minimum doses of few units for spasmodic dysphonia to maximum doses of hundreds of units for generalized spasticity. Whatever the dose, BoNT/A1 effects can be detected after 2–3 days from injection and reach its maximum value after 3–4 weeks. It maintains this level for a variable period of time depending on the anatomical site and then gradually declines to return to the original value within 3–4 months for skeletal nerve terminals and about 9–12 months for autonomic ones. Over the last decade, the high safety profile of BoNT/A-based drugs and the increasing understanding of the biology of the neurotoxin have resulted in significant growth of on-label indications but also favored the expansion of off-label use in a great variety of movement, urologic, gastrointestinal, secretory, dermatologic, and pain disorders (Table 1). Furthermore, in the coming years, the recent identification of diverse toxin types with different biological profiles and the availability of modified recombinant toxins are expected to expand even more in the future therapeutic use of BoNTs. Indeed, scientific challenges for BoNT drug development include modification of its duration of action, its needle-free delivery, and the design of BoNT hybrid drugs for specific target tissues.

Adverse Effects

Despite the remarkable safety profile of BoNT drugs due to their minimal migration from the site of injection, the possibility of local diffusion or potential leakage into the systemic circulation exists and depends on a number of factors including volume, dose, and injection technique. Most complications and unwanted effects of BoNT injections, such as weakness, are due to local diffusion and are generally limited to the adjacent muscles. Systemic adverse effects such as allergic reactions and generalized weakness are due to transport within the blood circulation and are very rare.

Contraindications to BoNT therapy are limited to a hypersensitivity to the toxin or an infection at

the site of injection, and there are no known drug interactions with botulinum toxin although caution is recommended when BoNT is used in patients treated simultaneously with aminoglycosides, anticholinergics, or other neuromuscular-blocking agents.

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Bradykinin

► Kinins

Brain Capillary Endothelial Cells

► Blood-Brain Barrier

Bremelanotide

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Synonyms

PT-141; VYLEESI

Definition

PT-141, or bremelanotide, is a melanocortin receptor (MCR) agonist that was approved by the FDA on June 21, 2019, for use in premenopausal women with generalized hypoactive sexual desire disorder (HSDD). HSDD is present in 1 out of 10 women. It is defined as low sexual desire that causes distress to an individual or relationship that is not due to interpersonal problems, the effects of drugs or medications, or the presence of a medical or psychiatric condition. In phase 3 clinical trials, bremelanotide was effective in treating HSDD but was accompanied by adverse events including nausea (40%), flushing (20.3%), and headache (11.3%). Currently, several studies of co-administering bremelanotide with anti-emetic (anti-nausea) drugs are ongoing in the hopes of seeing a reduction in the presence of adverse events (Dhillon and Keam 2019). Since its FDA approval, bremelanotide has been marketed by AMAG Pharmaceuticals under the brand name VYLEESI as a treatment for HSDD in premenopausal women. VYLEESI is a 1.75 mg subcutaneous injection that is taken at least 45 min before sexual activity (AMAG Pharmaceuticals Inc. 2019).

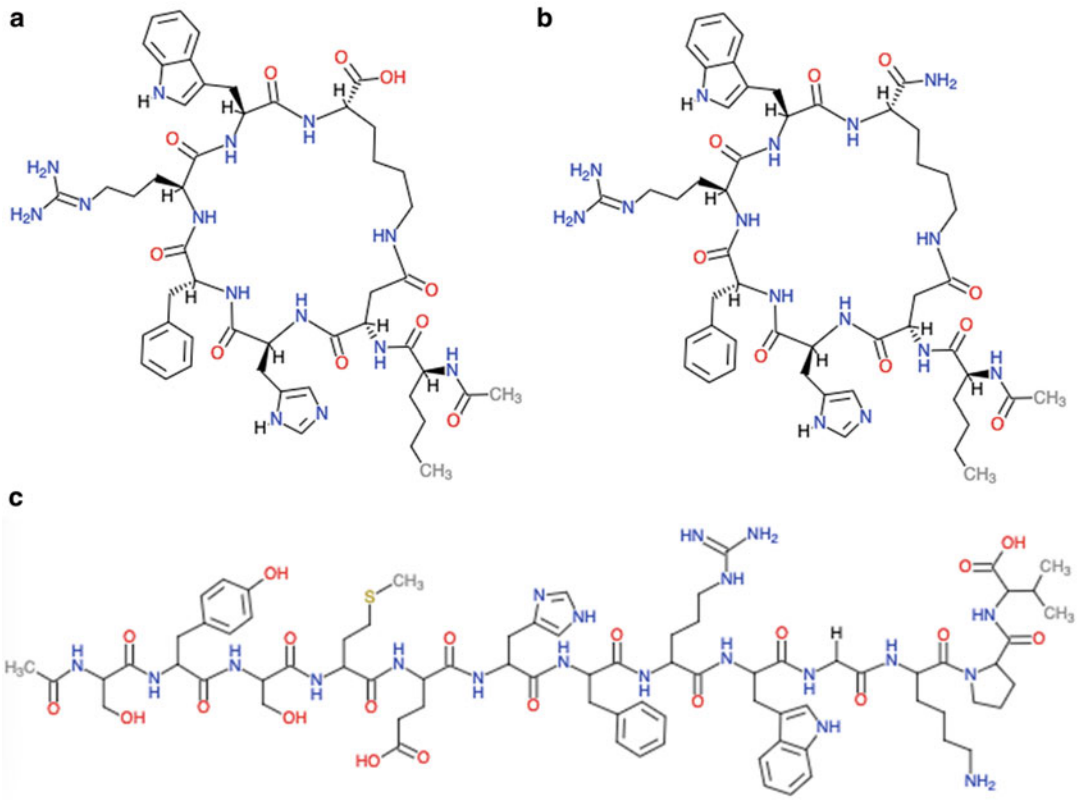
Although bremelanotide has been approved for use in women, it has also been studied as a treatment for men with erectile dysfunction (ED). ED is characterized as the inability to maintain an erection sufficient for sexual intercourse. The

primary treatment for men with ED is use of a phosphodiesterase 5 (PDE5) inhibitor such as Viagra. However, PDE5 inhibitors are not effective in all men. The prevalence of Viagra nonresponsive men has generated numerous phase 1 and phase 2 clinical trials investigating bremelanotide as an alternative or adjuvant treatment. Many phase 2 clinical trials have supported the efficacy of bremelanotide when used alone or when co-administered with PDE5 inhibitors (Hellstrom 2008). However, phase 1 clinical trials have demonstrated a host of adverse events including increased blood pressure (White et al. 2016). With the tight association between ED and cardiovascular disease, the increase in blood pressure elicited by bremelanotide has proven the biggest barrier preventing phase 3 clinical trials in men.

While bremelanotide has been investigated almost exclusively for its effects on reproductive behaviors, like other MC4R agonists, it also inhibits food intake. For example, bremelanotide has been used a positive control for food intake and body weight suppression in an investigation of other MCR-binding peptides in rats (Xin Chen et al. 2009). As mentioned, bremelanotide tends to induce nausea after acute administration in humans, which would be expected to produce food aversion.

Mechanism of Action

Bremelanotide is a cyclic heptapeptide lactam that is a functional analog of endogenous α-melanocyte-stimulating hormone (α-MSH) (Fig. 1a). α-MSH differs from bremelanotide as the former is a noncyclic tridecapeptide (Fig. 1c). Bremelanotide was originally derived from another synthetic α-MSH analog called melanotan



Bremelanotide, Fig. 1 **Melanocortin drugs:** (**a**) bremelanotide, (**b**) melanotan II, and (**c**) α-MSH

II which is primarily used as a sunless tanning agent. Although melanotan II has been shown to elicit erections, it is not ideal for treatment of sexual function due to a slow induction of erection (roughly 2 h). In contract, bremelanotide can induce erection as early as 45 min postinjection. Melanotan II's structure only differs from bremelanotide by the presence of a C-terminal amide (Fig. 1b) (King et al. 2007).

Bremelanotide is a melanocortin receptor (MCR) agonist. It non-selectively activates multiple MCR subtypes with the following order of affinity: MC1R, MC4R, MC3R, MC5R, and MC2R (AMAG Pharmaceuticals Inc. 2019). Not much is known about bremelanotide's mechanism of action, but some conclusions can be made based upon our current understanding of MCRs. MC1R is expressed by melanocytes found in skin and hair. Activation of MC1R by α-MSH leads to increased pigmentation, which reduces DNA damage from ultraviolet light exposure. MC1R activation is likely the cause of the focal hyperpigmentation prevalence seen after frequent dosing of bremelanotide. MC4R is prominently expressed in the central nervous system but is particularly concentrated in the PVN. In adult male Sprague-Dawley rats, immunostaining of c-fos (a protein expressed after neural activation) 2 h after bremelanotide treatment showed an increase in neural activation in the paraventricular nucleus (PVN) of the hypothalamus. In an in vitro study, bremelanotide treatment led to an increase in cAMP production in MC4R-expressing HEK-293 cells, which is associated with neural activation. These results indicate that bremelanotide primarily activates MC4R-expressing neurons in the PVN to mediate its effects on sexual function (King et al. 2007).

Various experiments have been done by Palatin Technologies to further understand the pharmacokinetic profile of bremelanotide. A 1.75 mg subcutaneous dose of bremelanotide has a mean plasma C_{max} of 72.8 ng/mL and a median T_{max} of about 1 h. When increased doses (0.3–10 mg) of bremelanotide are administered, C_{max} increases and plateaus at 7.5 mg in plasma. The bioavailability of bremelanotide was 100% with no difference between administration to the abdomen or thigh. The average volume of distribution is 25.0 L with 21% of bremelanotide binding to serum proteins. 1.75 mg of bremelanotide has a mean terminal half-life of roughly 2.7 h with a clearance of approximately 6.5 L/hr. A study utilizing a radiolabeled dose found that 22.8% of bremelanotide is excreted through feces and 64.8% is excreted in urine (AMAG Pharmaceuticals Inc. 2019).

Clinical Use (Including Side Effects)

Phase 1 Clinical Trials

Phase 1 clinical trials designed to test drug safety showed bremelanotide treatment leads to a transient increase in blood pressure (BP) in both men and women. For women, a randomized, double-blind, and placebo-controlled trial tested the effects of three doses of subcutaneous bremelanotide (0.75, 1.25, and 1.75 mg). The trial consisted of 397 premenopausal women with female sexual dysfunction. BP changes were not significant in women treated 0.75 mg of bremelanotide. Nevertheless, within 4 h, 1.25 mg of bremelanotide led to a significant increase in systolic BP by 2.4 mmHg while 1.75 mg led to a 3.1 mmHg increase. Additionally, in that same time period, diastolic BP significantly increased by 3.0 mmHg (1.25 mg) and 3.2 mmHg (1.75 mg). All three groups had a maximal heart rate decrease of roughly six beats per minute around 4–8 h after bremelanotide injection. Consistent with these results, a separate study on males receiving 4–6 mg of subcutaneous bremelanotide had a 4–5 mmHg increase in systolic BP. Bremelanotide was originally intended for intranasal delivery. However, intranasal bremelanotide is inconsistently absorbed into circulation leading to erratic systemic concentrations and unpredictable effects on BP (White et al. 2016). Intranasal administration was eventually replaced with subcutaneous injection. The above results are further supported by additional phase 1 clinical trials on women that were done by Palatin Technologies alongside the stage 3 clinical trials. These additional trials required by the FDA prior to approval of VYLEESI showed a maximal

increase of 6 mmHg in systolic BP, increase of 3 mmHg in diastolic BP, and decrease in heart rate of 5 beats per minute. The significant effects of bremelanotide occurred 2–4 h post dose and were completely gone within 12 h (AMAG Pharmaceuticals Inc. 2019).

Another phase 1 clinical trial tested the safety of bremelanotide when co-administered with alcohol. In this randomized, placebo-controlled, double-blind, three-period, three-way crossover study, 12 healthy female and 12 healthy male subjects drank a placebo beverage or 0.6 g/kg ethanol; 10 min later, a 20 mg dose of intranasal bremelanotide or a placebo was given. The quantity of ethanol consumed was three standard drinks (equivalent to three 1.5-ounce shots of 80-proof liquor). Incidence of flushing was similar between bremelanotide plus ethanol and bremelanotide alone. Additionally, incidence of headache was similar between bremelanotide plus ethanol and the ethanol alone. In all, alcohol consumption had no synergistic effects and did not impact the pharmacokinetic profile of bremelanotide (AMAG Pharmaceuticals Inc. 2019).

Phase 2 Clinical Trials

A critical phase 2 clinical trial provided evidence for the efficacy of bremelanotide treatment in women. This double-blind study used premenopausal women that were at least 21 years old who had been diagnosed with HSDD and/or female sexual arousal disorder for at least 6 months. The 327 participants were randomized to take bremelanotide 0.75, 1.25, or 1.75 mg or placebo. From baseline till the end of the 12-week study, an increase in 0.7 satisfying sexual events was reported in women taking 1.25 mg or 1.75 mg compared to a 0.2 increase in placebo ($p < 0.0180$). During further self-reported assessments, the study found that bremelanotide treatment lead to an increase in sexual desire and decrease in distress associated with sexual function (Dhillon and Keam 2019). The abundance of important phase 2 clinical trials provided enough support of bremelanotide's efficacy to begin phase 3 clinical trials in women.

In men with ED, two phase 2 studies analyzing the effects of bremelanotide provided evidence for its efficacy alone and when co-administered with the PDE5 inhibitor sildenafil. The first study was in sildenafil-resistant subjects, defined as having less than a 50% success rate of achieving an erection after taking 100 mg of sildenafil. Twenty-five men took a dose of 4 mg and 6 mg bremelanotide or a placebo before watching erotic videos. Erectile responses were measured using a RigiScan device during visual sexual stimuli. Subjects receiving 4 mg bremelanotide had an average duration of over 60% erectile rigidity for 28 min compared to 41 min in the 6 mg group and 6 min in placebo group. In the second study, 19 sildenafil-responders were assigned to take 25 mg of sildenafil with either 7.5 mg of intranasal bremelanotide or a placebo. Like the previous study, erectile responses were monitored using the RigiScan device during visual sexual stimuli. A statistically significant increase in the average duration of over 60% rigidity was seen in the bremelanotide/sildenafil group (≈67 min) compared to sildenafil alone (≈46 min). No additional side effects were reported from co-administration of bremelanotide and sildenafil. Thus, co-administration may lead to a synergistic effect on erectile function that improves quality of life in both sildenafil responsive and nonresponsive men. This synergy provides the potential for dose-reduction of both drugs while simultaneously reducing prevalence of adverse events (Hellstrom 2008).

A larger-scale phase 2 clinical trial of bremelanotide in men provided additional evidence of its efficacy for ED treatment. In this 12-week study, 728 nondiabetic men with ED were randomly assigned to take 5, 7.5, 10, 12.5, or 15 mg of intranasal bremelanotide or a placebo. At the beginning and end of the study, men took the International Index of Erectile Function (IIEF) questionnaire. Men treated with 7.5 mg of bremelanotide or higher showed a significant increase in their IIEF score. The Self-Esteem and Relationship (SEAR) questionnaire was also taken. From baseline to end of study, there was a significant increase in scores in the SEAR questionnaire for men taking bremelanotide. This indicates that

bremelanotide administration led to an improvement in self-esteem and in interpersonal relations (Hellstrom 2008). Overall, bremelanotide's efficacy in treatment of ED is well-supported; however, bremelanotide's effects on blood pressure has prevented continuation into phase 3 clinical trials.

Phase 3 Clinical Trials

Two identical, 24-week, randomized, double-blind, studies titled 301 and 302 occurred in premenopausal women with HSDD but are known collectively as the phase 3 RECONNECT studies. The phase 3 RECONNECT studies were completed by Palatin Technologies in 2017 and were included in a new drug application submitted in 2018. The results supported the FDA's approval of bremelanotide for premenopausal women with HSDD in 2019. For both studies, subjects were premenopausal women at least 18 years old, who were in a relationship for at least 6 months and had been diagnosed with HSDD for at least 6 months. The subjects received either 1.75 mg of bremelanotide ($n = 635$) or placebo ($n = 632$). Participants were instructed to self-administer the drug as needed 45 min before sexual activity. Roughly 40% of patients taking bremelanotide discontinued the study prior to its 24-week completion compared to 13–25% for placebo. The remaining participants, on average, took bremelanotide two to three times per month, no more than once a week. Two published efficacy assessments were made prior to the study as a baseline and after completion. The first assessment was the Female Sexual Function Index (FSFI) which composed of two questions. Participants were asked "Over the past 4 weeks, how often did you feel sexual desire or interest?" and "Over the past 4 weeks, how would you rate your level of sexual desire or interest?". The participants gave a rating for both questions from 1 (never or none) to 5 (always or very high). These questions are intended to document changes in sexual desire. The responses to these two questions were added together and the sum was multiplied by 0.6 to give scores ranging from 1.2 to 6. In Study 301, FSFI changed from baseline to end of study by 0.5 in bremelanotide versus 0.2 in placebo ($p < 0.0002$). Likewise, in Study 302, the bremelanotide-treated group changed by 0.6 versus 0.2 in placebo ($p < 0.0001$). The second assessment was the Female Sexual Distress Scale (FSDS) which asks, "How often did you feel: Bothered by low sexual desire?". Women responded on a scale of 0 (never) to 4 (always). This question was intended to assess the level of distress related to their low sexual desire. In Study 301, scores on the FSDS had decreased by 0.7 in the bremelanotide-treated group compared to placebo which decreased by 0.4 ($p < 0.00001$). Correspondingly, in Study 302, scores decreased in the bremelanotide-treated group by 0.7 compared to a 0.4 decrease in the placebo-treated group ($p < 0.0053$). Another test assessed the number of satisfying sexual events, but no differences were seen between bremelanotide or placebo treatment. Overall, these tests in the phase 3 RECONNECT studies provided evidence to the FDA of bremelanotide's efficacy (AMAG Pharmaceuticals Inc. 2019; Dhillon and Keam 2019).

The phase 3 RECONNECT studies also evaluated the safety of bremelanotide by analyzing the prevalence of adverse events (AEs). Of the common AEs, a majority were mild (31%) or moderate (41%) in intensity. The most common AEs of bremelanotide treatment (> 4% incidence) included nausea (40% vs. 1.3% in placebo), flushing (20.3% vs. 0.3% in placebo), injection site reactions (13.2% vs. 8.4% in placebo), headache (11.3% vs. 1.9% in placebo), and vomiting (4.8% vs. 0.2% in placebo). The most common bremelanotide-related AE was nausea (40%). This AE led to 13% of participants requiring an anti-emetic and 8% of participants leaving the study early. Notably, the incidence of nausea was 21% during the first dose of bremelanotide but declined to 3% after subsequent uses of the drug. The high number of women taking bremelanotide who prematurely discontinued the study and the lack of change in satisfying sexual events is likely attributable to the incidence rate of AEs, particularly nausea. For instance, 18% of participants left the study due directly to AEs (AMAG Pharmaceuticals Inc. 2019). Currently, a phase 1 study is planning to enroll 228 premenopausal women to understand the effects of co-administration of

bremelanotide with an antiemetic on the incidence of nausea (Dhillon and Keam 2019).

In men, bremelanotide's effects on blood pressure have been a major barrier preventing it from moving into phase 3 clinical trials for the treatment of ED. As mentioned (see *Phase Clinical Trials*), studies show that bremelanotide treatment leads to transient increases in both systolic and diastolic blood pressure (BP) in men and women (White et al. 2016). Although transient, this increase in BP is a health concern for people at high risk of cardiovascular disease, including people above the age of 50. ED correlates strongly with both age and cardiovascular disease risk, often developing in men over the age of 50; 94% of men with ED reported having their first problems with erection after 49 years of age (Bacon et al. 2003). Therefore, the number of men under the age of 50 with ED is a restricted demographic, making recruitment for phase 3 trials problematic and the potential drug market small. However, HSDD in women is not associated with the development of cardiovascular disease and is present in 8.9% of women between the ages 18 and 44 (Dhillon and Keam 2019). Therefore, bremelanotide can be marketed to a large demographic of premenopausal women for the treatment of HSDD with less risk.

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B

Bromodomain Inhibitors

▶ Epigenetics

Bronchial Asthma

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Synonyms

Asthma bronchiale

Definition

According to the Global Initiative for Asthma (GINA, http://www.ginasthma.org), asthma affects about 340 million people worldwide. Men of all ages are affected by this chronic airway disorder that – without appropriate treatment – limits quality of life and may be fatal.

Asthma is a chronic inflammatory disorder of the airways associated with recurrent airway obstructions, airway hyperresponsiveness, and airway remodeling. Symptoms of asthma include **wheezing**, coughing, chest tightness, and shortness of breathing, particularly at night or in the early morning. Airway obstruction is often reversible either spontaneously or after treatment.

Missing or inadequate treatment will result in airway remodeling.

Basic Mechanisms

The majority of pediatric and adult patients with asthma have so-called extrinsic (allergic) asthma (Quirt et al. 2018) (Fig. 1). These asthmatics have increased serum IgE levels and suffer from Type I allergy characterized by high serum IgE levels and blood eosinophils. In susceptible individuals **allergens** are presented by dendritic cells (DCs), which then lead to an IL-25, IL-33, and thymic stromal lymphopoietin (TSLP)-driven induction of T_H2 CD4+ T-lymphocytes. Subsequently, T_H2 cells produce the T_H2 cytokines IL-4, IL-5, and IL-13 that induce the maturation of naïve T-helper cells (T_H0) into further T_H2 cells and induce B-cells to produce specific IgE antibodies. Among these, especially IL-4 triggers the IgE production of B lymphocytes. Allergic asthma depends on the presence of IgE bound to high-affinity Fcε-receptors ($Fc\varepsilon_\varepsilon R$) especially on mast cells and to low-affinity Fcε-receptors on other cells such as eosinophils. Without IgE allergic asthma cannot occur. These responses can be downregulated by IL-10 from regulatory T-cells (Treg) or macrophages that modulate T_H2 cell maturation and increase tolerance to allergens. In addition, IL-17 from T_H17 cells may downregulate eosinophil chemotaxis (Barnes 2018).

There is also non-allergic asthma (Fig. 1) that involves innate lymphoid cells (ILCs). Here, neither IgE nor mast cells are required to induce or perpetuate the inflammatory response. However, pro-inflammatory mediators comparable to those in allergic responses are released from epithelial cells upon exposure to xenobiotics, pathogens, or toxins. These mediators, including IL-25, IL-33, and TSLP, favor the polarization of ILC cells to the type 2 phenotype (ILC2) that produce IL-5 and IL-13, responses that may be enhanced by IL-9 and prostaglandin D_2 (PGD_2). Again, IL-5 recruits eosinophils from the bone marrow and increases their survival in the bronchial mucosa of the airways, while IL-13 induces bronchial hyperresponsiveness and goblet cell hyperplasia (Lambrecht and Hammad 2015).

Cardinal Symptoms

The activation of mast cells by allergen initiates the asthma symptoms: within minutes the early allergic response (EAR), within hours the late allergic response (LAR), and within years and after repeated asthma episodes chronic airway inflammation, airway remodeling, and airway hyperresponsiveness.

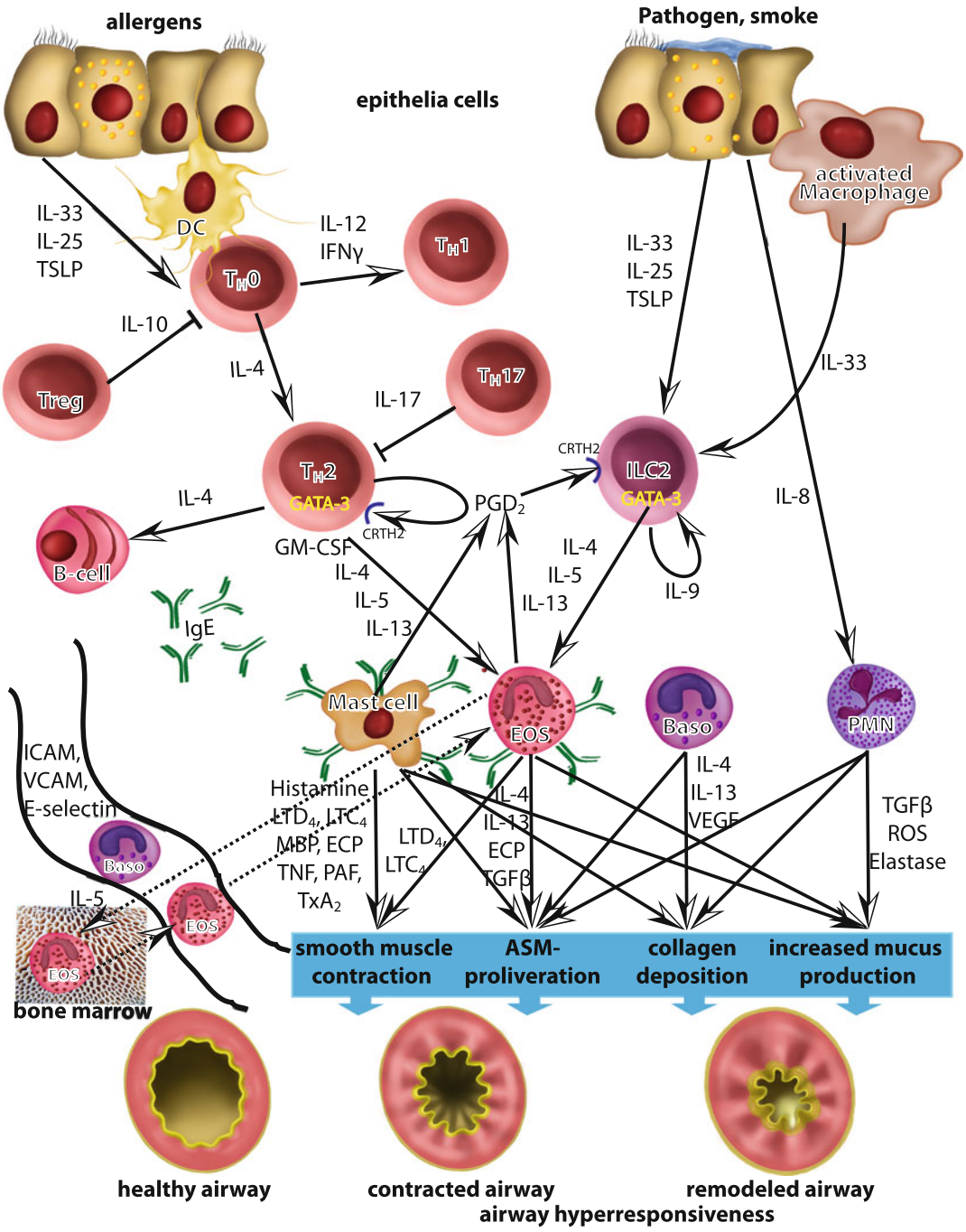
Early and Late Phase in Allergic Asthma

Immediate responses to allergen occur within minutes. Allergen-induced cross-linking of IgEs on mast cells causes degranulation of preformed (histamine, proteoglycans, serine proteases, TNFα) and secretion of newly formed mediators (prostaglandins, cysteinyl -leukotrienes, thromboxane, **platelet-activating factor**, cytokines). The mixture of histamine, prostaglandins, thromboxane, and cysteinyl leukotrienes causes airway smooth muscle contraction.

Frequently, the early allergic response is followed by a late-phase response (4–6 h later), caused by the pulmonary sequestration of immune cells, especially eosinophils, mast cells, and T-lymphocytes. This leukocyte recruitment depends on mast cell-derived mediators such as TNFα and various chemokines, as well as on the expression of adhesion molecules on leukocytes (e.g., VLA-4, CD11/18) and vascular endothelial cells (e.g., VCAM-1, ICAM-1, E-selectin). Products of these leukocytes have several functions and cause the second phase including bronchoconstriction, mucus secretion, and airway swelling. Finally, this leads to chronic inflammation and tissue destruction.

Inflammation

Airway inflammation is a characteristic clinical feature of asthma. The distinction between the LAR and chronic inflammation becomes more difficult as the disease progresses. Infiltrated leukocytes and macrophages release cytotoxic mediators such as **reactive oxygen species** and **cationic (basic) proteins** that cause epithelial damage and cytokines like IL-4, -5, -9, -13, -25, and -33 as well as alarmins like thymic stromal lymphopoietin (TSLP) that perpetuate the



Bronchial Asthma, Fig. 1 Pathophysiology of asthma. T-helper cells 2 (Th2) and innate lymphoid cells (ILC) take a central position in the pathophysiology of asthma. Various cytokines inactivate Th1 and Th17 cells, activate eosinophils, and trigger a class switch in B lymphocytes to make them produce IgE. These IgEs bind to mast cells activating mast cells, EOS, leukocytes, and macrophages. The mast cell mediators trigger the early allergic response, the late allergic response, the activation of Th2 cells, and other inflammation responses. The inflammatory response mobilizes eosinophils (EOS), neutrophils (PMN), macrophages, and lymphocytes that infiltrate the lung tissue and sustain the inflammation. Eosinophils release mediators that contribute to the EAR and cytotoxic agents that injure the epithelial layer. Repetitive and chronic inflammation leads to airway remodeling and airway hyperresponsiveness. Baso, basophils; DC, dendritic cell

inflammation. Sustained inflammation leads to airway hyperresponsiveness and airway remodeling.

Hyperresponsiveness

Airway hyperresponsiveness is an exaggerated airway narrowing in response to a variety of unspecific stimuli. It can be measured by bronchial provocation with histamine, methacholine, or adenosine. The reason for hyperresponsiveness may be stimulation of sensory nerves, located within the epithelium, that become easily accessible after denudation of the epithelium. This irritation of nerves leads to reflex smooth muscle contraction, mucus secretion, and edema formation. In addition, hyperresponsiveness can also be caused by IL-5, IL-13, major basic protein (MBP), eosin cationic protein (ECP), peptidoleukotrienes, and smooth muscle hypertrophy.

Bronchial Remodeling (BHR)

The term "airway remodeling" refers to structural changes in the airways and is the consequence of chronic airway inflammation. The structural changes involve a thickening of the airway wall that is induced by leukotriene C, VEGF, and tryptase from mast cells as well as TGFβ from eosinophils. IL-13 induces hyperplasia of goblet cells coupled with hypertrophy of submucosal glands. Increased vascularity of the airway wall amplifies mucus secretion and plasma protein leakage that are responsible for the formation of the characteristic mucus plugs that obstruct the airways. Other characteristic features are smooth muscle hypertrophy and hyperplasia and subepithelial fibrosis by growth factors. This explains the incomplete reversibility of airway narrowing in asthmatic patients.

Drug-Sensitive Asthma

Nonsteroidal anti-inflammatory drugs (NSAID) like aspirin affect about 10% of all asthmatics. This is a non-allergic response to aspirin and other agents that inhibit the cyclooxygenase. Mechanistically, the most likely explanation is a lack of bronchoprotective prostaglandin E_2 and shifting of arachidonic acid metabolism toward the leukotriene pathway, favoring the production of the bronchoconstrictive leukotrienes C_4 and D_4 and chemotactic leukotriene B_4.

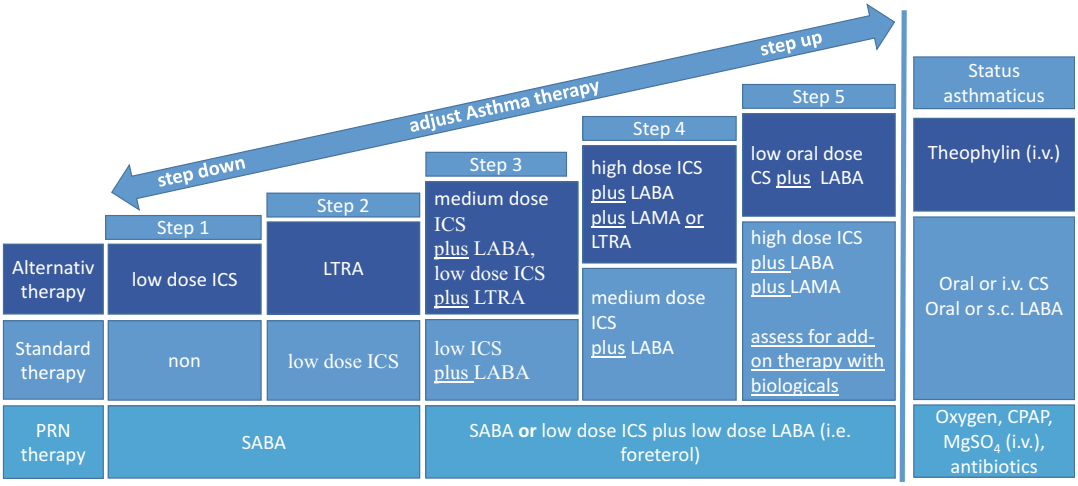
General Principles of Managing Asthma

In asthma management the long-term goals are to reduce and control reoccurring. This involves a continuous approval of the status of asthma including symptoms and lung functions, as well as regular adjustment of the medication in a step-up/step-down manner (Fig. 2, GINA).

Pharmacological Interventions

In general, pharmacological agents are divided into **relievers** that lead to a rapid improvement of breathing during asthma attacks (β2-agonists, anticholinergics) and **controllers** (steroids, antileukotrienes, biologicals) that control the disease by suppressing the inflammation and the exacerbations (Fig. 2). The basic pharmacological therapy is inhaled corticosteroids (ICS). GINA recommends that every adult and adolescent with asthma should receive ICS-controller medication. There are five steps for the pharmacological therapy of asthma, depending on the asthmatic state (Quirt et al. 2018; Israel and Reddel 2017; Patel et al. 2018; Barnes 2018) (Fig. 2). The classification of asthma in patients should be initially higher to guarantee a sufficient anti-inflammatory therapy. The importance in asthma therapy is a continuous monitoring of asthma symptoms. The overall aim in asthma therapy is to achieve adequate asthma control and risk reduction. Once patients have no symptoms, asthma is considered under control, and therapy may be adjusted accordingly (step down). The basic principle is a step-up and step-down strategy to obtain the lowest possible therapeutic treatment for adequate control in order to control the inflammation-driven progression and chronification of the disease with the least possible side effects.

Some 5–10% of patients are resistant to corticoid therapy; these patients may benefit from new antibody-based therapies (biologicals). At present, healing of asthma by pharmacological



Bronchial Asthma, Fig. 2 Therapeutic scheme for the stepwise pharmacological therapy of asthma. PRN, pro re nata; SABA, short-acting beta-2 agonists; LABA, long-acting beta-2 agonists; LAMA, long-acting muscarinic antagonists; LTRA, leukotriene-receptor antagonist; ICS, inhaled corticosteroids; CS, corticosteroids. (Modified from https://ginasthma.org/, 2019)

therapy is not possible, except for hyposensibilization therapies in case of allergic asthma.

Allergen-Specific Immunotherapy (SIT)

In allergic asthma, with well-known allergens and high levels of specific IgE, allergen-specific immunotherapy is an excellent therapeutic option. This therapy depends on the availability of allergens to induce immunological tolerance and on the severity of the disease. A variety of immunogenic proteins from allergens, such as grass, ragweed, house dust mite, some animal hair, and dander, are available for either subcutaneous or sublingual hypersensitization. The problem of this therapy is a risk of occasional anaphylactic shocks. Although the hypersensitization does usually not lead to a complete remission of symptoms or airway inflammation, it can tremendously reduce the amount of medication needed to control the disease.

β_2-Receptor Agonists

Beta-2-receptor agonists relax the bronchial smooth muscle by increasing intracellular cAMP levels that in turn decrease calcium levels and interfere indirectly with myosin light chain phosphorylation (critical for smooth muscle contraction). Beta-2-receptor agonists are very effective bronchodilators and can rescue patients from asthma attacks. They are classified according to their duration of action: Short-acting beta-2 agonists (**SABA**) like terbutaline, albuterol, and fenoterol have a short biological half-life of 2–6 h and are used as the only therapy in nonpersisting asthma and as a standby medication in all other stages of asthma. However, the GINA guidelines do not prefer SABA use alone, as this may increase the risk of exacerbations. For other levels of asthma, long-acting beta-2 agonists (**LABA**) such as salmeterol, bambuterol, and formoterol with half-lives of 15–18 h are used. Ultra-long-acting beta-2 agonists (**ULABA**) like indacaterol or olodaterol, with half-lives up to 24 h, are mainly used for the treatment of **COPD**.

Typical side effects of this class of drugs are interactions with cardiac β_1-receptors, leading to tremor, arrhythmias, and tachycardia.

Anticholinergics

Cholinergic smooth muscle contraction occurs mainly via M_3 receptors. The clinically used anticholinergics (like ipratropium bromide, tiotropium bromide) bind competitively to M_3, M_2, and M_1 receptors on airway smooth muscle and mucosal glands, but only tiotropium dissociates quickly

from the postsynaptic inhibitory M_2 receptors, contributing to its enhanced inhibitory effect and its prolonged actions (16 h instead of 6 h). However, the lag phase of 30 min after intake limits their usefulness in acute asthma attacks. The inhaled anticholinergics are rarely used in asthma, but frequently as a long-term treatment of COPD. Despite inhalation, typical parasympathetic side effects such as increased heart rate, soporific effects, and dry mouth may occur.

Anti-leukotriene Therapy

Leukotrienes have multiple roles in asthma. They cause bronchoconstriction, swelling, and mucus secretion (LTC_4, LTD_4) as well as eosinophil recruitment (LTE_4). Anti-leukotriene therapy is based either on inhibition of 5-lipoxygenase (zileuton; only approved in the USA) or on cys-LT1 receptor antagonists (**LTRA**, like montelukast, zafirlukast, and pranlukast). The anti-inflammatory potential of leukotriene antagonists is smaller than that of steroids. Patients who cannot or will not take inhaled steroids are possible candidates for these drugs. Anti-leukotriene therapy is particularly effective in the treatment of NSAID-sensitive and exercise-induced asthma.

Corticosteroids (CS)

As asthma is a chronic, inflammatory disease, steroids represent the most important and most frequently used medication. Already after the first treatment, steroids reduce cellular infiltration, inflammation, and the LAR, whereas changes in the EAR require prolonged treatment to lower the existent IgE levels. The mechanisms of steroid actions are complex and only incompletely understood. Besides their general anti-inflammatory properties (see chapter ▶ "Glucocorticoids"), like the reduction of IL-4, IL-5, and IL-13 production, the induction of IL-10 production from T-lymphocytes is also of particular importance for asthma therapy. The introduction of inhaled steroids (**ICS**) has dramatically limited the side effects of steroids and is considered one of the most important advancements in asthma therapy. Inhaled steroids (beclomethasone, budesonide, fluticasone, triamcinolone, mometasone) are used in all stages of asthma. Oral steroids are used in severe uncontrolled asthma, during an acute exacerbation for a short period of time (3–5 days) and for the treatment of **status asthmaticus**. Minor side effects of most inhaled steroids are hoarseness and candidiasis, while oral corticosteroids are associated with systemic side effects (including adrenal suppression, osteoporosis, and cataracts), which is mostly avoided by the prodrug steroid ciclesonide. Nevertheless, it has been recently shown that the use of oral steroids in severe asthma is associated with corticosteroid comorbidities in nearly all patients (Sweeney et al. 2016). Therefore, long-term high-dose systemic steroids should be avoided or reduced by the use of **biologicals** such as specific antibodies.

Biologicals

To avoid severe side effects of orally given steroids, biologicals are new therapeutic options. Monoclonal antibody therapies have shown to reduce the frequency of severe asthma (Barnes 2018).

Anti-IgE

Allergic or extrinsic asthma affects the majority of all asthmatics. The production of IgE by B-cells and the FcεR-dependent activation of mast cells, eosinophils, basophils, and macrophages are critical for the development of asthma. A clear correlation exists between circulating IgE levels and disease severity. Reduction of circulating IgE antibodies by a humanized mouse anti-IgE antibody (omalizumab) mitigates asthma symptoms. Owing to their long half-life, (bi)monthly subcutaneous antibody injections are sufficient to suppress IgE levels and to reduce asthma symptoms in pediatric and adult asthmatics. This biological is approved as an add-on therapy to steroids in severe asthma.

Antibodies Against IL-4, IL-5, and IL-13

Interleukin-5 (IL-5) is the essential cytokine for recruitment and increased lifetime of eosinophils. Mepolizumab, reslizumab, and benralizumab are three IL-5-targeting agents approved for the treatment of severe eosinophilic asthma (>400 cells

per μL). Reslizumab and mepolizumab are IL-5-scavenging antibodies, whereas benralizumab blocks the IL-5 receptor. Mepolizumab and benralizumab are injected subcutaneously every 4 weeks, whereas reslizumab is administered every 4 weeks via intravenous infusion.

IL-13 is one of the main cytokines responsible for bronchial hyperresponsiveness. Tralokinumab is an anti-IL-13 human immunoglobulin G4 monoclonal antibody, neutralizing IL-13 levels and developed for the treatment of severe, uncontrolled asthma. The STRATOS trials showed the safety and efficacy of subcutaneous bi- or 4-weekly tralokinumab injections. Tralokinumab may be beneficial in patients with high fractional exhaled nitric oxide, even though it lacks an ICS-sparing effect (Busse et al. 2019).

T-helper 2 (Th2) lymphocytes are induced and persist in allergic asthma. IL-4 is the essential cytokine for the polarization of TH_0 toward TH_2 cells. In addition IL-4 and IL-13 together induce goblet cell hyperplasia, smooth muscle proliferation, and mucus production. Dupilumab is a fully human monoclonal antibody that blocks the heterodimeric receptor complex consisting of the IL-4 receptor α-subunit and the IL-13 receptor α1-subunit. Dupilumab is approved for subcutaneous injections on a weekly or bi-weekly basis in patients with moderate to severe allergic asthma (≥12 years).

Rarely Used Therapeutics

At the beginning of the twentieth century, methylxanthines like **theophylline**, bamifylline, and elixophyllin were among the first therapeutic options for asthma patients. Acting at least in part as adenosine (A1)-receptor and phosphodiesterase (PDE) antagonists, they increase intracellular cAMP levels. Accumulation of intracellular cAMP by inhibition of PDE3 relaxes airway smooth muscle, and inhibition of **PDE4** reduces inflammation. PDE4 inhibitors (roflumilast, cilomilast) have shown some effectiveness in advanced clinical trials but are mainly used for the treatment of **COPD**. Today methylxanthines are rarely used, and when then as an additional therapy for uncontrolled asthma (oral) and for status asthmaticus (i.v.).

Also, **cromones** may be used to prevent asthma attacks, if taken continuously. They act as mast cell-stabilizing agents that prevent the release of mast cell mediators. Cromones have virtually no side effects, but today they are only rarely used, mostly in children and sometimes in adults who do not tolerate or who are not willing to take steroids.

Future Therapeutic Options

In COPD patients with eosinophilia, the GATA3-specific DNAzyme SB010 attenuated inflammatory response. Therefore, this therapy is approved for **COPD** with eosinophilia. Eosinophilia is also a common phenotype of allergic patients with asthma. Therefore this therapy may be a promising therapeutic option in asthmatic patients.

The chemoattractant receptor-homologous molecule (CRTH2) is a G-protein-coupled receptor expressed on various cell types including T_H2 cells, ILCs, eosinophils, mast cells, and basophils. Prostaglandin D_2, a main mediator of mast cells, is the endogenous ligand. CRTH2 antagonists are in clinical development for the treatment of asthma.

Patients with severe non-eosinophilic asthma usually don't benefit from biologicals against IgE, IL4, IL5, and IL13. Thymic stromal lymphopoietin (TSLP) is released as an alarmin by epithelial cells (see above) and is central to the regulation of innate immune cells, dendritic cells, T-cells, and B-cells. Tezepelumab is a human monoclonal antibody that binds TSLP. In phase 2 clinical trial, subcutaneous Tezepelumab injections every 4 weeks reduced asthma exacerbations significantly. A phase 3 trial has started in October 2018 (Barnes 2017, 2018).

Asthma therapy is continuously developing toward personalized medicine. Increasingly, the therapy takes into account asthma subsets, IgE and eosinophil levels, and comorbidities.

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Butyrylcholinesterase (EC 3.1.1.8) (BChE or BuChE): ChE

► Cholinesterases

C

C Kinase

► Protein Kinase C

Ca^{2+} Antagonists

► Ca^{2+} Channel Blockers

Ca^{2+} Channel Antagonists

► Ca^{2+} Channel Blockers

Ca^{2+} Channel Blockers

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Synonyms

Ca^{2+} antagonists; Ca^{2+} channel antagonists

Definition

Ca^{2+} is an important intracellular second messenger that controls several cellular functions including muscle contraction in smooth and cardiac muscle. Ca^{2+} channel blockers ("Ca^{2+} antagonists") inhibit depolarization-induced Ca^{2+} entry through one specific member of the voltage-gated Ca^{2+} channel family, so-called L-type channels. Inhibition of L-type channels in muscle cells of the cardiovascular system causes a decrease in blood pressure, a reduction of cardiac contractility, and has antiarrhythmic effects. Therefore, these drugs are used clinically to treat hypertension, myocardial ischemia, and cardiac arrhythmias.

Mechanism of Action

Voltage-gated Ca^{2+} channels are Ca^{2+}-selective pores in the plasma membrane of all electrically excitable cells, such as muscle cells, neurons, endocrine cells, and sensory cells. They open in response to membrane depolarization (e.g., an action potential) and permit the influx of Ca^{2+} ions along their electrochemical gradient into the cytoplasm. L-type Ca^{2+} channels, the pharmacological target of Ca^{2+} channel blockers, are one of the five different types of voltage-gated Ca^{2+} channels.

Ca^{2+} Channel Physiology

Ca^{2+} influx across the plasma membrane not only triggers Ca^{2+}-dependent intracellular signaling events but also supports membrane depolarization. In nerve terminals and sensory or endocrine

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cells, Ca^{2+} channels initiate neurotransmitter or hormone release, respectively (stimulus-secretion coupling). Ca^{2+} entry into the soma and dendrites of nerve cells leads to the activation of intracellular pathways affecting gene transcription (excitation-transcription coupling) and neuronal plasticity. In smooth muscle and cardiac myocytes, Ca^{2+} influx through these channels induces muscle contraction (excitation-contraction coupling).

Ca^{2+} Channel Function

Like other voltage-gated cation channels, Ca^{2+} channels exist in at least three states: A resting state stabilized at negative potentials (such as the resting membrane potentials of most electrically excitable cells) is a closed state from which the channel can open. The open state is induced by depolarization. Channels do not stay open indefinitely because they are "turned off" during depolarization by transition into an inactivated state. Inactivation is driven both by depolarization (voltage-dependent inactivation) and by the permeating Ca^{2+} ions (Ca^{2+}-dependent inactivation). Inactivation helps to fine-tune Ca^{2+} signaling and to prevent toxicity induced by cellular Ca^{2+} overload during prolonged stimulation. Once the cell repolarizes, inactivated channels return to the resting state and are now again available for opening. These gating mechanisms are mainly driven by the voltage-dependent conformational changes of the four voltage-sensing domains of the pore-forming α1 subunit (Catterall et al. 2019).

Organic Ca^{2+} channel blockers can inhibit Ca^{2+} flux not only by obstructing the open pore but also by "allosterically" stabilizing inactivated closed states. By delaying its transition to the resting state after repolarization, drugs can also delay the recovery from inactivation, i.e., increase the "refractory period" of these channels (Zamponi et al. 2015).

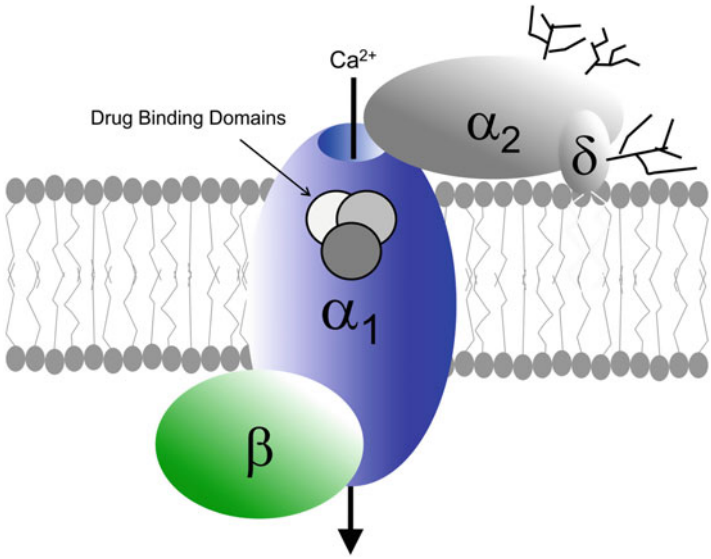
Ca^{2+} Channel Types

In order to accomplish these diverse physiological tasks described above, nature has created at least five different types of Ca^{2+} channels. These are termed L-, N-, P/Q-, R-, and T-type. Although they are all structurally similar (Fig. 1), they differ with respect to their biophysical properties and their tissue and subcellular distribution (Alexander et al. 2019). Some of them need only weak depolarizations to open and can conduct Ca^{2+} currents at membrane potentials below action potential threshold (e.g., low-voltage-activated T-type Ca^{2+} channels and Cav1.3 L-type channels). Others require stronger depolarizations and open during action potentials (e.g., high-voltage-activated N-, P/Q-, R-, and most L-type Ca^{2+} channels) (Alexander et al. 2019; Zamponi et al. 2015).

These channel types also differ with respect to their sensitivity to drugs. Only L-type Ca^{2+} channels are sensitive to the Ca^{2+} channel blockers widely known as "Ca^{2+} antagonists."

L-Type Ca^{2+} Channel Blockers

Organic blockers of L-type Ca^{2+} channels (also termed "Ca^{2+} antagonist") are widely used to treat hypertension and other cardiovascular disorders. They belong to the most frequently prescribed drugs worldwide. L-type Ca^{2+} channels are a major channel type mediating contraction in all types of muscle cells, including arterial vascular smooth muscle. Although they do not support fast neurotransmitter release from nerve terminals during short action potentials, L-type channels provide Ca^{2+} for neurotransmitter release in sensory (cochlear hair cells, retinal photoreceptors) and hormone release in endocrine cells (e.g., adrenalin and aldosterone release in adrenal chromaffin and adrenal cortical zona glomerulosa cells, respectively, insulin secretion in pancreatic β-cells). In neurons they drive Ca^{2+}-dependent signaling pathways (e.g., via the Ca^{2+}/calmodulin-dependent protein kinase CaMKII) important for coupling synaptic activity to gene transcription in neurons and contribute to synaptic plasticity, learning, and memory. Despite these multiple functions, in vivo therapeutic plasma concentrations of L-type Ca^{2+} channel blockers only cause relevant pharmacological effects in the cardiovascular system. Central nervous system side effects have not been reported, and inhibition of insulin secretion is only relevant during intoxications (Kumar et al. 2018). From the four L-type Ca^{2+} channel α1 subunit isoforms (Cav1.1–Cav1.4),

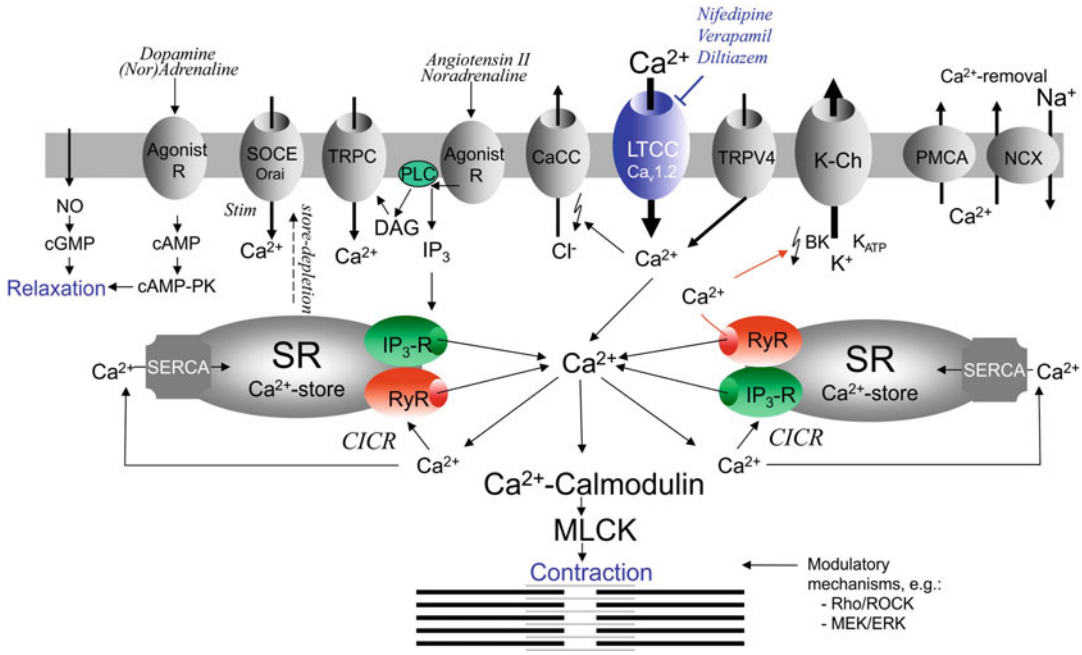


Ca^{2+} Channel Blockers, Fig. 1 Subunit structure of L-type Ca^{2+} channels: Most voltage-gated Ca^{2+} channels exist as hetero-oligomeric protein complexes. α1 subunits form the Ca^{2+}-selective ion pore and contain the voltage sensors of the channel. In the case of L-type Ca^{2+} channels, they also carry high-affinity binding sites for the different chemical classes of Ca^{2+} channel blockers (gray circles). The accessory α2-δ and β (and in some tissues also γ) subunits stabilize channel function and support their targeting to the plasma membrane. Notably, other proteins can associate with the channel allowing the formation of cell-specific signaling complexes important for channel targeting and modulation. The cryo-EM structure of the mammalian skeletal muscle (Cav1.1) L-type Ca^{2+} channel complex together with drugs bound to their binding domains within the α1 subunit has recently been reported (Zhao et al. 2019)

only Cav1.2 is expressed in arterial vascular smooth muscle and the ventricular myocardium. Cav1.3 is also expressed in the heart but serves mainly as a pacemaker channel in the sinoatrial node, a function enabled by its operating range at more negative potentials as compared to Cav1.2.

In the heart and in vascular smooth muscle, L-type channels are embedded in a network of other channels and transporters regulating intracellular Ca^{2+} signals and contractility. Simplified views of major signaling pathways are depicted in Figs. 2 and 3.

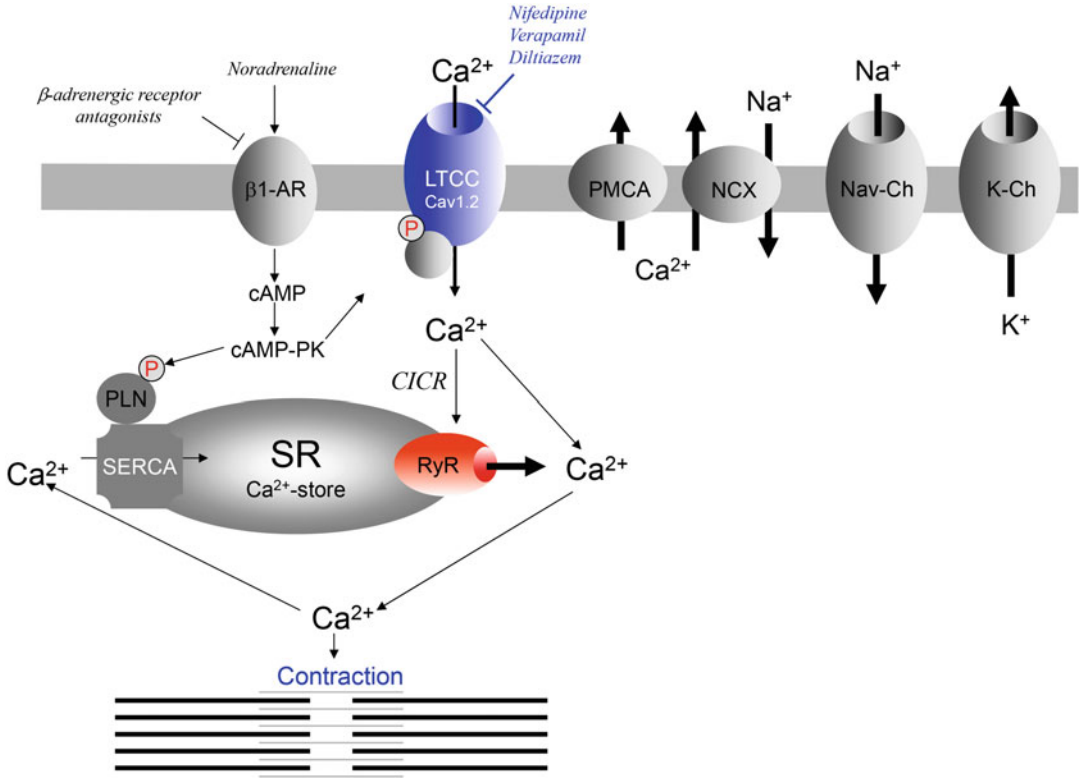
In arterial vascular smooth muscle, extracellular Ca^{2+} entry is mainly mediated by the opening of Cav1.2 L-type Ca^{2+} channels, which are a major determinant of myogenic tone, peripheral vascular resistance, and blood pressure. The inhibition of Ca^{2+} channels reduces depolarization-induced Ca^{2+} entry and therefore less Ca^{2+} is available for activation of calmodulin. Calmodulin activates myosin light chain kinase (MLCK) and thereby promotes actin-myosin interaction and contraction (Fig. 2). Vascular smooth muscle also contracts after stimulation of receptor-activated pathways, such as the pharmacologically relevant angiotensin II AT1 (by angiotensin II) and α1-adrenergic receptors (by, e.g., noradrenaline) and the IP_3-mediated release of Ca^{2+} through IP_3 receptors from sarcoplasmic reticulum (SR) stores (legend to Fig. 2 for details). Localized Ca^{2+} signals (such as sparklets, sparks, or Ca^{2+} waves) also play an essential role in orchestrating the interplay between Ca^{2+} entry and Ca^{2+} extrusion mechanisms (Brozovich et al. 2016). Agonist-induced contractions are much less sensitive to Ca^{2+} channel blockers. The differential contribution of depolarization-induced and receptor-activated contraction in different types of smooth muscle and under different pathophysiological conditions is one of the explanations why Ca^{2+} channel blockers are not effective smooth muscle relaxants in other disease states (such as bronchial asthma or urethral spasms).



Ca^{2+} Channel Blockers, Fig. 2 L-type Ca^{2+} channels in vascular smooth muscle: Simplified view of Cav1.2 L-type Ca^{2+} channel signaling as the pharmacological target of Ca^{2+} channel blockers in arterial smooth muscle. In contrast to cardiomyocytes, action potentials in smooth muscle are not carried by fast voltage-gated Na^{+}-channels, and membrane potential is more depolarized. Depolarizations are more long-lasting and induced by different mechanisms not further discussed here (including changes in intravascular pressure). Contraction requires the binding of Ca^{2+} ions to calmodulin, which then activates myosin light chain kinase (MLCK). MLCK phosphorylates the light chain of myosin, which initiates contraction. L-type Ca^{2+} channels opening in response to depolarization serve as a major global Ca^{2+} source for activation of this pathway. As a consequence, inhibition of these channels by specific blockers reduces contractility and can induce vasodilation. Alternatively, Ca^{2+} can be released from intracellular stores after activation of membrane receptors (e.g., of angiotensin II AT1 or α1-adrenergic receptors) coupled to inositol triphosphate (IP_3) production via phospholipase C (PLC). IP_3 opens Ca^{2+}-permeable IP_3 receptor (IP_3-R) channels present on the sarcoplasmic reticulum (SR) membrane. Ryanodine receptor Ca^{2+} channels (RyR) can also contribute to SR Ca^{2+} release. Receptor activation can also trigger Ca^{2+} entry, e.g., via diacylglycerol (DAG) through members of the TRPC channel family ("receptor-operated Ca^{2+} entry") or indirectly through intracellular store depletion. SR store depletion activates "store-operated Ca^{2+} entry" (SOCE) channels in the plasma membrane. These receptor-activated mechanisms are less sensitive to Ca^{2+} channel blockers. However, receptor activation and intracellular Ca^{2+} (e.g., through Ca^{2+}-activated chloride channels, CaCC) can depolarize the plasma membrane and may thus indirectly promote opening of Cav1.2 Ca^{2+} channels. Receptor-mediated activation of cAMP-dependent protein kinase (cAMP-PK) results in muscle relaxation through different mechanisms not depicted here. Activation of membrane potassium channels (K-Ch) can also oppose vasoconstriction by inducing membrane hyperpolarization. For example, Ca^{2+}-activated BK K^{+}-channels are activated by Ca^{2+} released from the SR by RyR. Note that both RyR and IP_3-R can be activated by Ca^{2+} itself ("Ca^{2+}-induced Ca^{2+} release," CICR), a positive feedback loop for Ca^{2+} release. TRPV4 channels appear to induce CICR through RyR and thus mediate membrane hyperpolarization. Major Ca^{2+} removal mechanisms involve the plasma membrane Ca^{2+} pump (PMCA) and the sodium-Ca^{2+} exchanger (NCX) in the plasma membrane and the SR Ca^{2+} pump in the SR (SERCA)

In the heart, Ca^{2+} entering through L-type channels during the action potential serves as a trigger ("trigger Ca^{2+}") for further Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum that initiates contraction (Fig. 3) (Eisner et al. 2017). β-Adrenergic receptor activation increases inotropy at least in part by cAMP-dependent phosphorylation of L-type channels or associated regulatory proteins, thereby increasing Ca^{2+} entry.

Three different chemical classes of organic Ca^{2+} channel blockers can be distinguished: Dihydropyridines (DHPs; prototype nifedipine),



Ca^{2+} Channel Blockers, Fig. 3 L-type Ca^{2+} channels in cardiac myocytes: Simplified view of the pharmacological action of L-type Ca^{2+} channel blockers in cardiac myocytes. In cardiac myocytes, L-type Ca^{2+} channels (Cav1.2) open when the plasma membrane is depolarized by an action potential carried along the muscle cells by the opening of voltage-gated Na^{+}-channels (Nav-Ch). The action potential duration is both dependent on the depolarizing effect of Ca^{2+} influx through L-type channels and the repolarizing action of multiple potassium channels (K-Ch). Ca^{2+} influx triggers massive release of Ca^{2+} (Ca^{2+}-induced Ca^{2+} release, CICR) by opening ryanodine-sensitive Ca^{2+} channels (RyR) in the sarcoplasmic reticulum (SR), resulting in an intracellular Ca^{2+} transient triggering contraction. Contraction is terminated by the rapid uptake of Ca^{2+} into the SR by SR Ca^{2+} ATPases (SERCA). β-Adrenergic receptor stimulation increases inotropy by phosphorylation (P) of phospholamban (PLN) and L-type channels (or associated proteins, Liu et al. 2020) through cAMP-dependent protein kinase (cAMP-PK). The resulting stimulation of Ca^{2+} influx and Ca^{2+} pump activity increases the load of Ca^{2+} in the SR stores. This leads to enhanced Ca^{2+} transients upon depolarization. Inhibition of Ca^{2+} influx through L-type Ca^{2+} channels by Ca^{2+} channel blockers decreases Ca^{2+} entry and SR load. Less Ca^{2+} influx and reduced release from the SR results in smaller Ca^{2+} transients and reduces inotropy. *PMCA* plasma membrane Ca^{2+} pump, *NCX* sodium-Ca^{2+} exchanger

phenylalkylamines (prototype verapamil), and benzothiazepines (prototype diltiazem). The drugs reversibly interact with their binding domains in a stereoselective manner and with dissociation constants in the (sub)nanomolar range (0.1–50 nM) (Striessnig et al. 1998). Radioligand binding, photoaffinity labeling, and site-directed mutagenesis studies (Hockerman et al. 1997; Striessnig et al. 1998) predicted that they all bind to adjacent, allosterically coupled, and partially overlapping sites close to or within the pore of the channel. These predictions have recently been confirmed in high-resolution structures of the drugs bound to the skeletal muscle Cav1.1 L-type channel (Zhao et al. 2019) and a Ca^{2+}-selective bacterial voltage-gated Na^{+}-channel (Tang et al. 2016).

Dihydropyridines (DHPs)

Widely used DHPs are amlodipine, lercanidipine, nisoldipine, nicardipine, nifedipine, and isradipine.

They do not act as pore blockers by binding directly within the ion-conducting pathway. Instead they act "allosterically" by stabilizing the inactivated state of the channel and do not require the channel to open in order to access their binding domain (Tang et al. 2016; Zhao et al. 2019). Inactivated channels are more likely to exist in arterial vascular smooth muscle because of its more depolarized membrane potential and longer-lasting depolarizations than in cardiac muscle. Moreover, the arterial smooth muscle channel differs slightly from the cardiac isoform due to alternative splicing of Cav1.2 α1 subunits, which also facilitates channel block by DHPs. As a consequence, DHPs inhibit the channels in arterial smooth muscle at lower concentrations than in cardiac muscle or in neurons (Ortner et al. 2017). Their clinically relevant actions therefore primarily result from their vasodilating properties in arterial smooth muscle (including the coronary arteries) and not from direct actions on the myocardium and the conduction system (i.e., antiarrhythmic and cardiodepressant effects), which are observed at higher concentrations in vitro or at toxic plasma levels.

Phenylalkylamines

Verapamil is the most widely used phenylalkylamine. At therapeutic plasma concentrations, verapamil inhibits not only Cav1.2 L-type channels in vascular smooth muscle but also in the heart. In addition to vasodilation, this results in negative chronotropic, dromotropic, and inotropic effects. Access to its binding domain is greatly enhanced by opening of the intracellular channel mouth (the so-called activation gate; Tang et al. 2016; Zhao et al. 2019). Once bound to the open state, it can also promote inactivated channel conformations. Verapamil also slows the recovery of channels from inactivation. This increases the "refractory period" of the drug-bound channel. As a consequence, the number of channels available for opening and Ca^{2+} influx decreases when the time between depolarizations shortens (i.e., when stimulation frequency increases). The open channel block and slowing of recovery explain why inhibition by a given verapamil concentration increases at higher heart rates (frequency-dependent block). Like the lidocaine block of voltage-gated Na^{+}-channels, the verapamil block of Ca^{2+} channels in the heart becomes more pronounced during tachyarrhythmias. Together these pharmacological effects allow its use not only for the treatment of hypertension but also of angina (limitation of oxygen consumption through its cardiodepressant action) and supraventricular tachyarrhythmias.

Benzothiazepines

Diltiazem is the only benzothiazepine in clinical use. Its binding domain in the channel overlaps with the one of verapamil (Tang et al. 2016; Zhao et al. 2019). Its molecular mechanism of action as well as its pharmacological effects also closely resembles those of phenylalkylamines.

All three classes of Ca^{2+} channel blockers also inhibit depolarization-induced contraction of venous smooth muscle in vitro. However, venous relaxation does not substantially contribute to the hemodynamic actions of Ca^{2+} channel blockers.

Clinical Use (Including Side Effects)

DHPs are potent arterial vasodilators and are therefore classified as "selective Ca^{2+} channel blockers with mainly vascular effects" (WHO ATC classification). At therapeutic plasma levels, they act on resistance vessels and therefore reduce peripheral vascular resistance, lower arterial blood pressure, and antagonize vasospasms in coronary or peripheral arteries. By reducing afterload, DHPs also reduce cardiac oxygen demand. Together with their vascular spasmolytic effect, this explains most of the beneficial actions of DHPs in angina pectoris. Most DHPs are only licensed for the therapy of hypertension, some of them also for the treatment of angina pectoris and vasospastic (Prinzmetal) angina. Long-acting DHPs are recommended as first-line drugs for the treatment of primary hypertension, either as monotherapy or as part of antihypertensive combination therapy (Whelton et al. 2018; Williams et al. 2018). There is solid clinical evidence demonstrating that their use reduces the risk of subsequent cardiovascular events.

Fast DHP-induced lowering of blood pressure results in compensatory sympathetic activation and a subsequent increase in heart rate and cardiac oxygen demand. This unfavorable effect has been mainly associated with the use of short-acting DHPs, such as immediate-release formulations of nifedipine, nitrendipine, or isradipine. The use of such formulations that cause fluctuations in plasma levels upon multiple dosing is discouraged. Instead, DHPs with slower onset and longer duration of action (amlodipine or extended-release formulations of, e.g., nifedipine, felodipine, isradipine) are recommended. Due to their vasodilating properties in the absence of negative inotropic actions, DHPs have also been evaluated as vasodilators in the treatment of heart failure with reduced ejection fraction in addition to standard therapy. Although long-acting DHPs (like amlodipine and extended-release felodipine) seem to be safe in these patients, they are not recommended for this indication.

Nimodipine, but not other DHPs, is also a potent inhibitor of nucleoside transport with actions similar to known nucleoside transport inhibitors such as dipyridamole. It is likely that this mechanism also contributes to the potent vasodilating properties of this DHP. Nimodipine is licensed for the prevention of vasospasms after subarachnoid hemorrhage.

DHPs are also used to treat vasospasms of peripheral arteries (e.g., Raynaud's phenomenon) and pulmonary hypertension.

Therapeutic doses of verapamil and diltiazem also exert negative inotropic, dromotropic, and chronotropic actions, in addition to their vasodilatory and spasmolytic properties. They are classified as "selective Ca^{2+} channel blockers with direct cardiac effects" (WHO ATC classification). As a consequence, compensatory tachycardia does not occur and heart rate may even decrease. Similar to β-adrenergic antagonists, verapamil and diltiazem inhibit exercise-induced increases in heart rate and myocardial oxygen consumption. Therefore both drugs are licensed for the treatment of angina, vasospastic angina, and hypertension. Due to their negative dromotropic and antiarrhythmic properties (see above), they are indicated for slowing AV conduction and to treat supraventricular arrhythmias. In patients with normal contractile function, the negative inotropic action of verapamil is partially compensated by the decreased afterload and improved myocardial perfusion. However, verapamil may decrease left ventricular function in patients with heart failure and reduced ejection fraction. Unlike β-adrenergic blockers, verapamil and diltiazem are not recommended in patients with heart failure and for the early treatment or secondary prevention of myocardial infarction.

C

Side Effects

Many unwanted effects are related to the vasodilatory effects of Ca^{2+} channel blockers, such as flushing, headache, dizziness, and hypotension. DHPs cause leg edema and ankle swelling upon chronic use, which is therapy-limiting in some patients. Constipation is a frequent side effect of verapamil likely due to its inhibitory action on intestinal smooth muscle. Bradycardia, atrioventricular block, or a decrease in left ventricular function are observed with verapamil (and to a lesser degree diltiazem), especially in patients taking β-adrenergic blockers or who have preexisting cardiac disease (impaired left ventricular function, atrioventricular block). Worsening of angina has also been observed with DHPs. This is most likely due to their effect on coronary resistance vessels resulting in "coronary steal" directing blood flow away from already hypoperfused regions. It may also be caused by the reactive sympathetic activation with an increase of heart rate and of cardiac oxygen consumption.

Epidemiological and case-control studies suggested that Ca^{2+} channel blockers cause increased risk for myocardial infarction, cancer, and gastrointestinal bleeding. The increased cardiovascular morbidity was associated with short-acting DHPs and with fast-release formulations of verapamil and diltiazem. It was explained by the unfavorable hemodynamic effects of short-acting drugs. Enhanced cardiovascular morbidity has not been consistently shown for long-acting formulations. An increased risk of cancer or of gastrointestinal bleeding was not confirmed in other large trials.

Ca^{2+} channel blockers cause no side effects expected from channel block in other tissues (e.g., cochlear inner hair cells, retinal photoreceptors, neurons, or pancreatic β-cells). Despite the dependence of insulin secretion on L-type Ca^{2+} channel function, impaired glucose tolerance is not a frequent side effect of Ca^{2+} channel blockers in clinical practice. The reason for the absence of such side effects may be explained by the lower bioavailability in these tissues, by different cellular activity patterns (affecting channel state- and frequency-dependent block), by alternatively spliced channel subunits conferring different drug sensitivity (see above), and by the existence of various L-type channel isoforms, which differ in drug sensitivity. Cav1.2 channels, the major L-type channel isoform in vascular smooth muscle and cardiac ventricular muscle, are more sensitive to Ca^{2+} channel blockers than Cav1.3 and Cav1.4 (see references in Ortner et al. 2017), the major isoforms expressed in cochlear inner hair cells and retinal photoreceptors, respectively.

Other Pharmacological Actions of Ca^{2+} Antagonists

Although the clinical benefit of Ca^{2+} channel blockers is due to inhibition of Cav1.2 channels as outlined above, inhibition of Cav1.2 or Cav1.3 L-type channels in other tissues may also be of therapeutic interest. In the brain both L-type channel isoforms are involved in neuronal signaling events underlying drug-taking behaviors. In preclinical models systemic isradipine treatment decreases cocaine relapse, a finding that will be pursued in clinical trials (Addy et al. 2018). Ca^{2+} influx through Cav1.3 channels appears to contribute to oxidative stress and neurodegeneration of substantia nigra dopaminergic neurons in Parkinson's disease (Guzman et al. 2018). This prompted the phase 2 and 3 STEADY-PD clinical trial program (Biglan et al. 2017). However, treatment with 5 mg immediate-release isradipine b.i.d. over 36 months was not neuroprotective, likely because brain drug concentration is too low to fully inhibit Cav1.3 channels in these neurons (Ortner et al. 2017).

Somatic de novo mutations in the pore-forming α1 subunit of Cav1.3 Ca^{2+} channels are frequently found in aldosterone-producing adenomas or adrenocortical aldosterone-producing cell clusters. These mutations activate Cav1.3 channels and drive autonomous Ca^{2+}-dependent aldosterone secretion resulting in primary aldosteronism (Azizan et al. 2013). The development of Cav1.3-selective inhibitors may therefore represent a nonsurgical treatment option for these patients.

Notably Cav1.3 channels also control cardiac pacemaking in the sinoatrial node but do not contribute to cardiac inotropy and arterial myogenic tone. Cav1.3-selective Ca^{2+} channel blockers may therefore also act as bradycardic agents lacking negative inotropic actions.

Cross-References

- ▶ Antihypertensive Drugs
- ▶ Voltage-Dependent Ca^{2+} Channels

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Ca^{2+}-Binding Proteins

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C

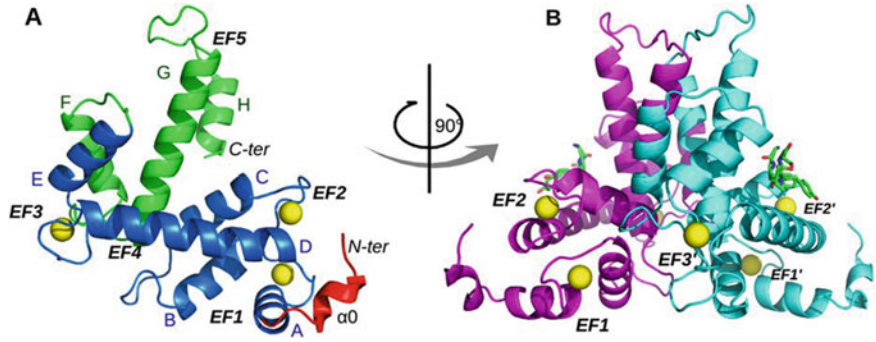
Synonyms

Allergy; Biomarkers; Calcium-binding; Cancer; Cardiomyopathy; Clinical chemistry; Diagnostics; Drug targets; EF-hand; Inflammation; Laboratory medicine; Neuropathology; Zinc-binding

Definition

After entering the cell, Ca^{2+} is reversibly complexed to specific Ca^{2+}-binding proteins which decipher the information carried by Ca^{2+} and pass it on to various targets regulating a myriad of biological functions. These proteins contain highly specific Ca^{2+}-binding sites, a characteristic helix-loop-helix structure called the EF-hand, with one Ca^{2+}-binding in the loop (Grabarek 2006; Schaub and Heizmann 2008). This highly conserved motif is found to date in a great number of Ca^{2+}-binding proteins grouped into distinct subfamilies (Haynes et al. 2012; Kawasaki and Kretsinger 2017). Figure 1 shows the structure of sorcin as an example of a penta-EF-hand calcium-binding protein expressed at high levels in several human cancers.

S100 proteins constitute the largest subgroup within the EF-hand protein family involved in many cellular activities (*see separate entry in this volume*). Protocols for studying the many facets of Ca^{2+}-binding proteins in cellular functions, their clinical relevance, and their use as diagnostic and prognostic biomarkers in laboratory medicine and as novel therapeutic drug targets, have been published (Heizmann 2019).



Ca^{2+}-Binding Proteins, Fig. 1 Overall structure of calcium-bound human Sorcin. (**a**) Monomer comprising a part of the flexible N-terminal domain with the alpha helical region (α 0 (red) and a calcium-binding domain divided in EF1–3 (blue) and EF4–5 (green). Calcium ions (yellow) are bound at EF1, EF2, and EF3. Helices (A-H) and EF-hands (EF1–5). (**b**) Dimer, pairing of EF4–5 of two monomers (cyan and magenta). The N-terminal hexapetide is shown in green sticks. (This research was originally published by Ilari et al. (2015); © Springer Nature, Scientific Reports)

Basic Characteristics and Clinical Relevance

Cardiology

The *cardiac troponin complex* is composed of troponin I (cTnI), troponin T (cTnT), and the Ca $^{2+}$-sensing troponin C (cTnC). The heart-specific isoforms of TnT and TnI were found to be organ-specific biomarkers of cardiac necrosis when released into the blood. More than a hundred mutations have been reported in all three subunits of cardiac troponin associated with inherited hypertrophic (HCM), dilated (DCM), and restrictive cardiomyopathies. Most mutations have been found in cTnT and cTnI, with only a few in cTnC in HCM and DCM. Some of these mutations directly affect the Ca^{2+}-sensitivity and Ca^{2+}-affinity of cTnC and normal heart functioning (Li and Hwang 2015).

Calmodulin regulates more than 100 different proteins in a Ca^{2+}-dependent manner (Ikura and Ames 2006). Mutations in calmodulin have been identified, linked to dominantly inherited ventricular tachycardia (CPVT) and severe cardiac arrhythmias (Nyegaard et al. 2012). These results indicate that calmodulin could be a therapeutic target for some cardiac disorders.

Parvalbumin was the first Ca^{2+}-binding protein to have its amino acid sequence and atomic 3D structure resolved (Kawasaki and Kretsinger 2017). Parvalbumin functions as a Ca^{2+}-buffer, enhancing relaxation of fast-contracting skeletal muscles in small mammals, but is absent from the human heart (Heizmann et al. 1982). Based on these results, parvalbumin was therapeutically introduced into the human heart to speed up relaxation after each heartbeat in diastolic heart failure (HF) to improve Ca^{2+}-handling and cardiac performance (Coutu et al. 2004).

Myosin light chain-2 (MYL2, also named MLC-2) exists as three isoforms in mammalian muscles, encoded by three distinct MLC-2 genes: MCL-2f, fast-twitch skeletal muscle isoform; MLC-2v, cardiac ventricular and slow-twitch skeletal muscle form; and MLC-2a, cardiac atrial form. Genetic loss-of-function studies of the cardiac forms in mice have demonstrated their key roles in cardiac function during early embryogenesis. In adults, MLC-2v phosphorylation regulates cardiac myosin cycling kinetics and normal cardiac function. Dephosphorylation of MLC-2v has been implicated in dilated cardiomyopathy and heart failure (Sheikh et al. 2015).

Calcineurin (Cn), a serine-threonine protein phosphatase, is a heterodimer consisting of two subunits: CnA and CnB (containing 4 EF-hands each). On activation, calmodulin binds as a third subunit. The immunosuppressive Cn inhibitors cyclosporine and FK506 can prevent cardiac hypertrophy and dilated myopathy. Constitutive overexpression of activated Cn leads to cardiac hypertrophy in mice, and genetic deletion of the

stress-responsive isoform of Cn (CnB beta) inhibits the development of myocardial hypertrophy. Cn levels, when measured in left ventricular samples of heart failure individuals, indicate that Cn may play a critical role in the progression of human heart failure (Wolska 2009).

CIB1 is a 22-kDa protein with 4 EF-hand domains, but only the third and fourth C-terminal domains bind calcium at physiological concentrations. CIB1 interacts with many signaling proteins including CnB. Ablation of CIB1 was found to protect the mouse heart from hypertrophy and the development of heart failure, suggesting that CIB1 could be a potential target for future therapeutic strategies (Heineke 2013).

Penta-EF-hand (PEF) protein family: calpains, sorcin, grancalcin, ALG-2, and peflins (Maki et al. 2002).

Calpains are a family of cysteine proteinases activated by Ca^{2+}. The human m-calpain is composed of a large 78–80 kDa catalytic subunit and a small 29 kDa regulatory subunit; each subunit contains five EF-hand motifs. Calpain activity is tightly controlled by its endogenous inhibitor calpastatin. Overactivation of calpain and mutations of calpain have been associated with cardiac and cerebral ischemia (Zatz and Starling 2005).

Sorcin undergoes Ca^{2+}-induced conformational changes which enable them to translocate from the cytoplasm to membranes and to interact with a variety of target proteins (Fig. 1). In the heart, sorcin modulates key regulatory processes such as the calcium-induced Ca^{2+}-release and excitation-contraction coupling. A natural F112L sorcin mutant was found to be associated with hypertrophic cardiomyopathy and hypertension. This F112L variant was unable to translocate to the membrane and showed a decrease in both Ca^{2+}-affinity and interaction with target proteins, resulting in an alteration of E-C coupling in the heart (Ilari et al. 2015; Collins et al. 2007) (Table 1).

Ca^{2+}-Binding Proteins, Table 1 Calcium-binding proteins in heart failure

Proteins	Disease association
Cardiac troponin	
TN-C	Mutations, hypertrophic cardiomyopathy
TN-T	Mutations, cardiomyopathies Biomarker, myocardial infarction
TN-I	Mutations, cardiomyopathies Autoantibodies, myocardial infarction
Calmodulin	Mutations, tachycardia, sudden cardiac death Arrhythmia, cardiac arrest in infants
Parvalbumin	Gene therapy to enhance cardiac performance
MLC-2	Dephosphorylation in dilated cardiomyopathy and heart failure
Calcineurin	Cardiac hypertrophy, heart failure
CIB1	Cardiac hypertrophy
Calpains	Mutations, cardiovascular diseases
Sorcin	Mutations, cardiac hypertrophy

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Oncology

Sorcin is overexpressed in many cancer cells as a result of the increased drug resistance to several chemotherapeutic agents (Shabnam et al. 2018). Multidrug resistance (MDR) is one of the major causes of treatment failure in pediatric acute leukemia (ALL), one of the most common types of cancer in children. Overexpression of sorcin is a prognostic marker for multidrug-resistant pediatric ALL, correlating with upregulated MDR1/P-glycoprotein (P-gp) expression in these patients. Sorcin may be a biomarker of MDR and

therapeutic target for reversing multidrug resistance in ALL and other types of cancers (Dabaghi et al. 2016).

CIB1 was also identified in cancer, considered to be a novel drug target in human tumors (Wang et al. 2017).

Swiprosin-1, an actin-binding protein also known as EF-hand domain-containing 2 *(EFHD2),* is overexpressed in various human cancer tissues, especially at highly invasive stages of malignant melanoma. Swiprosin-1 stimulates cancer invasion and metastasis through activating the Rho family of small GTPases suggesting that these proteins could be considered as a potential therapeutic target to prevent cancer invasion and metastasis (Hu et al. 2015). *EFHD2* expression was found to correlate with postsurgical recurrence of cancer in patients with stage I adenocarcinoma, and the multivariate Cox regression analysis revealed that EFHD2 expression was an independent clinical predictor for this cancer type (Fan et al. 2017).

Tescalcin is a Ca^{2+}- and Mg^{2+}-binding protein with one consensus EF-hand and three additional domains with EF-hand homology. Tescalcin shares sequence homology with the calcineurin-B homologous protein 3 (CHP3), can inhibit the phosphatase activity of calcineurin A, and has emerged as a regulator of cell differentiation and growth. Tescalcin is overexpressed in colorectal cancer (CRC) contributing to the invasive and metastatic activity. Patients with CRC showed increasing serum levels of CRC. The protein is therefore considered to be a potential diagnostic marker and oncotarget in CRC (Kang et al. 2016).

LETM1, a leucine zipper/EF-hand containing transmembrane protein, is located in the inner membrane of mitochondria. Elevated levels of expression have been correlated with carcinogenesis and tumor progression of breast cancer and were found to be an independent marker for a poor prognosis for patients with this type of cancer (Wang et al. 2015) (Table 2).

Ca^{2+}-Binding Proteins, Table 2 Calcium-binding proteins in cancer

Proteins	Disease association
Calmodulin	Autophagy in cancer, glioblastoma invasion, HRAS- and NRAS-driven cancers
Sorcin	Multidrug resistance, gastric, colorectal, breast cancers, childhood ALL [a]
CIB1 [b]	Tumor progression
Swiprosin-1/ EFHD2 [c]	Malignant melanoma, stage I lung adenocarcinoma
Tescalin	Colorectal cancer
LETM1 [d]	Breast and bladder cancers

References: Li et al. (2018) Cell Death Dis. https://doi.org/10.1038/s1419-017-02553-7; Nussinov et al. (2017) Biochim Biophys Acta. https://doi.org/10.1016/j.bbadis.2017.10032; Colotti et al. (2014) Molecules 19: 13976–13,989; Genovese et al. (2017) Cell Death Dis 8: e2950. https://doi.org/10.1038/cddis.2017.34; Dabaghi et al. (2016) Gent Test Mol Biomarkers 20:516–521; Tuo et al. (2017) Oncotarget 8:104258–104,271; Tong et al. (2015) Cell Biochem Biophys 72:453–459; Gong et al. (2014) Oncol Lett 8:2393–2398; Leisner et al. (2016) FASEB J 30:2640–2650; Wang et al. (2017) Cell Physiol Biochem 43:1413–1424; Hu et al. (2015) Oncotarget 6:13060–13,071; Fan et al. (2017) Sci Rep. doi:https://doi.org/10.1038/s41598-017-15,186-y; Kang et al. (2016) Tumor Biol 37:13843–13,853; Li et al. (2015) Int J Clin Exp Pathol 8:12893–12,900; Wang et al. (2015) Exp Mol Pathol 98: 254–259; Huang et al. (2017) Oncol Rep 38:2935–2940

[a]Acute lymphoblastic leukemia

[b]calcium and integrin-binding protein

[c]EF-hand domain-containing protein D2

[d]leucine zipper/EF-hand containing transmembrane-1

Inflammation and Allergies

Calpains, a family of cysteine proteases (Zatz and Starling 2005), are involved in the process of several inflammation-associated diseases and are considered as therapeutic targets for these diseases (Ji et al. 2016).

Calcineurin is a serine-threonine protein phosphatase (Wolska 2009). Calcineurin-NFAT signaling is essential for infection control, and calcineurin inhibitors, used in translation medicine (e.g., cyclosporine A and tacrolimus), are now being tested in various inflammatory conditions and autoimmune pathologies (Bendickova et al. 2017).

Parvalbumin isoforms are major fish allergens in humans. Approximately 70% of fish allergies, mediated by parvalbumin IgE antibodies, lead to respiratory and gastrointestinal symptoms. A FAST (food allergy-specific immunotherapy) project was

Ca^{2+}-Binding Proteins, Table 3 Calcium-binding proteins in inflammation and allergies

Proteins	Disease association
Calpain	Inflammation-associated diseases
Calcineurin	Inflammation and autoimmune pathologies
Parvalbumin	Fish allergy

References: Ji et al. (2016) Biomed Rep 5:647–652; Bendickova et al. (2017) EMBO Mol Med. doi:https://doi.org/10.15252/emmm.201707698; Freidl et al. (2019) Allergy doi:https://doi.org/10.1111/all.13994; Argiz et al. (2019) J Investig Allergol Clin Immunol. https://doi.org/10.18176/jiaci.0412

initiated to develop a safe and effective subcutaneous immunotherapy for fish allergy, using recombinant hypoallergenic carp parvalbumin, Cyp C 1. These results have now warranted first-in-man immunotherapy studies to further develop this new vaccine for a safe and effective therapy for fish allergy (Freidl et al. 2019) (Table 3).

Brain Injury and Neuropathology

The structures, functions, and their use as markers of different neuronal subpopulations of these neuronal calcium-binding proteins have been published (Heizmann 2019; Burgoyne et al. 2019; Schwaller 2019; Mundhenk et al. 2019). Proteins are involved in learning and memory (e.g., parvalbumin (Tripodi et al. 2019)) and are associated with various brain disorders (some listed in Table 4). Recently a novel mutation in CAPN1 (encodes calpain-1) was reported to cause a pure hereditary spastic paraplegia in an Italian family (Cotti Piccinelli et al. 2019).

Ca^{2+}-Binding Proteins, Table 4 Calcium-binding proteins in brain disorders

Proteins	Disease association
Calmodulin	Parkinson's disease
Parvalbumin	Schizophrenia, Alzheimer's disease, motor neuron disease
Calbindin D-28 k	Epilepsy, motor neuron disease
Calretinin	Short-term synaptic depression
NCS-1 [a]	Schizophrenia, bipolar disorder
VILIP-1 [b]	Alzheimer's disease
Calcineurin	Amyotrophic lateral, sclerosis, Alzheimer's disease
Calpain	Alzheimer's disease, spastic paraplegia
DREAM [c]	Huntington's disease, Alzheimer's disease

References: Martinez et al. (2003) J Biol Chem 278: 17379–17,387; Fachim et al. (2018) Epigenomics doi:10: 2217/epi-2017-0159; Albuquerque et al. (2015) Front Aging Neurosci 24;7:30. https://doi.org/10.3389/fnagi.2015.00030; Ince et al. (1993) Neuropath Appl Neurobiol 19(4); D'Alessio et al. (2019) Behav Neurol 2;2019: 7396793. https://doi.org/10.1155/2019/7396793; Bolshakov et al. (2019) Front Cell Neurosci 13;13;91. https://doi.org/10.3389/fncel.2019.00091; Bandura J, Feng (2019) Mol Neurobiol 56 (9):60–6094. https://doi.org/10.1007/s12035-019-1497-2; Schindler et al. (2019) Alzheimers Dement 15(5):655–665. https://doi.org/10.1016/j.jalz.2018.12.019; Ferri et al. (2004) J Neurochem 90:1237–1242; Lian et al. (2001) Exp Neurol 167:158–165; Veeranna et al. (2004) Am J Pathol 165:795–805; Lopez-Hutardo et al. (2019) Sci Rep. 13;9(1):7260. doi:https://doi.org/10.1038/s41598-019-43,677-7

[a]Neuronal calcium sensor-1
[b]Visinin-like protein-1
[c]Downstream Regulatory Element Antagonist Modulator

Drugs

Studies are in progress to develop drugs against calcium-binding proteins and their target proteins. For example, the binding of sorcin to doxorubicine, vincristine, paclitaxel, and cisplatin was investigated, and it was shown that binding of doxorubicin impairs cell death and increases drug resistance in cancer cells. Troponin-modulating drugs are being developed for future treatment of cardiomyopathy and heart failure. Blockade of DREAM activity by repaglinide or even better by IQM-PC300 is neuroprotective in Huntington's disease (Lopez-Hurtado et al. 2019).

Outlook

So far, only a small number of this largest family of Ca^{2+}-binding proteins (with over 200 human genes coding for proteins containing the EF-hand Ca^{2+}-binding motif) have been explored for their clinical relevance, and this does not include their intra- and extracellular targets/surface receptors or posttranslational modification (e.g., phosphorylation, myristoylation, or sumoylation). Some of

those proteins also bind Mg^{2+}, Zn^{2+}, and Cu^{2+} at position distinct from the Ca^{2+}-binding sites resulting in the modulation of their activities. Mutations have been reported in cardiac troponin, calmodulin, and other EF-hand proteins making them novel therapeutic targets for more selected interventions to improve the diagnosis and treatment of human diseases.

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Ca^{2+}-Induced Ca^{2+} Release (CICR) Channel

▶ Ryanodine Receptor

Ca^{2+}-Sensing Receptor (CaSR)

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Synonyms

Calcium-sensing receptor

Definition

CaSR is a G protein-coupled receptor highly expressed in the parathyroid glands, where it regulates the production and secretion of parathyroid hormone (PTH) in negative feedback way. CaSR can detect minor changes in serum calcium levels, and calcium is the primary ligand for the CaSR. Serum calcium $<1.1–1.2$ mmol/l promotes PTH release, which results in reabsorption of calcium by the kidney, intestine, and bone. Conversely, serum Ca^{2+} levels >1.2 mmol/l have the opposite effect (Fig. 1). CaSR is also expressed in numerous other tissues, where it has different functions. Defective calcium sensing by the parathyroid glands, associated to altered CaSR expression or function, results in altered divalent cation homeostasis, which can lead to long-lasting morbidity in patients.

Basic Characteristics

CaSR expressed in the parathyroid and its genetic variants. CaSR was first cloned from bovine parathyroid and characterized in 1993 by Brown and coworkers (Brown et al. 1993) who demonstrated a key role of CaSR in maintaining extracellular calcium levels by its ability to regulate PTH biosynthesis and release.

Calcium binding at the CaSR stimulates a Gq G-protein signaling leading to activation of phospholipase C pathway and prevents exocytosis of PTH. This event is accompanied by release of calcium from intracellular stores resulting in an increase in cytosolic calcium (Brown and MacLeod 2001).

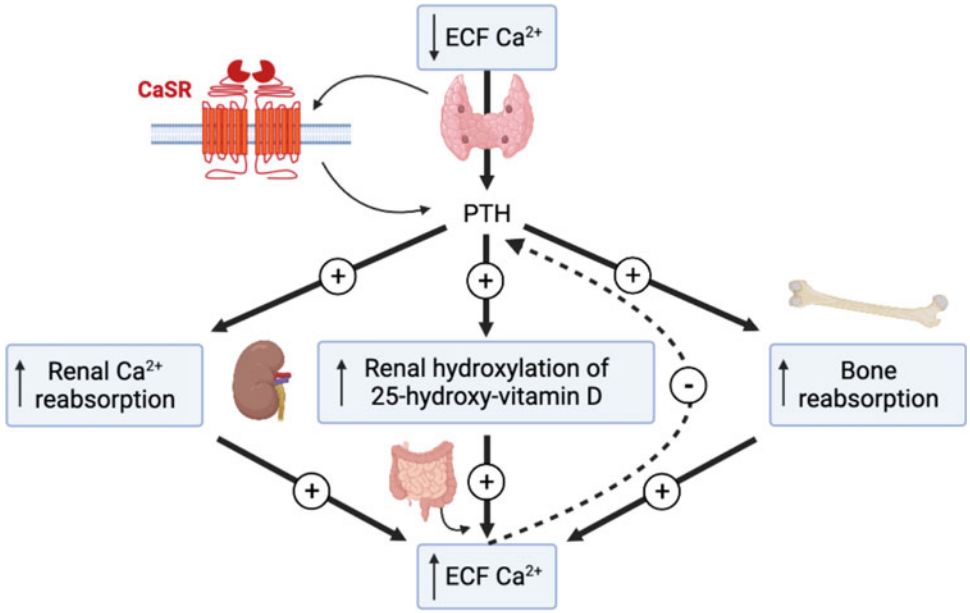
CaSR is however a pleiotropic receptor since different endogenous ligands can activate the receptor initiating multiple intracellular cascade in the same cell type or the same signaling in the different organs. Besides parathyroid, CaSR is expressed in many tissues involved in the regulation of extracellular calcium levels, such as the thyroidal calcitonin-secreting C-cells, kidney, bone, and intestine (Alfadda et al. 2014).

Nevertheless, several studies demonstrated the expression of CaSR in tissues not apparently involved in calcium homeostasis such as the brain, airways, blood vessels, breast, and liver.

Naturally occurring mutations of the CaSR gene in humans, as well as selective CaSR deletion in mice or some polymorphisms, are associated with severe alterations in calcium homeostasis.

Loss of function mutations of *CaSR* such as the two inherited conditions familial hypocalciuric hypercalcemia (FHH) and neonatal severe hyperparathyroidism (NSHPT) are characterized by altered calcium homeostasis (Pollak et al. 1993).

Conversely, the gain-of-function mutations of the CaSR such as autosomal dominant hypocalcemia (ADH) or type 5 Bartter syndrome are associated to hypocalcemia due to inhibition of



Ca^{2+}-Sensing Receptor (CaSR), Fig. 1 Role of the CaSR in calcium homeostasis
The CaSR is highly expressed in the parathyroid glands. The parathyroid CaSR detects reductions in extracellular calcium causing release of PTH. PTH increases resorption of calcium from bone, promotes urinary calcium reabsorption, and enhances expression of the biologically active 1,25-dihydroxyvitamin D that increases absorption of dietary calcium. CaSR expressed in the kidney acts independently of PTH to regulate urinary calcium reabsorption. Increases in calcium concentrations lead to negative feedback on the parathyroid glands, thereby inhibiting further PTH release. *CaSR* calcium-sensing receptor, *PTH* parathyroid hormone, *ECF* extracellular fluid

PTH secretion. Two examples of gain-of-function mutations of CaSR are N124K and R990G (Ranieri et al. 2013). N124K CaSR mutation causing ADH in humans is located in the extracellular domain, whereas the non-conservative polymorphism R990G is located in the intracellular tail of the receptor and in humans and is associated with primary hypercalciuria in patients. These two gain-of-function CaSR mutation sites are at opposite locations within the CaSR protein sequence; however functional analysis revealed comparable biological regulatory effects within cells. Specifically, functional expression of these mutations resulted in significantly higher calcium accumulation in the ER associated with higher SERCA expression and activity and reduced expression of the PMCA and basal cytosolic calcium (Ranieri et al. 2013).

Role of CaSR in the Cardiovascular System An interesting role for CaSR has been proposed as regulator of blood pressure and in vascular calcification. Both in vitro and in vivo evidences support this role. More precisely, a complex interplay between CaSR expressed in VSMC and in the endothelium has been suggested to play a critical role in the regulation of blood pressure (Loot et al. 2013). In vitro treatment with calcimimetics reduced calcification of vascular smooth muscle cells (VSMC). A mouse model of targeted CaSR deletion from VSMC displayed reduced endothelium contractility in aorta and mesenteric artery compared to wild-type animals in response to different stimuli (Schepelmann et al. 2016). More recently CaSR has been proposed as a new therapeutic target to pulmonary hypertension (Zhou et al. 2021).

Role of CaSR in the Bone The CaSR has a pivotal role in the bone and mineral metabolism (Hannan et al. 2018). In vivo and in vitro evidence demonstrated that CaSR regulates bone

remodeling by directly acting on bone cells. Osteoblasts and osteoclasts express functional CaSR and are able to sense changes in the extracellular calcium. During remodeling, local calcium released in the microenvironment can be sensed by CaSR of skeletal cells and in turn modulate bone turnover. Although calcilytics have failed thus far as anabolic therapies for osteoporosis, calcimimetic and calcilytic are under investigation for treating bone and mineral-related disorders.

Role of CaSR in Gut CaSR transcripts and proteins are abundantly expressed in the crypt and villous enterocytes of the small intestine as well as the surface epithelial cells of the large intestine.

CaSR is expressed in the gut and can be considered a nutrient-sensing receptor which activates multiple signaling pathways within the gut. Several evidences demonstrated that CaSR coordinates food digestion and nutrient absorption, regulates immune response, and promotes cell proliferation. Moreover CaSR promotes hormone secretion and reduces diarrhea and enhances intestinal barrier function (reviewed in Liu et al. 2018). CaSR has an emerging role in the maintenance of gut homoeostasis and protection of intestinal health.

Interestingly, recent evidence indicates that enterocytes use CaSR also to monitor luminal and extracellular calcium levels inducing paracrine and endocrine feedback responses to restrict calcium absorption (Chanpaisaeng et al. 2020).

Role of CaSR in the Kidney Several evidence demonstrated that the kidney is a calcium-sensing organ and senses changes in both luminal (urine) and serum calcium levels though the CaSR expressed in several nephron segments from the glomerulus to the inner medullary collecting ducts (Riccardi and Valenti 2016).

CaSR is expressed across the entire length of the nephron, with the highest expression within the TAL, and might directly contribute to the regulation of many aspects of renal function in a PTH-independent manner (Riccardi and Valenti 2016).

In the TAL, the CaSR is a major player for regulating urinary calcium excretion. CaSR activation by serum calcium in the TAL promotes urinary calcium excretion. Conversely, activation of the CaSR in the proximal tubule antagonizes the phosphaturic action of PTH and promotes proton secretion. In the collecting duct, CaSR activation decreases water reabsorption by inhibiting the tubular response to vasopressin and aquaporin-2 expression and trafficking (Ranieri et al. 2018) and increases proton excretion by stimulating proton pump activity. Therefore, CaSR activity in the proximal tubule and collecting duct can reduce the risk of calcium phosphate precipitation that is associated with the ability of the CaSR to enhance calcium excretion in the TAL.

CaSR as Pharmacological Target in Polycystic Kidney Disease CaSR activation has also been proven to correct some alterations found in cell models of autosomal polycystic kidney disease (ADPKD). Specifically, in human conditionally immortalized proximal tubular epithelial cells silenced for polycystin 1 (PKD1) or generated from a patient with ADPKD selective activation of the CaSR with calcimimetics increased cytosolic calcium, reduced intracellular cAMP and mTOR activity (Di Mise et al. 2018b), and rescue defective ATP mitochondrial production (Di Mise et al. 2018a), reversing the principal (ADPKD) dysregulations found in ADPKD.

CaSR as Pharmacological Target in Asthma CaSR has emerged as a potential therapeutic target for asthma (Yarova et al. 2015). Allergic asthma is associated to increases in certain polycations that activate CaSR in bronchi leading to activation of a signaling cascade characterized by increased intracellular calcium mobilization and p38 mitogen-activated protein kinase phosphorylation in airway smooth muscle (ASM) cells. In vivo treatment with calcilytics (CaSR antagonists) prevented these effects and reduced airway hyperresponsiveness and inflammation in allergen-sensitized mice in vivo indicating that calcilytics may represent effective asthma therapeutics.

Drugs

The development of pharmacological CaSR modulators calcilytics and calcimimetics has markedly

enhanced our understanding of the pathophysiology of the CaSR and has opened new possibilities in the clinical setting.

Calcimimetics Calcimimetic (such as cinacalcet) are able to rectify abnormal CaSR expression or function and have been successfully administered to adult patients to treat hyperparathyroidism secondary to kidney failure (Bushinsky et al. 2015). Calcimimetics correct CaSR function since they enhance CaSR sensitivity to extracellular calcium and reduce serum PTH levels by activating the CaSR expressed in the parathyroid gland and the kidney. There are two types of calcimimetics: type I are inorganic or organic polycationic agonists, and type II functions as positive allosteric modulators of CaSR activity. Cinacalcet belongs to the type II calcimimetic and, as mentioned, has been approved for the treatment of secondary hyperparathyroidism, a complication of end-stage renal disease. Conversely, the calcimimetic peptide AMG416 is being evaluated in case of secondary hyperparathyroidism in hemodialyzed patients with chronic kidney disease. AMG416 functions as a CaSR agonist and is administered intravenously. After a phase II clinical trial (Bushinsky et al. 2015), a phase III trial is now complete, but the data are not yet published.

Calcilytics Calcilytics function as CaSR antagonists and are being investigated for the treatment of autosomal dominant hypocalcemia with hypercalciuria caused by activating mutations of the *CaSR* gene conferring gain of function to the receptor (Letz et al. 2014). Basically, calcilytics are allosteric antagonists of the CaSR, and their action results in a rightward shift in the calcium concentration-response curve so that higher than normal calcium levels are required to suppress PTH release. Due to their ability to promote fluctuations in serum PTH which is known to have an anabolic effect on bone, calcilytics such as ronacaleret and JTT-305 were tested to treat age-related osteoporosis (Fitzpatrick et al. 2011). Unfortunately, those drugs were found to have no efficacy to improve osteoporosis. Nevertheless, calcilytics might be appropriate to treat hypercalciuric disorders caused by a gain of function variants of *CaSR*.

In clinical setting, the systemic treatment with allosterical modulators of the CaSR should take into account that CaSR is expressed in numerous tissues, and therefore there is the potential of adverse effects. As an example, in kidney transplant recipients, cinacalcet normalizes serum PTH and calcium concentrations, but long-term treatment might cause increase in urinary calcium excretion and the risk of renal calcium deposits that could compromise graft function. Conversely, calcilytics might be used to treat gain-of-function variants of the CaSR associated with hypercalciuria and stone diseases. However, an undesired effect would be the stimulation of PTH release, which might worsen hypercalciuria.

Undoubtedly the CaSR can be the target for therapeutic intervention for several inherited and acquired conditions, whereby the level of expression or function of the CaSR is altered. The next challenge will be to identify molecules and targeting strategies for tissue-specific CaSR expression.

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Cadherins/Catenins

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Synonyms

Calcium-dependent adhesion protein/plakoglobin

Definition

Cadherins (calcium-dependent adhesion proteins) are transmembrane proteins, which consist of an extracellular domain composed of cadherin repeats, a transmembrane domain, and a cytoplasmic domain that interacts with catenins and/or other cytoplasmic proteins.

Catenins are defined as cytoplasmic interaction partners of cadherins that form a chain of proteins ("catena," Latin for chain), which connects cadherins to the actin cytoskeleton.

Basic Characteristics

Structural Characteristics of Cadherins

Cadherins are a superfamily of Ca^{2+}-sensitive cell-cell adhesion molecules, which cause homophilic cell interactions. Cadherins can be divided into different subfamilies, namely, classical cadherins, desmosomal cadherins, protocadherins, and nonconventional cadherins (7TM cadherins, T-cadherin, FAT). Classical cadherins are often denoted by a prefix reflecting their principal expression domains; e.g., E is epithelial, N is neuronal, and P is placental. However, this classification is not stringent, as, for instance, E-cadherin can also be found in certain neuronal tissues and N-cadherin is also found in epithelial cells. Among the desmosomal cadherins, two subfamilies can be distinguished: the desmocollins 1–3 and the desmogleins 1–4.

The extracellular domain of cadherins consists of a variable number of a repeated sequence of about 110 amino acids. This sequence is termed the "cadherin repeat" and resembles in overall structure, but not in sequence, the Ig-like domains. The cadherin repeat is the characteristic motive common to all members of the cadherin superfamily. Classical and desmosomal cadherins contain five cadherin repeats, but as many as 34 repeats have been found in the FAT cadherin (see below). Cadherins are calcium-dependent cell adhesion molecules, which means that removal of Ca^{2+}, e.g., by chelating agents such as EDTA, leads to loss of cadherin function. The Ca^{2+}-binding pockets are made up of amino acids from two consecutive cadherin repeats, which form a characteristic tertiary structure to coordinate a single Ca^{2+} ion (Gumbiner 2005).

The classical cadherins are translated as precursor because they are N-terminally cleaved to reveal the mature proteins. This processing is required to activate the cell adhesion function of cadherins. Cadherins interact in trans (i.e., from opposite cells) via the most N-terminal cadherin repeats. A short amino acid sequence within this repeat, histidine-alanine-valine (HAV), has been implicated in mediating cell-cell contacts as HAV peptides can disrupt cadherin-dependent cell adhesion. Besides the trans-interactions of cadherins, the extracellular domains are also capable of forming cis-dimers through lateral amino acid contacts between cadherin molecules on one cell. This dimerization again mainly involves the first cadherin repeat. A zipper model based on the pattern of alternating cis- and trans-dimers for the adhesive interactions has been proposed (Gumbiner 2005).

Several nonconventional cadherins that contain cadherin repeats have been described, but they have specific features not found in the classical cadherins. Protocadherins constitute a big family of proteins with cadherin repeats at the extracellular domain, a transmembrane domain, and a cytoplasmic domain that differs from classical cadherins (Gumbiner 2005). The cadherin Flamingo, originally detected in *Drosophila*, contains seven transmembrane segments and in this respect resembles G protein-coupled receptors. The extracellular domain of Flamingo and its mammalian homologs is composed of cadherin repeats as well as EGF-like and laminin motifs. The seven transmembrane span cadherins have a role in homotypic cell interactions and in the establishment of cell polarity. The FAT-related cadherins are characterized by a large number of cadherin repeats (34 in FAT and 27 in dachsous). Their cytoplasmic domains can bind to catenins. T- (=truncated-) cadherin differs from other cadherins in that it has no transmembrane domain but is attached to the cell membrane via a glycosylphosphatidylinositol anchor (Gumbiner 2005).

Cytoplasmic Interactions of Cadherins

The cytoplasmic domains of cadherins bind to various proteins. The most C-terminal portion of classical cadherins directly associates with either β-catenin or the structurally related γ-catenin (more commonly called plakoglobin). β-Catenin/plakoglobin in turn binds to α-catenin, which is related to the cytoskeletal protein vinculin and associates with actin-binding proteins. Thus, β- and α-catenin provide a link of cadherins to the actin cytoskeleton (Gumbiner 2005). Another catenin, $p120^{ctn}$, binds to the cytoplasmic juxtamembrane domain of cadherins and appears to be involved in the cis-dimerization and clustering of cadherins. Based on their structure, β-catenin, plakoglobin,

and $p120^{ctn}$ belong to a protein superfamily, called the armadillo repeat family (Peifer et al. 1994). These proteins contain repeats of about 40 amino acids that were initially identified in armadillo, the Drosophila homolog of β-catenin. The armadillo repeats are built up of three α-helices that form a superhelix. The number of repeats varies between different members of the family; for instance, β-catenin contains 12 consecutive armadillo repeats. More distantly related members of the armadillo family include nonjunctional proteins, such as the importins, which are involved in nuclear protein import, and the tumor-suppressor APC. $p120^{ctn}$ is the founding member of a subfamily of armadillo proteins that include the plakophilins 1–3, p0071, ARVCF, and the nervous system-specific δ-catenin; the latter two are binding to the same region of cadherins as $p120^{ctn}$. Interestingly, the $p120^{ctn}$-related proteins are also found in the cell nucleus. $p120^{ctn}$ binds to the transcription factor Kaiso relieving its repressor activity, and plakophilin 2 is associated with RNA polymerase III complexes. Loss of $p120^{ctn}$ function in mice leads to activation of NF-κB signaling and inflammation. The desmosomal cadherins also associate with plakoglobin but not with β-catenin, and in addition they bind to plakophilins. Plakoglobin and plakophilins connect desmosomal cadherins to the desmosome-specific component desmoplakin, which links the complexes to the intermediate filament network (mainly cytokeratins in epithelial cells).

The cytoplasmic domains of protocadherins are unrelated to those of classical cadherins. They do not bind catenins, and it is not clear whether they are associated with the cytoskeleton (Gumbiner 2005). Some protocadherins contain a common cytoplasmic domain combined with different extracellular domains by differential pre-mRNA splicing.

Gene Organization

Several classical cadherin genes (e.g., E-, P-, VE-cadherins, and others) are found in a cluster on human chromosome 16 or on the syntenic mouse chromosome 8. The gene clustering is even more pronounced in the case of the desmosomal cadherins, which are all located on human chromosome 18q12 in relatively close vicinity to each other. Interestingly, the desmocollin and desmoglein genes (termed *DSC* and *DSG*) form two subclusters that have opposite transcriptional orientation. Protocadherins show a particularly striking genome organization. Subfamilies of protocadherins can be defined as those that share a common intracellular domain but differ in their extracellular domains (Gumbiner 2005).

Functional Characteristics

Classical and desmosomal cadherins are constituents of different types of intercellular junctions. E-cadherin, the classical cadherin of epithelial cells, is part of the adherens junction (zonula adherens), which is attached to a belt of actin via the catenins. As the name says, desmosomal cadherins are part of the desmosomes, which are rivet-like structures that make focal connections between cells. Desmosomes are characterized by a prominent intracellular plaque structure, which serves as an attachment point for intermediate filaments.

Functional studies show the adhesive role of cadherins (Gumbiner 2005). For example, cell-cell adhesion in vitro can be blocked by treatment of cultured cells with anti-cadherin antibodies, resulting in dissociation of the cell monolayer. Conversely, forced expression of cadherins by cDNA transfection in cells lacking these molecules leads to establishment of intercellular contacts. Thus, cadherins act as an adhesive glue that efficiently holds cells together. However, they may also have signaling function, as several in vitro studies show that cadherins influence differentiation and growth of cells. In vitro, cadherins can mediate the sorting out of cells, i.e., cells transfected with different cadherins separate from each other and forming homotypic aggregates. Accordingly, one of the main functions of cadherins might be in the delineation of tissue boundaries. Gene knockout experiments have revealed essential and cell-type-specific functions of cadherins in vivo. The knockout of the E-cadherin gene results in dissociation of cells of blastocyst-stage mouse embryos. The α-catenin knockout has a similar phenotype, while loss of plakoglobin leads to defects in cell adhesion of

heart muscle cells. The knockout of β-catenin does not affect cell junctions but rather has a Wnt signaling phenotype (see below), which is manifested at early stages of embryonic development. Apparently, plakoglobin, which is also present in the affected cells, can overtake the adhesive function of β-catenin under these circumstances. The mutation of desmosomal cadherins frequently leads to the disruption of skin layers. These phenotypes resemble those of the pemphigus blistering diseases in humans, which are caused by autoantibodies against desmocollins and desmoplakins (Spindler and Waschke 2014).

E-cadherin appears to play a major role in cancer as its expression is frequently downregulated in dedifferentiated, metastasizing tumors as well as during epithelial-mesenchymal transitions in embryonic development. E-cadherin gene expression is repressed by transcription factors of the Snail/Slug and ZEB1/ZEB2 families, as well as by Twist and E12/47 transcriptional repressors, which all bind to specific DNA elements in the E-cadherin promoter. E-cadherin inhibits invasion of tumor cells in in vitro systems and prevents tumor progression in vivo animal models. Moreover, E-cadherin has a role in contact-mediated inhibition of cell proliferation by its interaction with the Hippo signaling pathway (Mendonsa et al. 2018). Loss-of-function mutations of the E-cadherin gene occur in gastric carcinomas and certain types of breast tumors. The function of cadherins can be modulated by signaling pathways involving tyrosine kinases, which disrupt cell contacts, as well as by RhoA family GTPases. Conversely, E-cadherin can modulate the activity of these GTPases, although these signaling events have not been clearly defined in molecular terms so far (Gumbiner 2005).

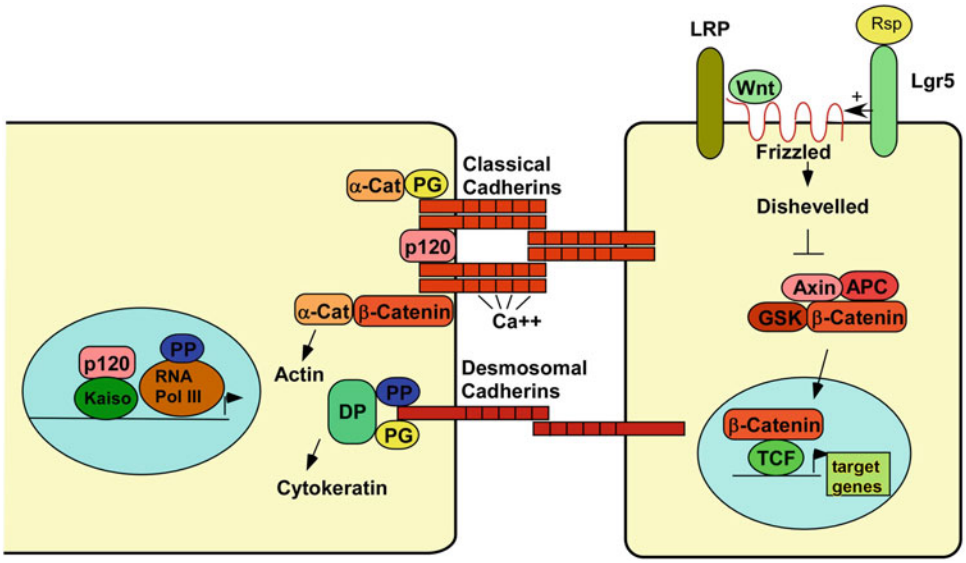
Protocadherins were shown to promote homophilic cell interaction via formation of cis- and trans-dimers in cell aggregation assays like classical cadherins (Gumbiner 2005). However, whether they reach a similar strength in cell-cell adhesion is not clear. Since there are so many members of the protocadherin family, which are mostly expressed in the nervous system, it has been speculated that protocadherins play discrete roles in setting up neuronal networks. Loss of protocadherins leads to various neuronal phenotypes including axonal patterning (Mountoufaris et al. 2018).

In *Drosophila*, *Fat* functions as a tumor-suppressor gene through interaction with Hippo signaling, and *dachsous* is involved in thorax, leg, and wing development. Several human and mouse *FAT* homologs have been identified, regulating various developmental steps involving cell proliferation (Blair and McNeill 2018).

β-Catenin in the Wnt Pathway

Besides its role in cell adhesion, β-catenin has an important function as a central signal transduction component in the Wnt pathway (Nusse and Clevers 2017). Wnts are a family of secreted glycoproteins that regulate a variety of developmental processes. Binding of Wnts to Frizzled receptors, which are seven transmembrane span proteins, induces stabilization of β-catenin. This pool of β-catenin is not associated with cadherins but accumulates in the cytoplasm and eventually enters the nucleus, where it teams up with transcription factors of the TCF family. TCFs bind to specific DNA sequences in Wnt-target promoters via an HMG box but lack transactivation domains. In the absence of β-catenin, TCFs behave as transcriptional silencers, in part, because they bind to diverse transcriptional repressors. β-Catenin lacks DNA-binding activity but contains strong transactivating sequences in its N- and C-terminal domains. Thus, when β-catenin binds to TCFs, a bipartite transcription factor is formed in which DNA-binding and transactivation domains reside on separate molecules. The TCF/β-catenin complexes can activate specific Wnt-target genes involved in determining cell fate and differentiation and inducing cancer (see below). For a list of Wnt-target genes, see www.stanford.edu/rnusse/wntwindow.html.

In the absence of Wnts, cytoplasmic "free" β-catenin is targeted for degradation by a multiprotein complex containing the scaffold component axin or the related protein conductin, the tumor-suppressor APC (adenomatous polyposis coli), and the serine/threonine kinases CK1 and GSK3β. When β-catenin binds to axin, it becomes phosphorylated by CK1 and GSK3β.



Cadherins/Catenins, Fig. 1 Overview of cadherin-mediated cell adhesion and the Wnt signaling pathway. Schematic representation of classical and desmosomal cadherins showing extracellular cadherin repeats and Ca^{2+}-binding sites and cytoplasmic interaction partners (the latter being depicted in the left cell only, which also shows association of $p120^{ctn}$ and plakophilin 2 with transcriptional regulators in the nucleus). The hypothetical cadherin zipper, which is made up of consecutive cis- and trans-dimers, is indicated (Gumbiner 2005). The basic outline of the Wnt pathway is shown in the right cell. LRP (lipoprotein receptor-related protein) is a coreceptor for Wnts. LGR5 (leucine-rich repeat containing G protein-coupled receptor5) is a receptor for Rsp (R-spondins), PG, plakoglobin, PP, plakophilin

Hyperphosphorylated β-catenin is recognized by the E3 ligase βTrCP/slimb, a component of the ubiquitination machinery, ubiquitinated, and finally degraded in proteasomes (Nusse and Clevers 2017). APC has several β-catenin and axin-binding sites and may function by sequestering free β-catenin and delivering it to the axin complex (Nusse and Clevers 2017). Thus, APC acts as a safeguard to prevent aberrant accumulation of β-catenin. Mutations of APC occur in up to 80% of colorectal carcinomas and lead to the formation of truncated proteins that are no longer able to interact with axin/conductin and to induce degradation of β-catenin. Therefore, these mutations result in the stabilization of β-catenin and in the formation of constitutive TCF/β-catenin complexes, which activate transcription of oncogenic target genes in a Wnt-independent fashion. In some colorectal tumors, and more frequently in other tumor types (e.g., hepatoblastomas), stabilization of β-catenin occurs through mutations of the critical serine or threonine residues normally phosphorylated by GSK3β. Thus, both genetically and functionally, β-catenin behaves as an oncogene, and the Wnt pathway has a major role in tumorigenesis. The pathway has also a prominent role in stem cell development in particular in the gut. In intestinal crypts, Wnts produced by surrounding mesenchymal cells called telocytes stimulate division of epithelial stem cells. In addition, secreted R-spondin proteins (Rsp) derived from myofibroblasts bind to receptors of the LGR (leucine-rich repeat containing G protein-coupled receptor) family such as LGR5 leading to the stabilization of Wnt receptors at the cell surface thereby enhancing Wnt signaling (Nusse and Clevers 2017) (Fig. 1).

Drugs

There are several potential approaches for pharmacological interference with the cadherin/

catenin system. Drugs that upregulate E-cadherin in tumors could be of potential benefit as part of a differentiation strategy to "normalize" epithelial cancers and to prevent metastasis formation. There are a number of substances reported to upregulate E-cadherin expression albeit in a rather indirect manner (Song et al. 2019). In contrast, more specific drugs targeting components of the Wnt pathway, e.g., by blocking the interaction of TCFs with β-catenin or raising the levels of negative regulator axin, have been identified and are candidates for interference with tumor growth. Moreover, certain diseases are associated with reduced Wnt signaling leading to efforts to increase activity of the pathway, e.g., by blocking GSK3 (Nusse and Clevers 2017).

Table appendix: Adhesion Molecules

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Calcidiol

► Vitamin D

Calciol

► Vitamin D

Calcitonin Family Receptors

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Synonyms

Adrenomedullin receptor; Amylin receptor; CGRP receptor

Definitions

The calcitonin (CT) receptor (CTR) and the CT-like receptor (CLR) are class B G protein-coupled receptors (GPCRs). These two GPCRs are able to interact with receptor activity-modifying proteins (RAMPs), and these interactions can influence all stages of the receptor lifespan. These receptors can be activated by several peptides, notably CT, CT gene-related peptide (CGRP), amylin, adrenomedullin (AM), and adrenomedullin 2/intermedin (AM2). Collectively this family regulates a diverse range of processes including appetite and satiety, neuromodulation, reproduction, development and maintenance of the cardiovascular and lymphatic systems, and maintenance of the skeletal system.

Basic Characteristics

Peptides

The CT peptide family comprises five structurally similar peptides: CT, CGRP, amylin, AM, and AM2. Since its discovery, AM2 has been referred to by two names – AM2 and intermedin (IMD). IMD has also been used as an alternative name for

melanocyte-stimulating hormone; to avoid confusion we recommend referring to this peptide as either AM2 or AM2/IMD but not IMD on its own. In humans and a number of other mammalian species (e.g., mice and rats), CGRP is found in two forms – αCGRP and βCGRP. In other species (such as pigs), βCGRP is not found, instead an alternative peptide known as the CT-receptor stimulating peptide is expressed (Hay et al. 2018).

These peptides have low amino acid sequence identity (Fig. 1a). However, they are united as a family by two structural features. These are: (1) a disulfide loop structure of 6–7 amino acids in the N-terminus formed by a disulfide bond between two cysteine resides, and (2) an amidated C-terminus. Removal of the disulfide loop creates antagonists with binding profiles that mimic the full length peptides; however, these antagonists often have lower affinities than the full length peptide (Hay et al. 2018). Removing the C-terminal amide also reduces the affinity of peptides for their receptors (Pioszak and Hay 2020). AM and AM2 are longer than the other peptides in the family due to the presence of an 14–15 amino acid extension prior to the disulfide loop structure (Fig. 1b). Synthetically created AM fragments which lack this extension have comparable pharmacologic and physiologic properties to the full-length AM, indicating that this region is not required for full AM function (Schönauer et al. 2017).

Receptors – Overview

This peptide family exerts its effects through two cell-surface class B GPCRs, CTR, and CLR. As class B GPCRs, CLR and CTR are made up of an extracellular N-terminus (~150 amino acids in length), seven membrane-spanning helices, and an intracellular C-terminus (~80 amino acids in length). Peptide activation of class B GPCRs occurs via the two domain model, in which the peptide C-terminus interacts with the receptor N-terminus to drive high affinity binding, which then allows the peptide N-terminus to interact with a binding pocket formed by the receptor transmembrane helices. This interaction between the transmembrane binding pocket and the peptide N-terminus stabilizes the receptor in a conformation that promotes signaling (Wootten and Miller 2020). CTR and CLR primarily couple to Gs proteins. This means that peptide activation results in the production of cyclic adenosine monophosphate (cAMP) within the cell; however, these receptors also activate other intracellular signaling pathways (Hay et al. 2018).

CTR can exist as one of many splice variants, displaying both species and tissue dependent expression. In humans the most well-characterized forms of CTR are $CTR_{(a)}$ and $CTR_{(b)}$, which are differentiated by the absence or presence of a 16 amino acid insert in intracellular loop 1, respectively. The presence of this insert affects signaling (Hay et al. 2018). CLR does not appear to display the level of variation associated with CTR.

Both CLR and CTR can interact with RAMPs. There are three RAMPs in humans. RAMPs are proteins of ~120–150 amino acids in length, with a large extracellular N-terminus (~100 amino acids), one membrane spanning helix, and a short intracellular C-terminal tail (~10 amino acids). CTR is able to traffic to the cell surface and act as a receptor

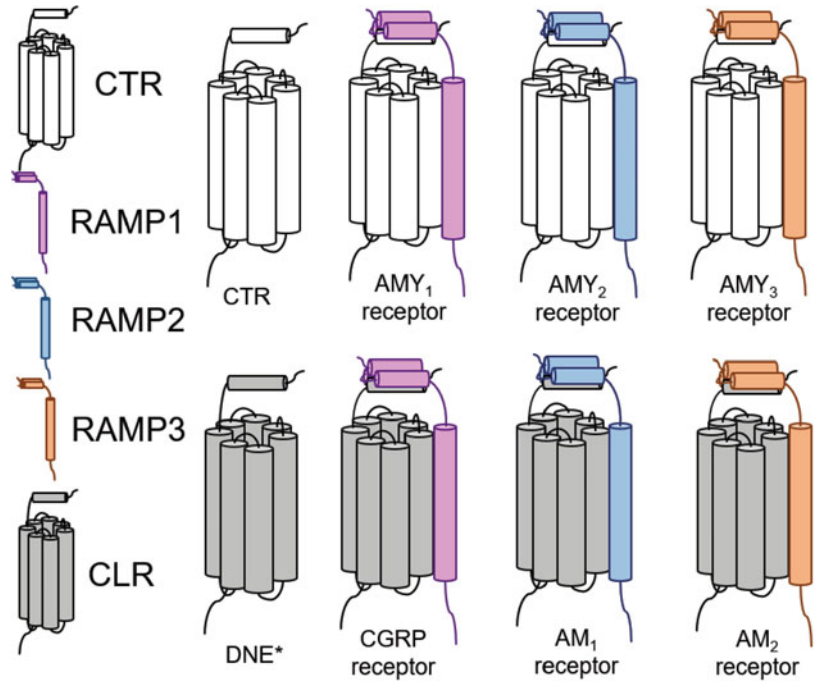
Calcitonin Family Receptors, Fig. 1 (**a**) Amino acid sequence alignment of human CT family peptides. In (**a**), AM and AM2 are shown as N-terminally truncated sequences. (**b**) shows a sequence alignment of the full-length AM and AM2 (53 amino acid form). Alignments performed in Geneious. Dark blue background with white text indicates an exact residue match, medium blue background indicates >80% similarity, and pale blue background indicates >60% similarity. All similarities were scored by Geneious. The C-terminal residue is amidated in all cases. The golden line above the sequences indicates the disulfide loop structure

without RAMP co-expression. In contrast, CLR is retained intracellularly when expressed alone, it is only when co-expressed with a RAMP that CLR can traffic to the cell surface and act as a receptor (Hay et al. 2018). Receptors formed through the interaction of CLR/CTR and RAMPs appear to exist as stable complexes for their entire lifespan (Poyner et al. 2002).

Receptor Composition and Pharmacology

The interactions between CLR/CTR and RAMPs create at least 7 distinct receptors. By interacting with RAMPs, CTR creates the AMY_1 (CTR:RAMP1), AMY_2 (CTR:RAMP2), and AMY_3 (CTR:RAMP3) receptors; the interaction of CLR with RAMPs creates the CGRP (CLR:RAMP1), AM_1 (CLR:RAMP2), and AM_2 (CLR:RAMP3) receptors (Fig. 2). CTR has (a) and (b) splice variants, meaning that the AMY receptors can also exist in (a) or (b) forms, that is, $AMY_{1(a)}$ and $AMY_{1(b)}$ receptors (Poyner et al. 2002).

Each receptor created through the interaction of CLR/CTR and RAMPs has a unique pharmacological profile. This paragraph, and Table 1, briefly summarizes the pharmacological profiles of the human receptors as defined by the ability of peptides to stimulate the production of cAMP. Alone, CTR is most potently activated by CT and more weakly activated by amylin. CGRP and amylin are the most potent peptides at the AMY_1 receptor at which they act with similar potencies. The AMY_2 receptor currently has a poorly defined pharmacological profile; however, it is likely that amylin is the most potent activator of this receptor. The AMY_3 receptor is most potently activated by amylin and more weakly activated by CGRP. The CGRP receptor is most potently activated by CGRP and is more weakly activated by AM and AM2. The AM_1 receptor is most potently activated by AM, followed by AM2 and then CGRP. At the AM_2 receptor, AM and AM2 are approximately equivalent, and both of these peptides are more potent than CGRP. It is worth highlighting that while the pharmacological profiles of these receptors are well defined, there is considerable overlap in peptide activity at these receptors, and the pharmacology can vary between species (Hay et al. 2018; Bower and Hay 2016).



Calcitonin Family Receptors, Fig. 2 Different receptors created through the interaction of CLR or CTR with RAMPs. DNE* – Does Not Express; this indicates that alone, CLR does not traffic to the cell surface and cannot act as a functional receptor

Calcitonin Family Receptors, Table 1 Relative rank order of peptide potency at defined human CTR- and CLR-based receptors

	CTR	CLR
Expressed alone	CT > amylin	Does not express on cell surface
+RAMP1	Amylin = CGRP	CGRP > AM = AM2
+RAMP2	Poorly defined (Amylin)	AM > AM2 > CGRP
+RAMP3	Amylin > CGRP	AM = AM2 >> CGRP

Rank orders are for cAMP production (Hay et al. 2019); ">" indicates an ~10-fold difference, while ">>" indicates an ~100-fold difference.

Molecular Understanding of Receptor Pharmacology

There are multiple interaction sites between CLR and RAMPs. The extracellular domain of the RAMP interacts with the extracellular domain of CLR. Additionally, the transmembrane domain of the RAMP interacts with CLR transmembrane helices 3, 4, and 5. It is presumed that a similar interaction mechanism exists between RAMPs and CTR (Pioszak and Hay 2020).

RAMPs influence the pharmacology of CLR and CTR by multiple complementary mechanisms. Residues within the RAMP extracellular domain augment the extracellular binding pocket of the receptor, providing a few key interaction points for residues in the peptide C-terminus. RAMPs also exert allosteric effects on CLR and CTR, causing the receptors to adopt subtly different conformations when complexed with different RAMPs. These conformational differences contribute to the binding specificity of the receptor. Conformational differences have been well defined for the extracellular N-terminus of these receptors and it is thought that RAMPs exert conformational effects across the entirety of CLR/CTR. It is likely that RAMPs also influence the conformational dynamics of CLR/CTR to influence specificity (Pioszak and Hay 2020).

Physiological Relevance of These Receptors

While there is much work on the physiological significance of this receptor family, the strong overlap in peptide activity at the different receptors means that it is difficult to conclusively link specific receptors to given physiological effects of individual peptides. Some headway has been made using animal models. However, because receptor components are involved in multiple receptor systems, and there is the possibility of functional redundancy in this receptor family, it is important to interpret these studies with care (Hay and Pioszak 2016).

CLR, RAMP1, RAMP2, and RAMP3 have been implicated in the lymphatic and cardiovascular systems, albeit to varying degrees. CLR and RAMP2 are critical and their deletion results in embryonic lethality, while mice lacking RAMP1 or RAMP3 survive to adulthood with less pronounced effects. RAMP1 and RAMP3 are also implicated in weight and metabolism, as mice lacking RAMP3 have a modest reduction in body weight in adulthood, and neuronal overexpression of RAMP1 is associated with decreased body weight and increased energy expenditure (Hay and Pioszak 2016). CLR is implicated in reproduction, as heterozygosity is associated with female subfertility (Serafin et al. 2020).

The effects of the peptides themselves are better defined. CT acts to reduce blood calcium levels and inhibit bone resorption. In many animals, CT is involved in physiological calcium homeostasis; however, in humans this effect is less established. Amylin is a neuroendocrine hormone which regulates energy homeostasis, nutrient influx, and satiety. CGRP causes powerful vasodilation, while also having roles in metabolism and pain sensation. AM is primarily known for its potent vasodilatory effects; however, this peptide also has regulatory roles in the lymphatic and reproductive systems and plays an important role in maintaining organ integrity. Like AM, AM2 is primarily known for its cardiovascular effects; this peptide also plays a role in renal function and has markedly high expression in the thyroid (Hay et al. 2018).

Drugs

In the past, drugs which targeted this family were typically analogues of the endogenous

Calcitonin Family Receptors, Table 2 FDA approved drugs that target the CTR/CLR receptor family

Name	Drug type	Mechanism	Disease(s)
Salmon CT	Peptide	Agonist of CTR	Hypercalcaemia and Osteoporosis
Pramlintide	Peptide	Agonist of CTR:RAMP complexes	Type 1 and 2 Diabetes
Erenumab	Antibody	Targets the CGRP receptor to inhibit function	Migraine (Preventative)
Eptinezumab	Antibody	Targets CGRP to inhibit function	Migraine (Preventative)
Fremanezumab	Antibody	Targets CGRP to inhibit function	Migraine (Preventative)
Galcanezumab	Antibody	Targets CGRP to inhibit function	Migraine (Preventative) and Cluster Headache (Preventative)
Ubrogepant	Small molecule	Antagonist of the CGRP receptor	Migraine (Acute)
Rimegepant	Small molecule	Antagonist of the CGRP receptor	Migraine (Acute)

peptides. Recently however, we have seen the successful development of nonpeptide small molecules and antibodies which target this family (Table 2).

CT-Based Drugs and Diagnostics

Analogues of human CT have been used clinically to treat hypercalcemia and osteoporosis. Salmon CT (which has ~50% amino acid sequence identity with human CT) has been approved as a therapeutic for the treatment of hypercalcemia since the 1970s; however, in practice its use is rare due to the rapid development of tolerance. A nasal spray formulation of salmon CT has been used for the treatment of osteoporosis in postmenopausal women for whom other treatments are not suitable; however, the efficacy of this treatment has been called into question. Additionally, circulating CT levels are used as a diagnostic marker for medullary thyroid cancer, with high CT levels being linked to the presence of tumors (Wells et al. 2016).

Amylin-Based Drugs

The combination of effects attributed to amylin has made it a target for the treatment of diabetes and other metabolic diseases. Pramlintide is an FDA-approved injectable peptide analogue of amylin that is used as an adjunct to insulin for the management of type 1 diabetes and insulin-requiring type 2 diabetes. Patients inject pramlintide before each meal as well as injecting insulin. At present these two treatments cannot be delivered in one injection due to differences in formulation requirements (Bower and Hay 2016). Pramlintide is also linked to decreases in body weight; this is currently being explored through the development of a new amylin mimetic, AM-833, which is in clinical trials for obesity management.

CGRP-Based Drugs

CGRP has long been targeted for the treatment of migraine. Initial progress was made with the development of nonpeptide small molecule antagonists of the CGRP receptor (the –gepant family of drugs); however, developments were delayed due to concerns of hepatotoxicity. Recent progress in this field has led to the development of ubrogepant and rimegepant. These drugs are now approved by the FDA for the treatment of migraine and are taken orally at migraine onset for acute migraine treatment.

Alongside these small molecules, antibodies targeting the CGRP peptide (eptinezumab, fremanezumab, and galcanezumab) or the CLR:RAMP1 complex (erenumab) have been approved by the FDA for the preventative treatment of migraine. These antibodies are delivered by injection either monthly (erenumab, fremanezumab, galcanezumab), or every 3 months (eptinezumab, fremanezumab). Galcanezumab has also been approved for the preventative treatment of cluster headache (Dubowchik et al. 2020).

AM-Based Drugs and Diagnostics

Recently, an antibody which targets the AM peptide (adrecizumab) completed phase II clinical trials for the treatment of sepsis with promising results. This same antibody may also be useful for the treatment of congestive heart failure; a phase II clinical trial is being developed to investigate its utility in this disease (Voors et al. 2019). In addition, mid-regional pro-AM (a stable by-product of AM production in the body) is gaining popularity as a diagnostic tool for cardiovascular disease and as a prognostic marker for septic shock (Nishikimi and Nakagawa 2018; Onal et al. 2018).

Cross-References

- ▶ Adrenomedullin/Intermedin
- ▶ Amylin
- ▶ G-Protein-Coupled Receptors

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Calcitriol

- ▶ Vitamin D

Calcium-Binding

- ▶ Ca^{2+}-Binding Proteins
- ▶ S100 Proteins

Calcium-Dependent Adhesion Protein/Plakoglobin

- ▶ Cadherins/Catenins

Calcium-Sensing Receptor

▶ Ca^{2+}-Sensing Receptor (CaSR)

Canakinumab

▶ Interleukin-1 (IL-1)

Cancer

▶ Ca^{2+}-Binding Proteins
▶ S100 Proteins

Cancer Immunotherapy

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Synonyms

Immune treatment; Immuno-oncology; Immunotherapy

Definition

Cancer immunotherapy identifies a type of cancer treatment that acts by exploiting the immune system. The term cancer immunotherapy can identify whether a treatment capable of activating the host immune system or be used to describe components of the immune system (synthetic, autologous, or allogeneic) that are administered to the host. Immunotherapy can be distinguished as follows: (1) active, i.e., exerts its effect only by engaging with the host immune system, or (2) passive, i.e., is designed to have a direct antineoplastic activity (Galluzzi et al. 2014).

Examples of active immunotherapy are monoclonal antibodies (MoAb) that can modulate immune effector cells activation or vaccines. Passive immunotherapy includes MoAb directed against protein expressed on the surface of cancer cells or the infusion of genetically modified immune cells (mainly T cells).

Basic Mechanism

Monoclonal Antibodies

Monoclonal antibodies (MoAbs) are laboratory-produced molecules designed to bind to antigens that are generally more numerous on the surface of tumor cells than on healthy tissues. MoAbs can exert their effect in different ways and can be divided into five main classes according to their preeminent mechanism of action: (1) MoAbs inhibiting signaling pathways required for the survival of cancer cells; (2) MoAbs capable of activating lethal receptors expressed on the surface of cancer cells; (3) MoAbs targeting tumor-associated antigens (TAA), thus opsonizing the cancer cell and activating antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC); (4) bispecific T cell engagers (BiTe), formed by single-chain variable fragments (scFv) of two distinct MoAb one targeting an antigen expressed on the tumor cell and the other a T cell surface antigen, hence engaging the T cell in the recognition and subsequent killing of cancer cells; and (5) MoAbs coupled with toxins or radionuclides, the so-called immune conjugates (Galluzzi et al. 2014). MoAbs of the last class are clearly a form of passive immunotherapy, in which the molecule coupled with the MoAb exerts a direct antitumor effect. The monoclonal antibody enables the delivery of the coupled drug directly to the target cell, minimizing off-target effects. Examples of this class of MoAbs are brentuximab vedotin, an anti-CD30 monomethylauristatin E conjugate used in the treatment of Hodgkin lymphomas and CD30 anaplastic lymphomas and belantamab mafodotin, an anti-BCMA (B cell maturation antigen) auristatin F conjugate currently under investigation for

multiple myeloma. TAA targeting MoAbs (class three) and BiTes (class four) directly engage the host immune system, thus classifying themselves as active immunotherapy. Example of class three is the anti-CD20 MoAb rituximab, the first antibody to be approved for the treatment of B cells non-Hodgkin Lymphomas. The effect of BiTes resides on their ability to bring the T cells in contact with the cancer cell, bypassing the need for antigen presentation through the major histocompatibility complex (MHC-I). The prerogative of BiTe of engaging a T cell response without the need of MHC enables the activation of all T cells not restricted to a specific T cell receptor. At present blinatumumab is the only BiTe construct approved for the treatment of acute lymphoblastic leukemia (ALL), but other BiTes against other malignancies are being developed. Regarding MoAbs of the first two classes, the precise mechanism of action is less clear. Although these MoAbs have traditionally been classified as passive immunotherapy, a growing body of evidence suggest that they might also promote ADCC and stimulate the host immune system by enhancing the activity of B- and T-lymphocytes, while inhibiting regulatory T cells, working as an active immunotherapy. A list of monoclonal antibodies and their main mechanism of action is reported in Table 1.

Immune Checkpoint Inhibitors

See ▶ "Immune Checkpoint Blockade".

Cellular Immunotherapy

Adoptive cell transfer or adoptive cell therapy (ACT) is the term used to indicate a type of immunotherapy characterized by the infusion in the host of lymphocytes. ACT involves the collection of circulating lymphocytes, their selection or modification in vitro, and the reinfusion in the host usually after a lymphodepleting therapy. Different types of ACT have been studied over the years: reinfusion of tumor infiltrating lymphocytes after ex vivo expansion and activation, reinfusion of NK cells or infusion of T cells genetically engineered with T cell receptor to be tumor specific. Currently the main approach of this kind of therapy relies on the use of chimeric antigen receptor (CAR) T cells. A CAR is a synthetic construct where a scFv recognition domain is linked to the cluster of differentiation 3 zeta (CD3ζ) portion of the T cell receptor (TCR), through a transmembrane domain (usually CD28). The scFv domain recognizes specific antigen on the cell surface without the need of MHC presentation, and activates the T cell through the CD3ζ domain. Examples of scFv domains currently approved for use in the clinic or undergoing clinical studies are anti-CD19, anti-B cell maturation antigen (BCMA), and anti-SLAMF7. The CD3ζ portion of the CAR contains immune tyrosine activation motifs (ITAMs), which, upon phosphorylation, activate the signaling cascade of the TCR. In second- and third-generation CAR-Ts the ITAMs are modified to contain signaling endodomains, such as CD28, 4-1IBB, and inducible T cell co-stimulator (ICOS). These signaling endodomains mimic the co-stimulation that is provided by the antigen-presenting cell in physiological condition, making the CAR-T cell more efficient in cell killing. More recently fourth- and fifth-generation CAR-Ts have been developed, including inducible expression of inflammatory cytokines or signaling domains from cytokines receptors (Benmebarek et al. 2019). Upon engagement of the CAR, an immunological synapse (IS) between the CAR-T cell and the cancer cell is formed. Once the IS has been formed CAR-T cells induce cell death by exocytosis of cytotoxic granules containing perforine and granzyme. Perforine enables granzyme to enter the cytoplasm of the tumor cell by forming pores on the cell membrane. Once in the cytoplasm, granzyme induces both caspase-dependent and independent apoptosis by activating caspases such as caspase 3 and BH3-only members of the BCL-2 family such as Bid (BH3-interacting domain). The IS formed during CAR-T cell engagement differs from "normal" IS, as they are smaller and show a more disorder pattern of activating clusters. As a result CAR-T cells have an earlier release of granzyme and perforine, shorter receptor–initiated signal duration, and a faster detachment from the target cell. Despite the signal being shorter, it has a higher strength compared with physiological TCR signaling. Interestingly, the amount of lytic granules

Cancer Immunotherapy, Table 1 Monoclonal antibodies and their class of action. For monoclonal antibodies active against immune checkpoint see chapter ▶ "Immune Checkpoint Blockade"

Antibody	Disease indication	Target/Class of action	Approval
Rituximab	B cell non-Hodgkin's lymphomas	CD20, Class III	1998 (EU), 1997 (USA)
Trastuzumab	Breast cancer	HER2, Class I	2000 (EU), 1998 (USA)
Ibritumomab tiuxetan	B cell non-Hodgkin's lymphomas	CD20, Class V	2004 (EU), 2002 (USA)
Cetuximab	Colorectal cancer	EGFR, Class I	2004 (EU), 2004 (USA)
Bevacizumab	Colorectal cancer	VEGF, Class I	2005 (EU), 2004 (USA)
Panitumumab	Colorectal cancer	EGFR, Class I	2007 (EU), 2006 (USA)
Ofatumumab	B cell non-Hodgkin's lymphomas	CD20, Class III	2010 (EU), 2009 (USA)
Brentuximab vedotin	Hodgkin's lymphoma, Anaplastic large cell lymphoma	CD30, Class V	2012 (EU), 2011 (USA)
Pertuzumab	Breast cancer	HER2, Class I	2013 (EU), 2012 (USA)
Ado-trastuzumab emtansine	Breast cancer	HER2, Class V	2013 (EU), 2013 (USA)
Obinotuzumab	Chronic lymphocytic leukemia	CD20, Class III	2014 (EU), 2013 (USA)
Ramucirumab	Gastric cancer	VEGFR2, Class I	2014 (EU), 2014 (USA)
Blinatumomab	Acute lymphoblastic leukemia	CD19, CD3 Class IV	2015 (EU), 2014 (USA)
Dinutuxumab	Neuroblastoma	GD2, Class III	2015 (EU), 2015 (USA)
Necitumumab	Non-small cell lung cancer	EGFR, Class I	2015 (EU), 2015 (USA)
Elotuzumab	Multiple myeloma	SALMF7, Class III	2016 (EU), 2015 (USA)
Daratumumab	Multiple myeloma	CD38, Class III	2016 (EU), 2015 (USA)
Inotuzumab ozogamycin	Hematological malignancies	CD22, Class V	2017 (EU), 2017 (USA)
Gentuzumab ozogamycin	Acute myeloid leukemia	CD33, Class V	2018 (EU), 2017 (USA)
Mogamuizumab	Cutaneous T cell lymphoma	CCR4, Class I	2018 (EU), 2018 (USA)
Moxetumomab pasudotox	Hairy cell leukemia	CD22, Class V	2018 (USA)
Polatuzumab vedotin	Diffuse large B cell lymphoma	CD79b, Class V	2019 conditional approval (EU), 2019 (USA)
Enfortumab vedotin	Urothelial cancer	Nectin-4, Class V	2019 (USA)
Trastuzumab deruxtecan	HER2+ Breast cancer	HER2, Class II	2019 (USA)
Sacituzumab govitecan	Triple negative breast cancer	TROP-2, Class V	Under review
Isatuximab	Multiple myeloma	CD38, Class III	Under review
Tafasitamab	Diffuse large B cell lymphoma	CD19, Class III	Under review
Oportuzumab monatox	Bladder cancer	EpCAM, Class V	Under review
Belantamab mafodotin	Multiple myeloma	BCMA, Class V	2020 conditional approval (EU), 2020 (USA)
Naxitamab	Neuroblastoma	GD2, Class III	Under review
Margetuximab	HER2+ breast cancer	HER2, Class I	Under review

delivered is comparable with those of cytotoxic T cells (Davenport et al. 2018). In addition to this fast acting killing mechanism, CAR-T cells can induce the apoptosis of the target cell by activating tumor necrosis factor (TNF) family receptors such as Fas via the expression on their membrane of ligand of the TNF family. The Fas/FasLigand (FasL) axis engagement leads to the activation of caspase 8 and pro-caspase 8. Active caspase 8 is able to induce apoptosis through the activation of caspase 3. Following receptor engagement FasL expression is upregulated in CAR-T cells, effectively enabling the killing also of antigen-negative tumor cells (Hong et al. 2018).

CAR-T cells currently used in the clinic are generated in autologous fashion, upon viral transduction and subsequent expansion of patient's derived peripheral leukocytes. Studies are currently ongoing to produce allogeneic CAR-T cells, to overcome production limitations due to ineffective transfection or ineffective in vitro expansion of patient-derived T cells.

Vaccines

Vaccines are the prototype of active immunotherapy. Differently from vaccines developed to prevent infective disease, cancer vaccines are not though to prevent the development of cancer, but are administered to patients with overt tumors to activate the host's immune system against the malignant cells. Different type of vaccines have been developed over the years, including autologous patient-derived immune cell vaccines, tumor antigen–expressing recombinant virus vaccines, peptide vaccines, DNA vaccines, and heterologous vaccines derived from tumor cell lines (Guo et al. 2013). Autologous tumor cell vaccines are prepared using patients-derived tumor cells that are reinjected in the patient usually after irradiation and combination with an immunostimulatory agent such as the Bacillus Calmette-Guérin (BCG). To overcome the availability of specimen that limits the applicability of autologous vaccines, allogeneic vaccines made of three or more established tumor cell lines have been developed. In addition to this approach peptide and DNA-based vaccines have also been developed. Peptide vaccines comprise peptide from a defined TAA; in contrast with autologous or allogeneic vaccines, which can activate the immune system against the tumor as a whole, peptide-based vaccines recognize only one or few epitopes of the chosen TAA. An additional limitation is the poor immunogenicity of TAA. To improve immunogenicity an approach consists in generating peptide vaccines by autologous tumor lysates that are then merged with immunostimulatory adjuvants or chaperons. DNA-based vaccines utilize bacterial plasmid to deliver tumor antigens. The vectors transfect somatic or dendritic cells (DC), resulting in indirect antigen presentation or cross-priming of DC, which in turn activates the immune response against the tumor. Viral vectors can also be used to deliver TAA, usually combined with immunomodulatory molecules. Messanger RNA can also be administered to induce an immunogenic response (Guo et al. 2013). A well-studied type of cancer vaccination is vaccine developed directly from patient's DC. Patient's derived monocytes or hematopoietic stem cells (HSCs) are induced to differentiate and maturate through treatment with various cytokines. DCs are then loaded with different types of TAA and reinfused in the patient, in order to induce immune responses that are tumor specific (Perez and De Palma 2019).

Others

Other forms of cancer immunotherapy include immunomodulatory drugs such as thalidomide and its derivate, immunostimulatory cytokines such as interferons (IFN) and interleukine 2 (IL-2), and even some conventional chemotherapy. All these treatment have shown to be able to modulate the host immune system, acting as an active cancer immunotherapy. Thalidomide was the first immunomodulatory drug to show effectiveness in hematologic malignancies. Its effect was mainly thought to occur via modulation of TNF-α. To reduce thalidomide side effect and increase antineoplastic activity lenalidomide and pomalidomide were later developed. These drugs not only have a direct antitumor effect, but are also active on the host immune system. Immunomodulatory drugs enhance antigen-specific T cell cytolysis and stimulate the production of IL-2 that can expand antigen-specific memory effector T cells. Furthermore immunomodulatory drugs can increase NK activity and can increase the production of granzyme B and FasL expression. The immunomodulatory drug lenalidomide also increased the secretion of IFN-γ by NKT cells (Davies and Baz 2010). Recently the effect of immunomodulatory drugs was shown to be dependent on their binding to the E3 ubiquitin ligase cerebron (CRBN) and consequent down regulation of IKAROS family zinc finger 1 (IKZF1), IKZF3, and interferon regulatory factor-4 (IRF-4) (Lopez-Girona et al. 2012). IKZF1 and IKZF3 regulate lymphocytes development

and differentiation and IKZF3 can control the apoptosis of T cells. IRF-4 is important in the activation of both innate and adaptive immune system by negatively regulating toll-like-receptor (TLR) signaling. Downregulation of IKZF1, IKZF3, and IRF-4 might account at least in part for the immunomodulatory properties of these drugs. Conventional chemotherapeutic also can have immunomodulatory effects. Chemotherapy can induce immunogenic cell death, and some drugs such as 5-fluoruracil and gemcitabine, when used in low doses and with a metronomic fashion, are able to stimulate the host immune system as an off-target effect (Galluzzi et al. 2014).

Pharmacological Relevance (Including Side Effects)

The discovery of immunotherapy has dramatically changed cancer treatment. The implementation of MoAb into treatment of hematologic and solid tumors was able to increase progression-free survival as well as overall survival. The first MoAb, the anti-CD20 rituximab, revolutionized the treatment of non-Hodgkin's lymphoma and forms now the backbone of almost all lymphoma treatment. More recently, MoAb anti-CD30, anti-CD38, and anti-SLAMF7 have been developed to treat Hodgkin lymphoma and anaplastic T cell lymphomas (anti-CD30) or multiple myeloma (anti-CD38, anti-SLAMF7). Like the anti-CD20, these drugs as well revolutionized treatment of hematologic malignancies and are rapidly moving forward in the first-line treatment of these diseases both as single agent as well as being part of combination therapy. MoAb are also exploited in the treatment of solid cancer, such as the MoAb anti-VEGF (used in gastrointestinal malignancies) or the MoAb anti-EGFR (used in head and neck cancer and gastrointestinal tumors). Similarly to what is seen in hematologic malignancies monoclonal antibodies are being associated in a wide variety of anticancer treatment as part of polychemotherapeutic schemas.

BiTe therapy has proven to be extremely effective in relapsed/refractory acute lymphoblastic leukemia, and is now under investigation in other hematologic malignancies as well as in solid tumors. The main hurdle of BiTe treatment is the short half-life of the compounds, which requires repeated or continuous infusion of the drugs. Research is ongoing to produce BiTe with a longer half-life (e.g., by coupling them with heavy-chain fragments) and more manageable administration schedule (Liu et al. 2017).

The first two CAR-T cell products were approved in 2017 and 2018 in the USA and in 2018 in Europe for the treatment of relapsed/refractory diffuse large B cell lymphoma (DLBCL) in adults and relapsed/refractory ALL in children. Approval was based on the impressive results of phase II clinical trials showing an overall response rate of 82% (ALL) and 52% (DBLCL) with 45% of ALL patients still in complete remission after 2 years and a median progression free survival that in the case of DBLC has not yet been reached. Interestingly the survival curves showed a plateau, suggesting that a proportion of patients with relapsed/refractory disease might be cured with this treatment approach. The success obtained by CAR-T cells in the treatment of DLCBL and ALL prompt their use also in other hematologic malignancies and in solid tumors. Trials are ongoing investigating the role of CAR-T cells in multiple myeloma, chronic lymphocytic leukemia, and gastrointestinal cancers (Kruger et al. 2019).

Despite being extremely interesting, the development of vaccines to treat cancer has so far not fulfilled the promised expectation. DC vaccines are safe and immunogenic, however clinical responses so far have been disappointing. This is likely due to the immunosuppressive tumor microenvironment that limits DC's function (Perez and De Palma 2019). The high production costs, especially of personalized vaccines, further limits their applicability. The possibility to combine vaccine strategy with ▶ "Immune Checkpoint Blockade" might help to overcome the immunosuppressive tumor microenvironment and increase vaccines efficacy (Mougel et al. 2019).

Immunomodulatory drugs have changed treatment paradigm of the hematologic malignancy multiple myeloma (the second most frequent hematologic malignancy after non-Hodgkin lymphomas), and form now the backbone of almost all anti-myeloma treatment.

The development of immunotherapy, especially of CAR-T cells and of BiTes, has seen the rise of new toxicity profiles different from what expected with conventional chemotherapy. Due to the activation of the immune system a wide range of reaction can develop, according to the type of immunotherapy used. Cytokine release syndrome (CRS) and neurological toxicities are the main nonhematological adverse events seen with the use of BiTes and CAR-T treatment. They have been reported in 5 to up to 50% of patients according to study and treatment indication. CRS is typically an early event, which develops within the first 7–14 days after CAR-T infusion. It is characterized by fever, gastrointestinal symptoms, headache, hypotension, dyspnea, and arthralgia. According to the degree of severity can be treated with steroids, inhibitors of interleukin-6 (IL-6) or even lymph depleting chemotherapy such as cyclophosphamide. Neurological toxicity can develop as an early event, usually in association with CRS, or as a later event occurring when the symptoms of CRS start to improve. Neurological toxicity is characterized by encephalopathy, aphasia, cognitive defects, seizures, EEG alteration, and edema seen in MRI. CAR-T cells are usually found in the liquor of these patients. Neurological toxicity is generally managed with high-dose steroids, as the use of IL-6 inhibitors has shown disappointing results in this indication. IL-6 could be administered in case of neurological toxicity with concomitant CRS. IL-1 inhibitors could also have a role in the management of toxicities (Brudno and Kochenderfer 2019). The tyrosine kinase inhibitor dasatinib can interfere with the lymphocyte-specific protein tyrosin kinase (LCK), thus inhibiting the phosphorylation of CD3ζ. Due to this inhibition, dasatinib can act as an "off switch" abrogating CAR-T cell function and proliferation (Mestermann et al. 2019). Further CAR-T products with build-in "off switch" that can induce programmed apoptosis in case of uncontrolled side effects are currently under development. In addition of these side effects, patients receiving CAR-T can develop prolonged leukocytopenia and thrombocytopenia that might require transfusion and antibiotic prophylaxis.

Cross-References

▶ Immune Checkpoint Blockade

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Cardiac Arrest

▶ Myocardial Infarction

Cardiac Glycosides

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Synonyms

Cardiac steroids; Cardiotonic steroids; Digitalis

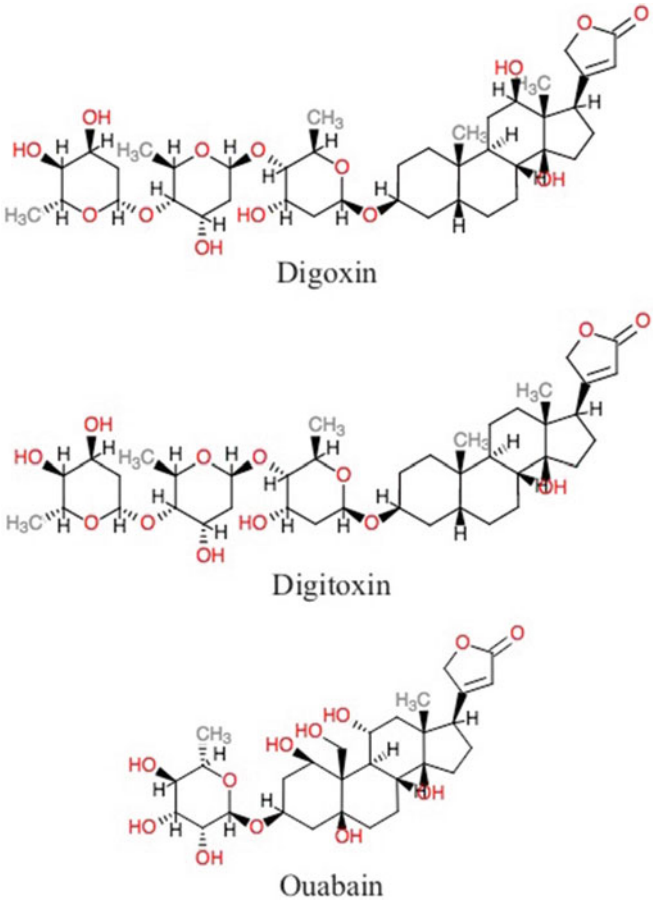
Cardiac glycosides are pharmacologically active compounds derived from different plants and the toad venom. Furthermore, these endogenous compounds evidently control blood pressure, and disturbances in their physiological levels have been observed in several diseases, including myocardial ischemia and infarction, diabetes, and cancer. Cardiac glycosides act on the membrane-bound sodium-potassium-adenosine triphosphatase (Na^+/K^+-ATPase) pump. The most important cardiac glycosides are digoxin, digitoxin, and ouabain (Fig. 1).

Cardiac glycosides are chemically characterized by a steroid nucleus comprised of four fused rings of 17 carbons (cyclopentanoperhydrophenanthrene nucleus in a *cis-trans-cis* configuration), with an unsaturated lactone ring at C17, and a sugar moiety (glycone portion) coupled through a hydroxyl group at C3. Usually, the glycone portion is composed of one to three units of digitoxose, glucose, thevetose, rhamnose, cymarose, gentiobiose, and oleandrose. There are two classes of cardiac glycosides, cardenolides, and bufadienolides, chemically distinguishable by the lactone ring. Cardenolides, derived from plants, contain the butyrolactone ring, a five-membered ring with a double bond. On the other hand, bufadienolides, found in some toad venoms and plants, have an α-pyrone ring, a six-membered ring with two double bonds.

Notably, some chemical features affect the inhibitory activities of Na^+/K^+-ATPase. The presence of a hydroxyl group at C14 of the steroid nucleus is essential for Na^+/K^+-ATPase activity. Furthermore, the type and number of sugar units in the aglycone portion affect the relative binding affinity of cardiac glycosides. Digoxin, digitoxin, and ouabain have a higher binding affinity than their aglycone counterparts. A rhamnose residue provides nearly an 18-fold higher affinity in comparison to compounds without the sugar moiety. The presence of digitoxose provides a lower binding affinity than cymarose. Moreover, binding affinity is not always related to the inhibitory activity. Modifying the lactone ring from five to six atoms could reduce the binding affinity but increase the inhibitory activity.

Digitoxin is almost completely absorbed following oral administration, whereas the intestinal absorption of digoxin is 60–80%, and that of ouabain is 3%. Protein binding of digitoxin is more than 90%, with digoxin about 30% protein bound. Serum protein binding is about 20% for digoxin, 97% for digitoxin, and 3% for ouabain. Peak concentration is achieved in 1–3 h for digoxin and 0.5–4 h for digitoxin. The biotransformation of cardiac glycosides is predominantly mediated by hepatic microsomal enzymes, with the elimination half-life of digoxin ranging from 36 to 48 h. Ouabain demonstrates an elimination half-life 11 h, which is 7–9 days for digitoxin. The principal elimination route for digoxin is through urinary excretion, with 50–70% eliminated as the parental compound in urine after intravenous administration. The major route of digitoxin elimination occurs through biliary excretion. Hence, impaired kidney function affects the elimination of digoxin but not digitoxin.

The gut bacteria *Eggerthella lenta* may reduce the lactone ring of digoxin, generating dihydrodigoxin, an inactive metabolite, decreasing drug bioavailability (Haiser et al. 2014).



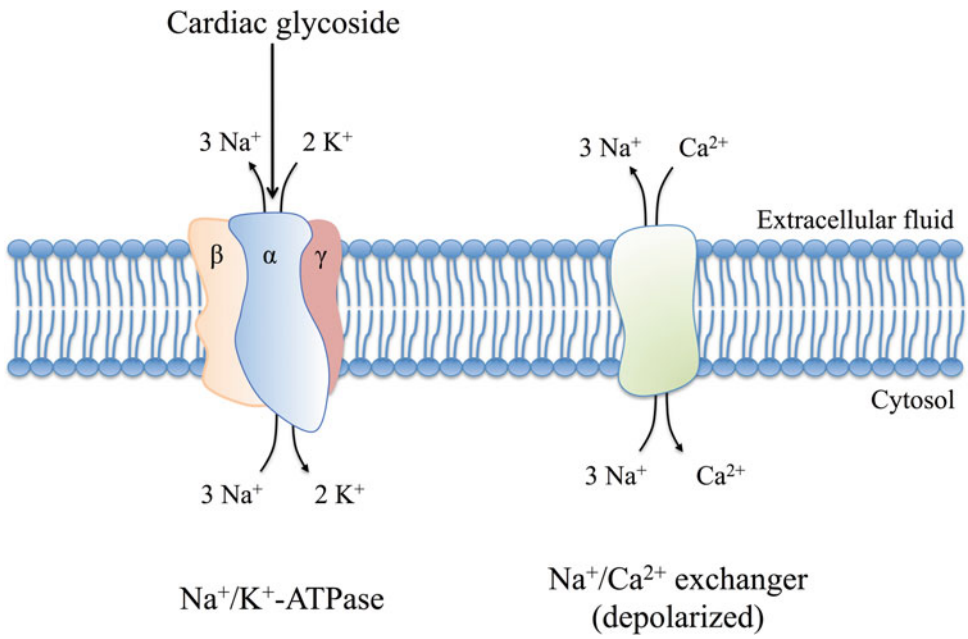
Cardiac Glycosides, Fig. 1 Cardiac glycosides – molecular structure of digoxin, digitoxin, and ouabain

Mechanism of Action

Cardiac glycosides act in the cardiac muscle cells to inhibit the activity of Na^+/K^+-ATPase, which is an entropy-driven reversible reaction (Fig. 2). This enzyme is a P-type pump that physiologically maintains low levels of intracellular Na^+, by actively transporting Na^+ outside and K^+ into cells in a 2:3 stoichiometry. Na^+/K^+-ATPase is composed of an αβ heterodimer and an accessory FXYD regulatory (γ) subunit. The α subunit demonstrates a molecular weight of approximately 112 kDa and 10 transmembrane segments. Particularly, the binding sites of Na^+, K^+, ATP, and cardiac glycosides are present within this subunit. The β subunit, with a molecular weight ranging from 40 to 60 kDa, contains one transmembrane domain and several extracellular glycosylation sites. There are four isoforms of the α subunit (α_1, α_2, α_3, and α_4) and three isoforms of the β subunit (β_1, β_2, and β_3). These isoforms demonstrate a differential distribution within the body as follows:

- α_1: throughout the organism
- α_2: heart, muscles, placenta, cartilage, adipocytes, and some areas of the brain
- α_3: muscles, heart, placenta, ovaries, erythrocytes, and neurons
- α_4: testis
- β_1: throughout the organism
- β_2: heart, muscles, cartilage, nervous system, and erythrocytes
- β_3: brain, cartilage, erythrocytes, liver, lungs, retina, and testis

The α_1 isoform is markedly abundant in cardiomyocytes compared to the α_2 isoform, but



Cardiac Glycosides, Fig. 2 Cardiac glycosides – schematic representation of the Na^+/K^+-ATPase

α_2 has a greater significance in cardiac muscle contractility by regulating the cytosolic Ca^{2+} levels. Digoxin and digitoxin present a higher binding selectivity for α_2 and α_3 than α_1, whereas ouabain has higher binding selectivity for α_1 than α_2.

In the most accepted model for the pump reaction cycle, Na^+/K^+-ATPase can be presented in two states, E_1 and E_2P. In the E_1 state, the enzyme associated with ATP and Mg^{2+} at the cytoplasmic side has three Na^+ ions. Next, it undergoes a conformational change hydrolyzing ATP into ADP, preserving the released inorganic phosphate attached to the enzyme. As Na^+ ions are transported outside the cell, the enzyme alters its conformation from the E_1 to the E_2P state. Na^+/K^+-ATPase in the E_2P state has a low affinity for Na^+, with a high affinity for K^+ present in the extracellular fluid. Two K^+ ions bind to the enzyme, which is dephosphorylated and has another bound ATP. Na^+/K^+-ATPase undergoes another conformational change, releasing K^+ into the cytosol and returning to the E_1 state (Crambert et al. 2000; Wasserstrom and Aistrup 2005; Katz et al. 2010).

Cardiac glycosides inhibit Na^+/K^+-ATPase, stabilizing it in the E_2P transition state. Intracellular Na^+ is maintained at physiological levels by action of the Na^+/Ca^{2+} exchanger, which in depolarized stage transporting Na^+ outside and Ca^{2+} into cells. Finally, the intracellular Ca^{2+} levels are enhanced, which increases its uptake by the sarcoplasmic reticulum via the $SERCA_2$ transporter. Thus, large amounts of Ca^{2+} are released after the depolarizing impulse reaches the myocyte, causing a more vigorous contraction. Beyond the positive inotropic effect, cardiac glycosides decrease the heart rate by increasing the refractory period of the atrioventricular node.

Clinical Use

Digoxin, as well as digitoxin in some countries, is used in the treatment of mild to moderate heart failure (NYHA III-IV) and cardiac arrhythmia owing to its positive inotropic and antiarrhythmic effects. Experimental studies have demonstrated the potential therapeutic uses of cardiac glycosides, including the treatment of some tumors (Botelho et al. 2019), viral infections (Amarelle and Lecuona 2018), and autoimmune diseases (Huh et al. 2011).

Furthermore, digoxin and digitoxin are used in association with other drugs in the management of mild to moderate heart failure related to left ventricular systolic dysfunction. These drugs increase the left ventricular ejection fraction, consequently increasing cardiac output and lowering the pulmonary capillary wedge pressure. At low doses, digoxin is responsible for neurohormonal modulation, improving the parasympathetic tone and suppressing sympathetic tone and the renin-angiotensin-aldosterone system. Parasympathetic activity is evidenced by the augmented heart rate variability, whereas the sympathetic effects are characterized by reduced serum norepinephrine levels and enhanced carotid sinus baroreflex sensitivity. In case of the renin-angiotensin-aldosterone system, digoxin reduces the plasma renin activity without affecting the aldosterone levels. The clinical signs and symptoms of heart failure are improved, mainly in patients who remain symptomatic regardless of being treated with other drugs (Ambrosy et al. 2014; Konstantinou et al. 2016).

In patients with atrial fibrillation, controlling the heart rate indicates beneficial effects on the quality of life, decreasing morbidity and the risk of developing tachycardia-induced cardiomyopathy. Even though β-blockers and nondihydropyridine calcium channel blockers are the most frequently used drugs, digoxin effectively reduces the ventricular heart rate. However, digoxin is not indicated in patients with pre-excitation and atrial fibrillation as it can induce ventricular fibrillation. Heart rate control is initiated at least 1 h after intravenous administration, peaking about 6 h later. Following prolonged oral maintenance dosing, the ventricular rate is reduced at rest, but not during exercising. The association of digoxin with β-blockers or nondihydropyridine calcium channel blockers raises the rate of control during exercise; this association may be useful in patients with concomitant heart failure. Notably, increased mortality has been mainly reported with long-term use. For heart rate control, digoxin is initially administered intravenously over 24 h, with an oral formulation prescribed for maintenance therapy (January et al. 2014).

Additionally, digoxin can be used for the management of paroxysmal supraventricular tachycardia (PSVT), especially in patients who do not undergo catheter ablation and who fail or cannot be prescribed preferred therapies, such as β-blockers, diltiazem, verapamil, or a class Ic antiarrhythmic agents (Page et al. 2016).

The digoxin dose must be adjusted in patients with renal dysfunction, in the elderly, and in case of concomitantly used drugs that reduce its elimination. Amiodarone, dronedarone, propafenone, quinidine, spironolactone, and verapamil increase blood digoxin concentrations as these drugs inhibit P-glycoprotein, which is responsible for the renal elimination of digoxin. Erythromycin, omeprazole, and tetracycline increase digoxin absorption, also increasing blood digoxin concentrations. In such patients, determining serum digoxin has been recommended to ensure adequate dosing (January et al. 2014).

Cardiac glycosides must be cautiously used owing to their narrow therapeutic window. The mechanism of toxicity is associated with excessive Na^+/K^+-ATPase inhibition. Excessive intracellular Ca^{2+} overloads the storing capacity of the sarcoplasmic reticulum. Transient increases in Ca^{2+} give rise to transient inward depolarizing currents, consequently delaying afterdepolarizations that can generate extrasystoles and ventricular arrhythmias. Notably, the symptoms observed in cases of poisoning include anorexia, nausea, vomiting, diarrhea, headache, fatigue, drowsiness, and visual disturbances. Several cardiac dysrhythmias can be observed, including sinus bradycardia, premature ventricular complexes, first-degree atrioventricular block, bigeminy, atrial tachycardia with atrioventricular block, and ventricular fibrillation. Death may occur following cardiac arrest (Konstantinou et al. 2016; Botelho et al. 2019).

Cross-References

► Antiarrhythmic Drugs

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Cardiac Infarction

▶ Myocardial Infarction

Cardiac Steroids

▶ Cardiac Glycosides

Cardiomyopathy

▶ Heart Failure
▶ Ca^{2+}-Binding Proteins
▶ S100 Proteins

Cardiotonic Steroids

▶ Cardiac Glycosides

Carfilzomib (Kyprolis)

▶ Proteasome Inhibitors

Catechol-*O*-Methyltransferase

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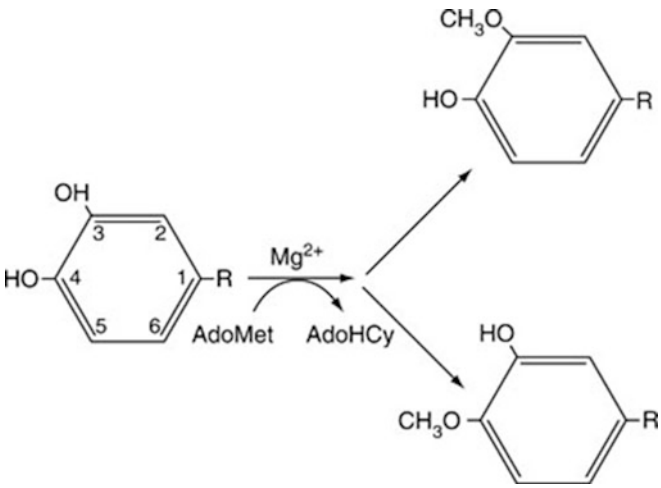
Synonyms

COMT; COMTase; EC 2.1.1.6

Definition

Catechol-*O*-methyltransferase (COMT) is a widespread enzyme that catalyzes the transfer of the methyl group of *S*-adenosyl-L-methionine (AdoMet) to one of the phenolic group of the catechol substrate (Fig. 1). High COMT activity is found in the liver, kidney, and gut wall (Guldberg and



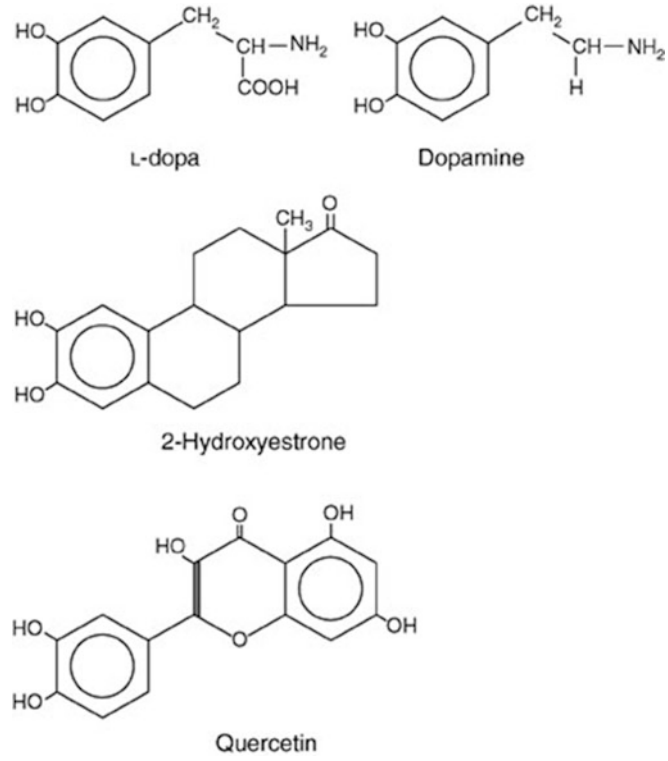
Catechol-*O*-Methyltransferase, Fig. 1 The basic function of COMT. Enzymatic *O*-methylation of the catechol substrate to 3-methoxy (major route) or 4-methoxy (minor route) products in the presence of Mg^{2+} and *S*-adenosyl-methionine (AdoMet)

Marsden 1975; Männistö and Kaakkola 1989). A single *COMT* gene codes for two separate enzymes, soluble (S-COMT) and membrane-bound (MB-COMT) forms. S-COMT contains 221 amino acids. MB-COMT has an additional amino-terminal extension of 43 (rat) or 50 (man) amino acids. The hydrophobic 17 and 24 amino acid residues in rat and man, respectively, form an α-helical transmembrane domain that serves as a membrane anchor; otherwise the two proteins are similar. MB-COMT is associated with intracellular membranes, not with cell membranes. Also S-COMT is strictly intracellular locating either in cytoplasm or nucleus. Synthesis of recombinant S-COMT in *Escherichia coli* and MB-COMT in insect cells, using baculovirus vectors, has helped to clarify the biochemistry, physiology, and pharmacology of COMT (Männistö and Kaakkola 1999). The active site of COMT consists of the AdoMet-binding domain and the catalytic site. S-COMT is abundant in peripheral tissues, while MB-COMT prevails in the brain. The catalytic site is formed by a few amino acids that are important for the binding of the substrate, water, and Mg^{2+} and for the catalysis of *O*-methylation. The Mg^{2+}, which is bound to COMT only after AdoMet binding, improves the ionization of the hydroxyl groups. The lysine residue (Lys144), which accepts the proton of one of the hydroxyls, acts as a general catalytic base in the nucleophilic methyl transfer reaction. Mice lacking totally COMT (knockout mice) or only S-COMT or MB-COMT have been developed. These animals breed quite normally suggesting that COMT is not a vital enzyme. Interestingly, MB-COMT-deficient male mice exhibit schizophrenia-associated behavioral abnormalities such as aggressive behavior and reduced prepulse inhibition. Both male and female mice move normally, but they are sensitized to acute pain and have impaired short-term memory. It appears that of the two COMT isoforms, MB-COMT is critical for normal behavior, and its function in COMT-dependent brain areas cannot be entirely substituted by S-COMT (Männistö and Kaakkola 1999; Tammimäki et al. 2016).

Mechanism of Action

COMT *O*-methylates catecholamines and other compounds having a catechol structure including catecholoestrogens (Fig. 2). The two isoforms of COMT may have distinct roles: MB-COMT, a high-affinity isoform of COMT, is supposed to be partially responsible for the termination of dopaminergic and noradrenergic synaptic neurotransmission. S-COMT, on the other hand, is a high capacity enzyme isoform being mainly responsible for the elimination of biologically active or toxic, particularly exogenous, catechols and some hydroxylated metabolites. During the first trimester of pregnancy, COMT present in the placenta protects the developing embryo from hydroxylated compounds. COMT also acts as an enzymatic detoxicating barrier between the blood

Catechol-*O*-Methyltransferase, Fig. 2 Some substrates of COMT



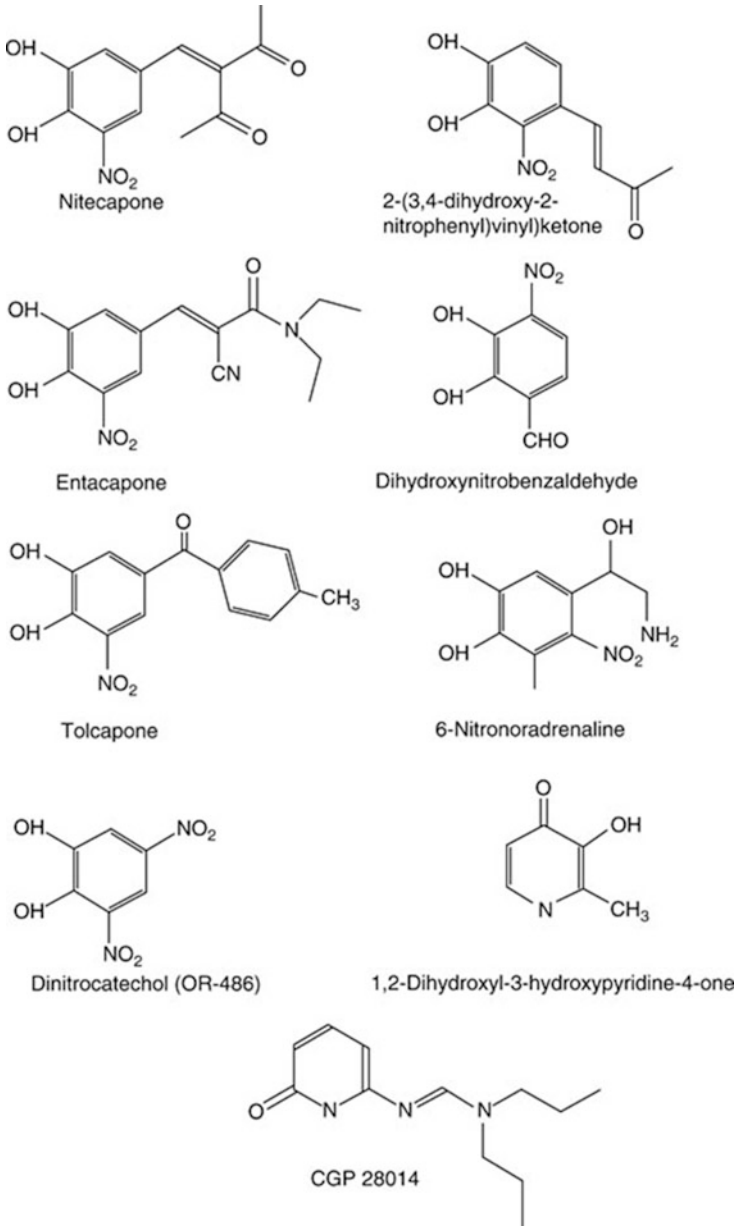
and other tissues, shielding against the detrimental effects of hydroxylated xenobiotics. COMT may serve some unique or indirect functions in the kidney and intestine tract by modulating the dopaminergic tone. The same may be true in the brain: COMT activity may regulate the amounts of active dopamine particularly in frontal cortical areas of the brain and therefore be associated with mood and other mental processes. COMT has several genetic one-nucleotide polymorphisms of which the most important one is caused by a change of valine amino acid to methionine leading to thermolability even at +37 °C and a significant decrease of COMT activity. Low COMT activity is connected to improved cognitive functions, probably due to increased dopaminergic activity. However, from population studies there is some evidence that certain psychiatric illnesses (e.g., schizophrenia, rapidly fluctuating depression) and even breast cancer in postmenopausal women may be slightly increased in people having low COMT activity (Männistö and Kaakkola 1999).

COMT Inhibitors

Early COMT inhibitors, like gallates, tropolone, and U-0521 (3′,4′-dihydroxy-2-methyl-propiophenone), have IC_{50} and K_i values in the micromolar range or higher but may still be practical in vitro tools. However, owing to unfavorable pharmacokinetics and toxicity, their clinical use is not possible (Guldberg and Marsden 1975).

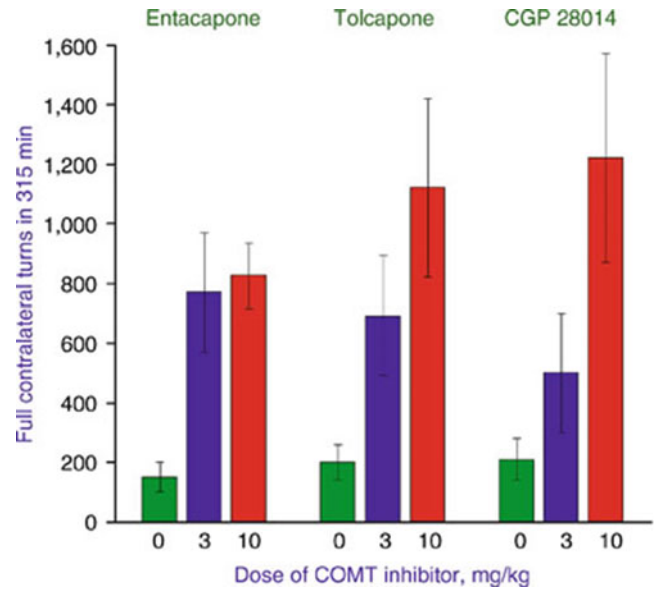
First second-generation COMT inhibitors were developed by three laboratories in the late 1980s. Apart from CGP 28014, nitrocatechol is the key structure of the majority of these molecules (Fig. 3). The current COMT inhibitors can be classified as follows: (i) mainly peripherally acting nitrocatechol-type compounds (entacapone, nitecapone, BIA 3–202, opicapone) (ii) broad-spectrum nitrocatechols having activity both in peripheral tissues and the brain (tolcapone, Ro 41–0960, dinitrocatechol, vinylphenylketone), and (iii) atypical compounds, pyridine derivatives (CGP 28014, 3-hydroxy-4-pyridone and its derivatives), some of which are not COMT inhibitors in

Catechol-*O*-Methyltransferase, Fig. 3 Chemical structures of some inhibitors of catechol-*O*-methylation



vitro but inhibit catechol *O*-methylation by some other mechanism. The common features of the new compounds are excellent potency, low general toxicity, and activity through oral administration. Their biochemical properties have been fairly well characterized. All nitrocatechols are fast acting, reversible, but tight-binding enzyme inhibitors. The extra long-lasting COMT inhibition of opicapone can be explained by its particularly tight binding. Most of these compounds have an excellent selectivity in that they do not affect any other enzymes studied (Männistö and Kaakkola 1989, 1999; Katsaiti and Nixon 2018).

Tolcapone, Ro 41–0960, and opicapone are longer acting than entacapone and nitecapone in in vivo studies in rats and man. All types of COMT inhibitors prolong the L-dopa-induced turning



Catechol-*O*-Methyltransferase, Fig. 4 Rat model of Parkinson's disease. Comparison of entacapone, tolcapone, and CGP 28014 in the rat turning model of Parkinson's disease (Tammimäki et al. 2016)

behavior of rats having unilateral nigral lesions (Fig. 4). This has generally been used as a reliable rat model of Parkinson's disease. It is noteworthy that the peripherally acting compounds entacapone and opicapone are practically as effective as the broad-spectrum compound tolcapone. This suggests that the majority of the beneficial action is peripheral in origin, evidently through enhanced bioavailability of L-dopa.

Clinical Use (Including Adverse Effects)

The main clinical use of COMT inhibitors is as adjunct (or additional adjunct) in the therapy of Parkinson's disease. The standard therapy of Parkinson's disease is oral L-dopa (as a drug levodopa) given with a dopa decarboxylase (DDC) inhibitor (e.g., carbidopa and benserazide), which does not reach the brain. When the peripheral DDC is inhibited, the concentration of 3-*O*-methyldopa (3-OMD), a product of COMT, in plasma is many times that of L-dopa. Since the half-life of 3-OMD is about 15 h, compared to about 1 h for L-dopa, the concentration of 3-OMD remains particularly high during chronic therapy, especially if new slow release levodopa preparations are used. A triple therapy (L-dopa plus DDC inhibitor plus COMT inhibitor) will evidently substitute the present double therapy in the coming years. A fixed combination preparation containing all three active drugs is indeed getting very popular worldwide.

COMT inhibitors rescue L-dopa and improve the brain entry of L-dopa by decreasing 3-OMD formation in peripheral tissues. The dose of L-dopa could be decreased, compared with the present combination therapy. Dose interval of L-dopa could also be prolonged. Further, COMT inhibitors should decrease fluctuations of dopamine formation in the brain.

Clinical studies, available only for entacapone, tolcapone, and opicapone, support preclinical findings. A dose-dependent (100–800 mg) inhibition of the COMT activity of the erythrocytes can be seen after entacapone and tolcapone. However, effective and sufficient dose levels of both entacapone and tolcapone, given concomitantly with L-dopa and DDC inhibitors to patients with Parkinson's disease, appear to be 100–200 mg. However, the treatment strategies of entacapone and tolcapone differ: entacapone is a short-acting compound that is given with each dose of L-dopa, and COMT activity may even recover between the doses. Tolcapone, as a longer-acting compound, is given three times a day, and the aim is to keep

COMT inhibited most of the time. Opicapone is so long-acting that it is given only once a day (Männistö and Kaakkola 1989, 1999; Katsaiti and Nixon 2018).

Since several adrenergic drugs, having a catechol structure, are also COMT substrates, it is possible to prolong or even potentiate in some cases their actions by COMT inhibitors. Such drugs include bronchodilating compounds (adrenaline, isoprenaline, rimiterol), dopamine agonists (dobutamine, fenoldopam, apomorphine), and antihypertensive drugs (α-methyldopa). It is possible to potentiate interactions with endogenous catecholamines during stress and exercise and adverse drug interactions with, e.g., exogenous noradrenaline and the drugs mentioned above. Fortunately, interaction studies in animals and man have not been able to substantiate this threat. Evidently, the capacity of S-COMT in the peripheral tissues is so high that only a minor general COMT inhibition can ever been achieved. Estrogens are easily hydroxylated to catecholoestrogens, which serve as COMT substrates. The consequence of preventing the major metabolic pathway of catecholoestrogens by COMT inhibitors requires further studies; it is possible that quinone-forming pathways are activated (Männistö and Kaakkola 1989, 1999).

In patients having Parkinson's disease, both entacapone and tolcapone potentiate the therapeutic effect of L-dopa and prolong the daily ON time by about 1 h. In the clinic, COMT inhibitors have been well tolerated, and the number of premature terminations has been low. In general, the incidence of adverse events has been higher in tolcapone-treated patients than in entacapone-treated patients. The main events have comprised of dopaminergic and gastrointestinal problems (Männistö and Kaakkola 1989, 1999; Katsaiti and Nixon 2018).

Dopaminergic overactivity causes an initial worsening of levodopa-induced dyskinesia, nausea, vomiting, orthostatic hypotension, sleep disorders, and hallucinations. Tolcapone has been associated with diarrhea in about 16–18% of cases and entacapone in less than 10% of cases. Diarrhea has led to discontinuation in 5–6% of patients on tolcapone and in 2.5% of those on entacapone. Urine discoloration to dark yellow or orange is related to the color of COMT inhibitors and their metabolites. Elevated liver transaminase levels are reported in 1–3% of patients treated with tolcapone but very rarely, if at all, in patients treated with entacapone or opicapone. Three cases of acute, fatal fulminant hepatitis have been described in association of tolcapone where more than 100,000 patients have been treated. In addition, a few potentially fatal neurological adverse reactions, including neuroleptic-like malignant syndrome, have been described. Because of these serious adverse drug reactions, tolcapone marketing was temporarily suspended in Europe and Canada in 1999. Now tolcapone is again available in most markets, but certain precautions and a regular follow-up of liver function need to be obeyed. So far, no restrictions of the use of entacapone and opicapone have been proposed (Männistö and Kaakkola 1989, 1999; Katsaiti and Nixon 2018).

Cross-References

- ▶ Synaptic Transmission
- ▶ α-Adrenergic System

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Cell Cycle Checkpoints

► DNA Damage Response

Cell Cycle Regulation

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Definition

The eukaryotic somatic cell cycle is defined by an order of two major tasks a dividing cell has to complete: replication of its DNA during the DNA synthesis phase (S phase) and segregation of the duplicated DNA onto two newly formed daughter cells during mitosis (M phase). These two events are timely separated by two so-called gap phases (G1 and G2 phases) during which cell growth and preparation for the main tasks takes place. S, G1, and G2 phases are typically termed interphase, while mitosis can be further subdivided into subphases, prophase, metaphase, anaphase, and telophase, which are followed by cytokinesis, the actual division of the cytoplasm.

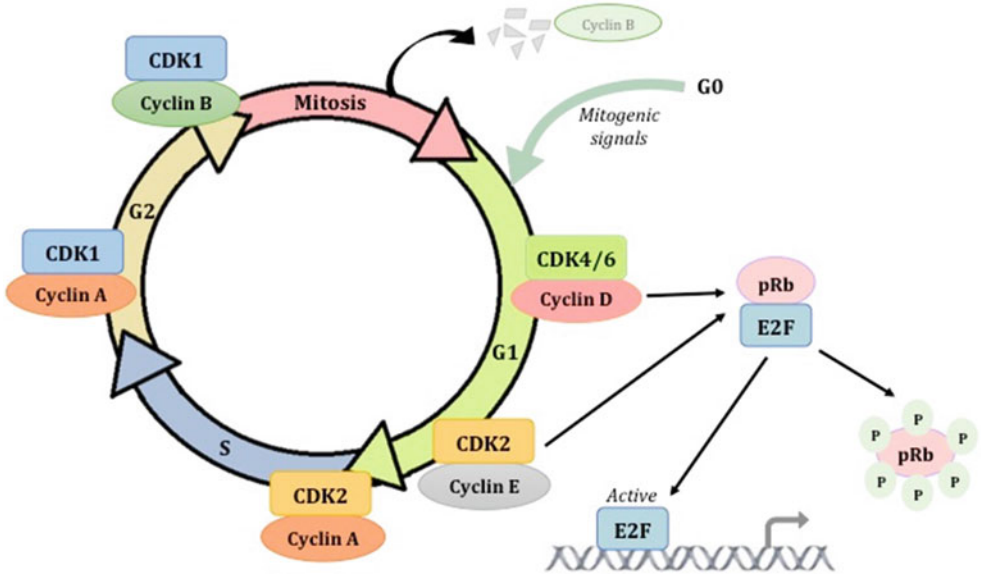
Basic Mechanisms

Upon encountering a mitogenic signal, a cell can enter the cell cycle, triggering a combination of events. The cell cycle progresses in an ordered fashion and typically lasts about 24 h in a human somatic cell. The fidelity of cell cycle events highly depends on various regulatory mechanisms that enable coordinated progression through the cycle. How a cell monitors the cell cycle and how cancer cells surpass these regulatory mechanisms is still incompletely understood and remains a subject of intense research. It has become clear in recent years that the mechanisms involving the trigger forces and control mechanisms of the cell cycle have been conserved throughout evolution. These mechanisms involve complex regulatory networks of proteins and are termed the cell cycle control system and cell cycle checkpoints, which ensure the correct order and timing of a faithful execution of the cell cycle. These systems constitute of a series of biochemical mechanisms and can broadly be categorized as:

1. Reversible protein phosphorylation
2. Regulated ubiquitin-dependent protein degradation
3. Transcriptional control mechanisms
4. Regulation by CDK inhibitors
5. Cell cycle checkpoint mechanisms

Cell Cycle Regulation by Protein Phosphorylation

Periodic activation and inactivation of protein activity play a dominant role in driving the different phases of the cell cycle. Central to this process are a class of protein kinases known as cyclin-dependent kinases (CDKs). CDKs generally consist of a catalytic subunit (the CDK) and a regulatory subunit, called cyclin, and catalyze the transitions from one cell cycle phase into another. The concentrations of CDKs remain constant throughout the cell cycle, but the levels of cyclins oscillate, thereby enabling the formation of active CDK-cyclin complexes at different time points during the cell cycle. In fact, various CDK-cyclin complexes are formed at different cell cycle stages and serve as a driving forces for cell cycle progression (Fig. 1). The first evidence for the key role of CDKs in cell cycle regulation goes back to the discovery of mitosis (maturation) promoting factor (MPF), which drives interphase cells into mitosis, subsequently leading to the idea that certain oscillating activities are responsible for the initiation of mitosis. MPF was first discovered in frogs and later in starfish oocytes and other animals such as mouse and surf clam. A large number of genetic experiments in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* using various cell division cycle (cdc) mutants then revealed that MPF is a heterodimeric protein kinase complex. The complex was shown to be

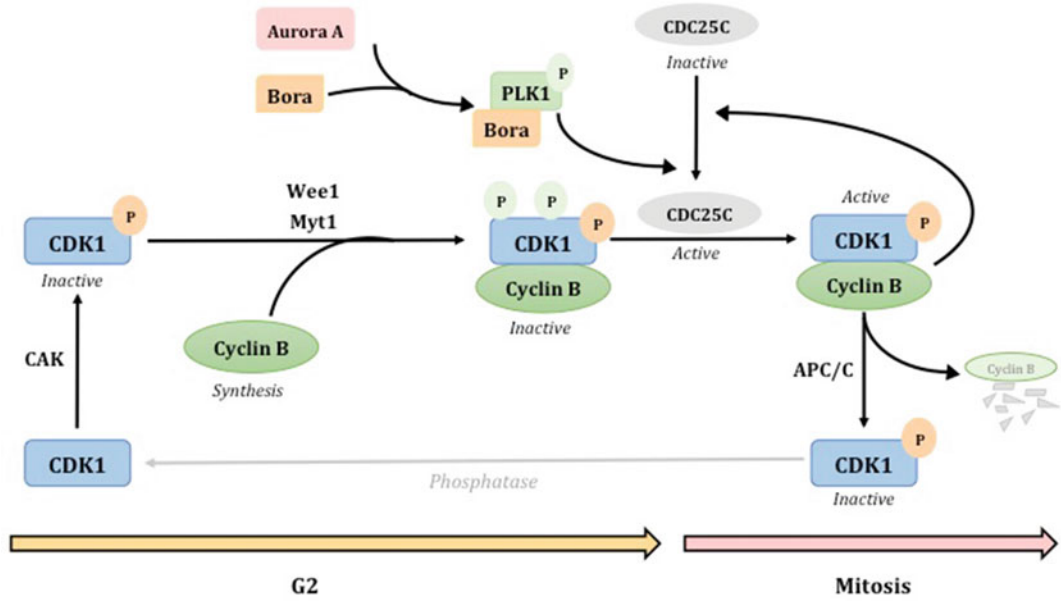


Cell Cycle Regulation, Fig. 1 Cell cycle regulation by cyclin-dependent kinases (CDKs). Different cyclins bound to different CDKs promote the transition from one cell cycle phase to another. CDK-dependent phosphorylation of Rb is required to release active E2F transcription factors, which promotes entry into S phase

constituted by CDK1 (CDC28 and cdc2 in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, respectively) and cyclin A or cyclin B. However, it later became clear that this complex can contain also additional components. The discovery of CDK1 was the first step to the identification of multiple CDKs such as CDK1, 2, 4, and 6 and different cyclins, including cyclin A, B, E, and D and their roles in different cell cycle stages. To date, more than 20 different CDKs and 30 cyclins are known in human cells. Not all of them are involved in cell cycle regulation (Li et al. 2015).

The CDK comprising CDK1 and cyclin B serves as a key trigger for mitosis (Fig. 2). In late S and G2 phase of the cell cycle, cyclin B accumulates in the cell and binds to CDK1, leading to formation of a in principle functional kinase complex. However, full activation of CDK1-cyclin B requires phosphorylation at threonine 161 on the CDK1 subunit, which is catalyzed by a CDK-activating kinase (CAK). Since CDK1-cyclin B complexes are only fully activated when cells enter mitosis, preformed CDK1-cyclin B complexes need to be kept inactive until the actual G2 to M transition is reached. For this, two phosphorylations at residues threonine 14 and tyrosine 15 (in mammals) are induced by the kinases Wee1 and Myt1, respectively, in G2 phase, and these phosphorylations block CDK1 activity. This inactive CDK1-cyclin B complex was also termed as pre-MPF and can be activated just prior to mitosis by removing both the inhibitory phosphorylation sites. This action is performed by CDC25C, a member of the CDC25 family of dual-specificity phosphatases, and leads to rapid CDK1 activation at the start of mitosis (Fig. 2). Consequently, at the G2/M transition, CDC25 activity increases while, Wee1 and Myt1 kinase activities decrease; this is at least partly caused by a positive feedback loop involving CDK1 itself (Li et al. 2015). Thus, initial activation of CDK1 (which occurs at centrosomes) supports its maximal activation at the beginning of mitosis.

More recently, it became clear that CDK1 may promote its own activation by stimulating another important serine/threonine kinase, called as polo-like kinase (PLK1, in mammals). Activation of PLK1 in turn relies on the phosphorylation of its activation loop at Thr-210 within its catalytic domain. This is performed by another serine/



Cell Cycle Regulation, Fig. 2 Regulation of CDK1 activity at the G2/M transition. Preformed kinase complexes consisting of CDK1 and cyclin B are kept inactive by two inhibitory phosphorylations mediated by Wee1 and Myt1 kinases. Activation of CDK1-cyclin B is triggered by CDC25C-mediated dephosphorylation at the G2/M transition. Active CDK is required for entry into mitosis, which is further promoted by Aurora A-mediated activation of PLK1 that also promotes CDK1 activation. CDK is inactivated at the end of mitosis by ubiquitin-dependent protein degradation of cyclin B

threonine kinase Aurora A along with its co-factor Bora. In late G2, CDK1 phosphorylate Bora, and this promotes an interaction between PLK1 and Bora, a step important for the Aurora A-mediated PLK1 activation (Joukov and De Nicolo 2018). The Aurora A/PLK1/Bora axis functions in a spatio-temporal manner, serving not only as a mitotic switch but also operating throughout mitosis by phosphorylating many different proteins involved in the execution of mitosis (Fig. 2).

Cell Cycle Regulation by Ubiquitin-Mediated Protein Degradation

To ensure proper progression of the cell cycle, a cell cycle control system is present in eukaryotic cells, which centers around the periodic formation of CDK-cyclin complexes. The protein levels of cyclins oscillate in a cell cycle-dependent manner, which are the result of transcriptional regulation, stability of their mRNA, and their protein stability. Decline in different cyclin proteins present at certain cell cycle stages is achieved by proteasome-mediated protein degradation. Timely degradation of cyclins results consequently in inactivation of the respective CDK1, which cannot be reactivated, thereby ensuring irreversibility of the cell cycle progression.

Protein proteolysis plays a critical role throughout the cell cycle and in particular in mitosis at transition from metaphase to anaphase. While high levels of cyclin B are required for full activation of CDK1 and for the entry into mitosis, cyclin B levels start to drop at the metaphase to anaphase transition, leading to an irreversible inactivation of CDK1, which contributes to the segregation of sister chromatids during anaphase and is required for the exit from mitosis. The decrease in cyclin B levels is due to regulated ubiquitin-mediated protein degradation by the 26S proteasome. Cyclin B is "tagged" by multiple copies of ubiquitin, a process termed as polyubiquitination, which serves as a prerequisite for its recognition and subsequent degradation by proteasome. Ubiquitination requires three

enzymatic activities including a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). UbcH10 is the relevant ubiquitin-conjugating enzyme involved in cyclin B ubiquitination, and a multi-subunit protein complex known as anaphase-promoting complex or cyclosome (APC/C) serves as a ubiquitin ligase for cyclin B ubiquitination (Borg and Dixit 2017). During mitosis, the APC/C activity requires the co-activator protein Cdc20 or (later in mitosis) Cdh1. These subunits contribute to substrate recruitment via their WD40 repeat domains. The WD40 repeats help in recognizing a destruction motif (defrost) within their substrates, typically a destruction (D-) box or KEN box or both. In addition to mitotic cyclins, the APC/C is involved in the destabilization of various other mitotic target proteins including "securin." The degradation of securin at the metaphase to anaphase transition is required for sister chromatid separation by activating the protease "separase" that causes the release of cohesins from the two sister chromatids in order to allow sister chromatid separation to happen. At the end of mitosis, the APC/C dissociates from its activator Cdc20 and binds to its other activator Cdh1, thereby maintaining its activity as the cell enters G1 phase. APC/C^{Cdh1} maintains G1 and blocks premature entry into S phase by catalyzing S and M phase-specific cyclin destruction. Moreover, the APC/C controls the degradation of regulators of the S phase until the assembly of pre-replication complexes has taken place. APC/C is also active in non-dividing and differentiated cells, suggesting its role beyond the cell cycle (Peters 2006).

In addition to protein proteolysis during mitosis, ubiquitin-mediated protein degradation is also required at the G1 to S transition and during the S phase. At this stage, an E3 ubiquitin ligase complex termed SCF (consisting of *S*kp1, *C*ullin, *F*-box protein, and Rbx1 subunits) is functional. The F-box subunit of SCF acts as a substrate recognition subunit that specifically binds to proteins exposing phosphorylated degron sequence motifs. It then binds to Skp1 using its F-box motif and brings substrate in close proximity of a functional E2 ubiquitin-conjugating enzyme. The SCF is involved in the ubiquitination of several proteins including the CDK inhibitor $p27^{KIP1}$, cyclin E, and the transcription factor E2F1. Thus, SCF-mediated ubiquitination contributes to proper G1 to S transition of the cell cycle (Li et al. 2015; Borg and Dixit 2017).

Together, it is now well recognized that ubiquitin-mediated protein degradation is an important regulatory mechanism throughout the cell cycle for an irreversible inactivation of various cell cycle regulators, including cyclins, mitotic regulators, CDK inhibitors, and transcription factors.

G1/S Transition and Transcriptional Control

The transition from G1 phase into S phase is one of the most studied cell cycle transitions. There is a transcriptional wave and tight regulation at G1/S, also known as "restriction point," which represents a point of commitment to cell division. This transition requires mitogenic signals and extensive transcriptional changes. The mechanism of G1 to S transcriptional program is well-established and depends on the E2F family of transcription factors, which is regulated by CDK-cyclin activity. E2Fs are the key transcription factors involved in gene expression required for the entry and execution of S phase as well as for later cell cycle progression. When cells enter the cell cycle from quiescence (G0), cyclin D (different types of cyclin D1, D2, and D3), cyclin E, and cyclin A are synthesized sequentially in a mitogen-dependent manner. Cyclin A and cyclin E preferentially activate CDK2, whereas D type cyclins activate either CDK4 or CDK6. The regulatory principles described for CDK1-cyclin B at G2/M apply similarly to the cyclin A/E-CDK2 and CDK4/6-cyclin D complexes. They also acquire an activating and two inhibitory phosphorylations, which are removed at or near G1/S transition by CDC25A, belonging to CDC25 family of dual-specificity protein phosphatases (Bertoli et al. 2013).

During the early G1 phase (and also during G0) of the cell cycle, activating E2F transcription factors (e.g., E2F1 and E2F2) are bound and thereby inhibited by hypophosphorylated retinoblastoma protein (Rb) – a so-called pocket protein. Rb

functions as a transcriptional inhibitor of the activating E2F transcription factors, thereby preventing expression of genes required for S phase and, thus, inhibiting entry into the S phase. In addition, repressive E2F transcription factors (e.g., E2F4 and E2F5) bind to p130 and p107 – another set of pocket proteins, which are transported into the nucleus, where they bind together with repressive E2Fs in order to prevent gene expression. During progression of G1, cyclin D-dependent CDK activity increases, followed by an increase in cyclin E- and cyclin A-dependent kinase activities, leading to hyperphosphorylation of pocket proteins. As a result, Rb can no longer bind to activating E2Fs resulting in the activation of E2F transcriptional activity. On the other hand, phosphorylation of p130 and p107 releases E2F4 and E2F5, and their repressive function at S phase gene promotors is abrogated (Bertoli et al. 2013; Giacinti and Giordano 2006). Hence, CDK-mediated phosphorylation of pocket proteins causes activation of a complex transcriptional program required for progression into S phase of the cell cycle (Fig. 1).

During the S phase, the genome has to be replicated completely and only once per cell cycle to maintain genomic integrity and prevent tumorigenesis. Replication is initiated at specialized sites on DNA called "replication origin." The first step of replication involves assembly of a pre-replication complex at replication origins, a process referred to as "origin licensing." This takes place already in late mitosis and in early G1 phase when CDK activity is low. The pre-replicative complex consists of CDC6, ORC/CDT1, and MCM2-7 proteins. The chromatin "licensed" for replication is then guided to the S phase by the phosphorylation of CDKs that are active at the G1/S phase transition. Upon entry into the S phase, pre-replicative complexes are transformed into active complexes, leading to the establishment of replication forks by unwinding origins and recruiting the DNA synthesis machinery including DNA polymerases. Upon accomplishment of DNA replication within a replicon, MCM proteins dissociate from the chromatin, and CDK2 activity during S phase prevents its reloading onto chromatin. This restricts the initiation of replication to only once per cell cycle (Pack et al. 2019).

Cell Cycle Control by CDK Inhibitors

CDK activities can be regulated by CDK inhibitor proteins (CKIs). CKIs are small proteins, which can be subdivided into two distinct groups: the INK4 proteins (inhibitors of CDK4) and CIP/KIP family of inhibitors (CDK-interacting protein/kinase inhibitory protein).

Proteins of the INK4 protein family including $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$ specifically inhibit CDK4/6-cyclin D complexes and, thereby, can block the transition from G1 to S phase of the cell cycle by preventing phosphorylation of pocket proteins. This leads to a cell cycle stop, preventing further proliferation of cells. The loss of $p16^{INK4a}$ is a well-established tumor suppressor protein, whose loss results in the deregulation of CDK4/6-Rb- $p16^{INK4a}$ axis (Giacinti and Giordano 2006; Pack et al. 2019).

Despite similarities in their structure and biochemical activities, regulation of each of the INK4 proteins differs significantly. $p16^{INK4a}$ is often induced in response to expression of oncogenic proteins to counteract transformation and hyperproliferation. Elevated levels of $p16^{INK4a}$ are also detected during cellular senescence, which results in delayed or abrogated cell cycle progression. $p15^{INK4b}$, however, is induced by growth inhibitory factors such as TGFβ signaling, where it plays an essential role in mediating the TGFβ triggered G1 arrest. $p18^{INK4c}$ and $p19^{INK4d}$ have roles for permanent exit from the cell cycle associated with terminal differentiation.

The KIP/CIP family represents the second group of CDK inhibitor proteins. These proteins are typically induced upon cellular stress signals (e.g., after genotoxic damage) and function as biological brakes of the cell cycle. They bind to preformed cyclin-CDK complexes (preferentially to CDK1 and CDK2) and render them inactive by blocking their substrate access. The best characterized of these inhibitors is $p21^{CIP1}$ that acts as part of the DNA damage checkpoint. It is transcriptionally activated by p53 in response to DNA

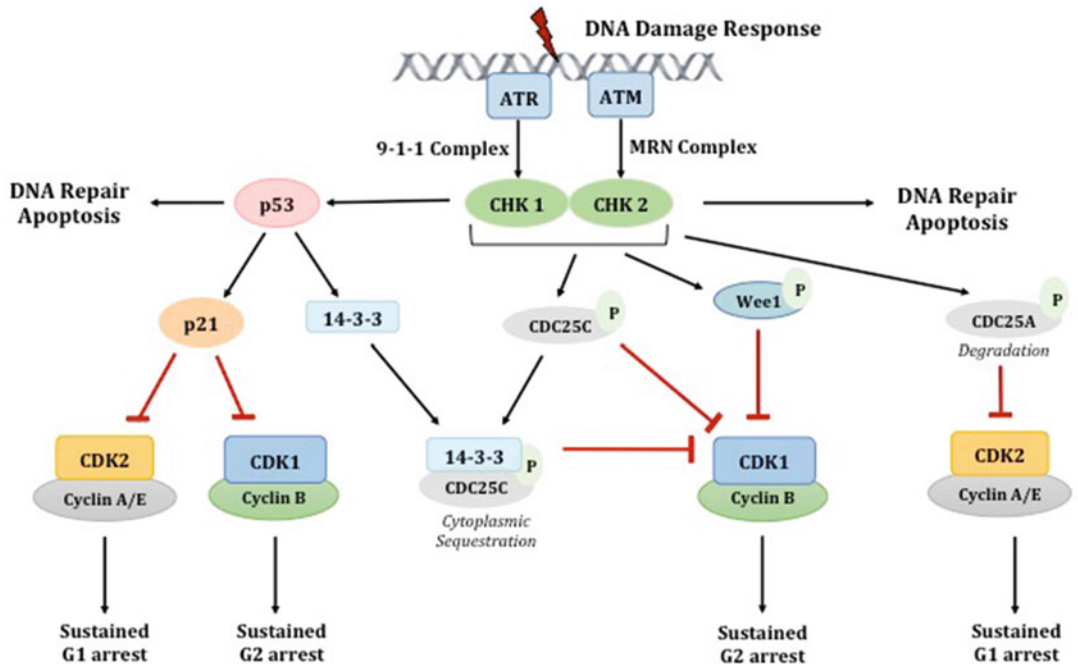
damage. Accumulated $p21^{CIP1}$ binds directly with CDK2-cyclin A/E and CDK1-cyclin A/B and inhibits their kinase activities, resulting in a cell cycle block at G1/S (where CDK2 is essential) and at the G2 to M transition (where CDK1 is essential). Upon cellular stress or genotoxic damage, the $p21^{CIP1}$-mediated cell cycle block provides a time window for, e.g., DNA repair, whereupon $p21^{CIP1}$ levels decline and cell cycle progression can resume. Another member of the KIP/CIP family of CDK inhibitors is $p27^{KIP1}$, which controls deregulated proliferation by inhibiting cyclin E-CDK2. More recently, it became clear that $p27^{KIP1}$ has also multiple non-canonical functions unrelated to its role in inhibiting CDKs during the cell cycle. Low levels of $p27^{KIP1}$ are caused by enhanced protein degradation and appear to be associated with tumorigenesis and poor prognosis. This is also underlined by studies in $p27^{KIP1}$ heterozygous mice showing a haplosufficiency for tumor suppression (Pack et al. 2019).

Cell Cycle Checkpoints

The healthy survival of cells depends on proper cell cycle progression and the ability to transmit the genetic information properly onto two daughter cells. Any errors in these processes are strongly associated with diseases, most notably with cancer. The faithful transmission of genetic information depends on proper DNA replication during the S phase, the absence of DNA damage throughout the cell cycle, and the equal distribution of sister chromatids during mitosis. The error-free progression of the cell cycle and faithful chromosome segregation are monitored at various transition phases by so-called cell cycle checkpoints.

DNA Damage and Replication Checkpoint

The DNA damage checkpoint pathway is among the best-understood checkpoint pathways (Fig. 3). The first step upon detection of DNA damage is the activation of either ATR or ATM kinases, depending on the nature of the damage. Generally, these kinases bind directly at the site of DNA



Cell Cycle Regulation, Fig. 3 The DNA damage checkpoint. In response to DNA damage cells activate p53-dependent and independent checkpoint pathways leading to cell cycle arrest at G1/S and G2/M allowing DNA repair. If the cellular damage cannot be repaired, cells induce apoptosis

damage and activate the effector kinases CHK1 and CHK2, which are responsible for halting the cell cycle in order to allow repair to occur. Typically, ATR is recruited to single-stranded DNA (ssDNA), which can be the result of single DNA strand breaks or of stalled replication forks during S phase. With this, ATR also acts as part of the so-called replication checkpoint. Upon occurrence of ssDNA, replication protein A (RPA) coats and stabilizes the single-strand site. This allows the recruitment of the ATR-interacting protein (ATRIP) to RPA, which is a prerequisite for the subsequent binding of ATR to the single-strands DNA site. The establishment of the ATRIP-ATR complex at the site of ssDNA causes the recruitment of RAD9-HUS1-RAD1 complex, also called the 9-1-1 complex, which stimulates ATR's kinase activity. One of the most important substrates of ATR is CHK1, whose phosphorylation also involves a protein called Claspin. Upon its activation, CHK1 becomes mobile and phosphorylates numerous substrates. One of the key substrates is the CDC25A phosphatase, which is inactivated (and in some cases degraded) upon CHK1-mediated phosphorylation, thereby preventing the activation of CDKs. This results in cell cycle block before or in S phase but also prevents entry into mitosis. In this way, the ATR-CHK1 axis also ensures that cells only enter mitosis when DNA replication is completed (Awasthi et al. 2015).

Upon detection of double-strand breaks (DSBs) of the DNA, the ATM kinase fulfills an important function as part of the DNA damage checkpoint. Activation of ATM at the site of damage requires the MRE11-RAD50-NBS1 (MRN-) complex localized at DSBs. ATM kinase activity is stimulated by the MRN complex, initiating a DNA damage response by phosphorylating the effector kinase CHK2. Similar to CHK1, activated CHK2 halts the cell cycle by phosphorylating and inactivating CDC25 phosphatases. Phosphorylation of CDC25C was shown to cause not only its catalytic inactivation but also its cytoplasmic sequestration mediated by binding to 14-3-3 sigma proteins. Overall, this prevents the activation of CDK2 and CDK1 at G1/S and G2/M transitions, respectively, leading to a stop of the cell cycle at these cell cycle transitions. In addition to targeting CDC25 phosphatases, both, ATM-CHK2 and ATR-CHK1, are able to phosphorylate and to activate the transcription factor p53. Activation of p53 induces the expression of a large number of target genes including *CDKN1A*, which encodes $p21^{CIP1}$, a CDK inhibitor protein. Accumulation of $p21^{CIP1}$ protein in response to DNA damage results in a sustained cell cycle arrest at both the G1/S and G2/M transition by directly inactivating CDK2 and CDK1 kinases, respectively. p53 also induces the transcription of DNA repair genes and genes responsible for the induction of apoptosis. The latter ensures that a damaged cell will rather die than transmitting damaged DNA onto newly formed daughter cells (Pack et al. 2019; Awasthi et al. 2015). Thus, p53 is a central regulator of the DNA damage response (DDR). Tumor cells frequently lack functional p53, which can directly contribute to tumorigenesis by promoting the accumulation of DNA damage and genomic instability.

Additionally, other upstream regulators of CDK1-cyclin B such as PLK1 or Aurora A seem to be also part of a DNA damage response. They also contribute to the cell cycle arrest at G2/M transition, independent of p53. Upon DNA damage, recruitment of Aurora A to the PLK1/Bora complex is inhibited to prevent PLK1 activation. This additional layer of regulation assures repression of pro-mitotic machinery in response to DNA damage (Joukov and De Nicolo 2018; Bruinsma et al. 2017). However, the detailed regulation of the PLK1-Aurora A axis in the DNA damage response is not completely understood.

Spindle Assembly Checkpoint

An important cell cycle checkpoint pathway that monitors the proper execution of mitosis is the spindle assembly checkpoint (SAC). The SAC is activated in the early phases of a normal unperturbed mitosis and monitors the proper alignment of chromosome. During a normal mitosis, progression into anaphase is inhibited until all chromosomes are bi-orientated and properly attached to the mitotic spindle. The SAC involves several proteins that are conserved from yeast to mammals; the core components include MAD1,

MAD2, BUB1, BUB3, BUBR1, and MPS1, which are specifically recruited to kinetochores only when kinetochores lack proper attachment to microtubules of the mitotic spindle. The latter is the case in the early phases of an unperturbed mitosis but also upon damage in mitosis (e.g., upon interference with microtubule function) that affects chromosome alignment. Binding of the SAC proteins at kinetochores is required to generate a diffusible mitotic checkpoint complex (MCC), which consists of MAD2, BUB3, and BUBR1. The MCC binds and inhibits a large ubiquitin ligase complex known as the anaphase-promoting complex or cyclosome (APC/C) in complex with its activator protein CDC20. The APC/C complex is involved in the degradation of various mitotic proteins, most notably securin and cyclin B. Securin is an inhibitor of separase, a protease that cleaves the cohesion ring complex in order to trigger sister chromatid separation at the metaphase to anaphase transition of mitosis (Peters 2006; Musacchio 2015). Thus, the APC/C is required for the onset of anaphase to allow the further progression of mitosis. Consequently, the blockage of APC/C activity by the SAC ensures that anaphase is initiated only upon complete chromosome alignment and, hence, prevents premature chromosome segregation, which is essential to maintain chromosomal stability. Since the APC/C also mediates the degradation of cyclin B leading to the inactivation of CDK1 at the end of mitosis, the SAC prevents premature exit from mitosis (Musacchio 2015).

Pharmacological Intervention to the Cell Cycle

Regulators of the cell cycle are frequently altered in human cancer often resulting in uncontrolled high CDK activities. A promising anti-cancer therapy is therefore the inhibition of CDKs using pharmacological CDK inhibitors that lead to a proliferative arrest of cells. Since prolonged cell cycle arrest results in the induction of apoptosis, the use of CDK inhibitors is a plausible strategy to eliminate tumor cells. In addition, other kinases or regulators of the cell cycle might be suitable targets as potential cancer therapeutics. Cell cycle checkpoints also represent potential targets for anti-cancer drugs. Cells damaged by genotoxic agents or by spindle poisons activate the appropriate checkpoint and halt the cell cycle until the damage is repaired. When these checkpoints are abrogated, cells progress through mitosis or the S phase prior to repair of the genotoxic damage, which promotes cell death. Hence, drugs abrogating cell cycle checkpoints were shown to sensitize cells to chemotherapy.

Pharmacological CDK Inhibitors

The rationale behind CDK inhibition during anti-cancer treatment is to stop hyperactive cell cycles and to inhibit the activity of cyclins that are frequently overexpressed in human cancer. Currently, various CDK inhibitors are evaluated in clinical trials. The first generation of CDK inhibitors included the broad inhibitor flavopiridol, which inhibits CDK1, CDK2, CDK4, CDK6, CDK8, and CDK9, leading to cell cycle arrest in G1 and G2 phases. Other investigated drugs are roscovitine, a purine-based CDK inhibitor, and paullone and indirubin, which are both selective and potent inhibitors of CDKs and GSK-3. With the aim to increase selectivity, specificity, and overall potency, a second generation of CDK inhibitors has been developed. Of these inhibitors, dinaciclib (MK-7965) has been extensively studied in the clinic. It was developed as a highly potent and specific inhibitor of CDK1, CDK2, CDK5, and CDK9. Other inhibitors include, olomoucine, a purine-based drug with high specificity for CDK1, CDK2, CDK5, and CDK7. Purvalanol B is more potent than olomoucine and selectively inhibits CDK1 and CDK2 inducing G2/M arrest. Palbociclib, a CDK4/6 inhibitor, is one of the first drugs to receive approval for first-line treatment of estrogen receptor-positive (ER+) breast cancer (Asghar et al. 2015). All these drugs arrest (tumor) cells in G1, S, or G2, and a prolonged cell cycle arrest is followed by the induction of apoptosis. While most of these CDK inhibitors have reasonable

toxicity profiles, specificity and selectivity remain an issue.

Pharmacological Cell Cycle Inhibitors

Many cell cycle regulatory kinases are overexpressed in human cancers and are therefore prime targets for chemotherapy to be targeted selectively in tumor cells. Several kinases that regulate mitotic progression are tested as anticancer drug targets. These include the Aurora and polo-like kinases (PLKs). First-generation Aurora kinase inhibitors showed off-target effects against other kinases, which led to high toxicity in cells. Second-generation Aurora kinase inhibitors were, however, highly specific and highly potent against rapidly dividing cancer cells. Hesperadin AT9283 and MLN8287 are examples targeting both Aurora kinase A and B. Another way to target mitotic progression of cancer cells is the inhibition of PLK1, which was shown to be frequently overexpressed in cancer cells and has been considered as a "druggable target." PLK1 inhibitors include rigosertib, volasertib, GSK461364, and B12536, all of which mainly targeting ATP-binding domain of PLK1. In addition to kinases, some non-kinase molecules acting in mitosis have been considered as suitable cancer therapeutic targets. For instance, the activity of mitotic kinesin Eg5 (KSP,kinesin 5) can be inhibited by small molecules (e.g., monastrol, KSP-IA) leading to the formation of monopolar mitotic spindles that prevent further progression of mitosis. The first small molecule inhibitor of KSP to be in clinical trials is ispinesib. These inhibitors usually result in mitotic defects associated with the induction of apoptosis (Otto and Sicinski 2017). However, it is currently unclear whether the efficacy of these new anti-mitotic drugs is superior over classical microtubule-targeting drugs like taxanes or *vinca* alkaloids.

Pharmacological Abrogation of Cell Cycle Checkpoints

The introduction of DNA damage leads to the activation of the DNA damage checkpoint resulting in G1 and G2 cell cycle arrest. Overriding this cell cycle block and inappropriate induction of S phase or mitosis, respectively, results in cell death in most cases. Thus, drugs that facilitate these transitions might sensitize cells to chemotherapy. Prime targets for checkpoint abrogating drugs are the ATM-CHK2 and ATR-CHK1 kinases. ATM can be inhibited by several methylxanthine derivatives (e.g., caffeine and pentoxifylline). The ATR inhibitor, NU6027, abrogates checkpoint signaling in both S and G2/M phase of the cell cycle. However, the cytotoxicity of these drugs limits their use in patients. In contrast, UCN-01, a staurosporine-derived drug, acts as a potent inhibitor of the CHK1 kinase and efficiently abrogates the G2 checkpoint upon DNA damage. The forced entry into mitosis in the presence of DNA damage results in apoptosis, which is also often referred to as mitotic catastrophe. Several clinical trials are now focusing on second-generation CHK1 inhibitors (e.g., SCH900776), which have shown promising results in the clinic. Recently, inhibitors targeting Wee1 kinase have also been considered as checkpoint abrogator. Wee1 kinase regulates G2/M transition by inhibiting CDK1 and halts cell cycle progression upon DNA damage. In order to exploit this property, the Wee1 inhibitor AZD1775 is being used in preclinical and clinical studies. AZD1775 is a pyrazolopyrimidine derivative that sensitizes (cancer) cells to chemotherapy and radiation therapy by disrupting the checkpoint at the G2/M transition (Otto and Sicinski 2017).

In contrast to the DNA damage checkpoint, the mitotic spindle assembly checkpoint is essential for cell viability. Therefore, targeting kinases of the spindle checkpoint might be a valid strategy for anti-cancer treatment. It is assumed that upon inhibition of the SAC, massive chromosome missegregation during mitosis is induced, which results in a lethal level of aneuploidy. On the other hand, drugs that target microtubules and the mitotic spindle directly are intensively used for many years in the clinic and are among the most effective drugs with a broad spectrum of activity in many cancer types. These drugs include microtubule stabilizers like taxanes (e.g., paclitaxel, docetaxel) and microtubule destabilizers like *vinca* alkaloids (e.g., vinblastine, vincristine), which affect the dynamic

properties of microtubules and the mitotic spindle leading to cell cycle arrest in mitosis and subsequent cell death (Otto and Sicinski 2017). Interestingly, a combination of anti-microtubule drugs and inhibitors of the spindle assembly checkpoint (e.g., MPS1/TTK inhibitors) were shown to show higher efficacy than either drug alone (Janssen et al. 2009) indicating that also combinations of different anti-cell cycle drugs might be beneficial for anti-cancer treatment.

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Cellular Senescence

▶ Senescence

Cephalosporins

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Synonyms

7-ACA derivatives; Beta-lactam antibiotics; Derivatives of the 7-aminocephalosporanic acid

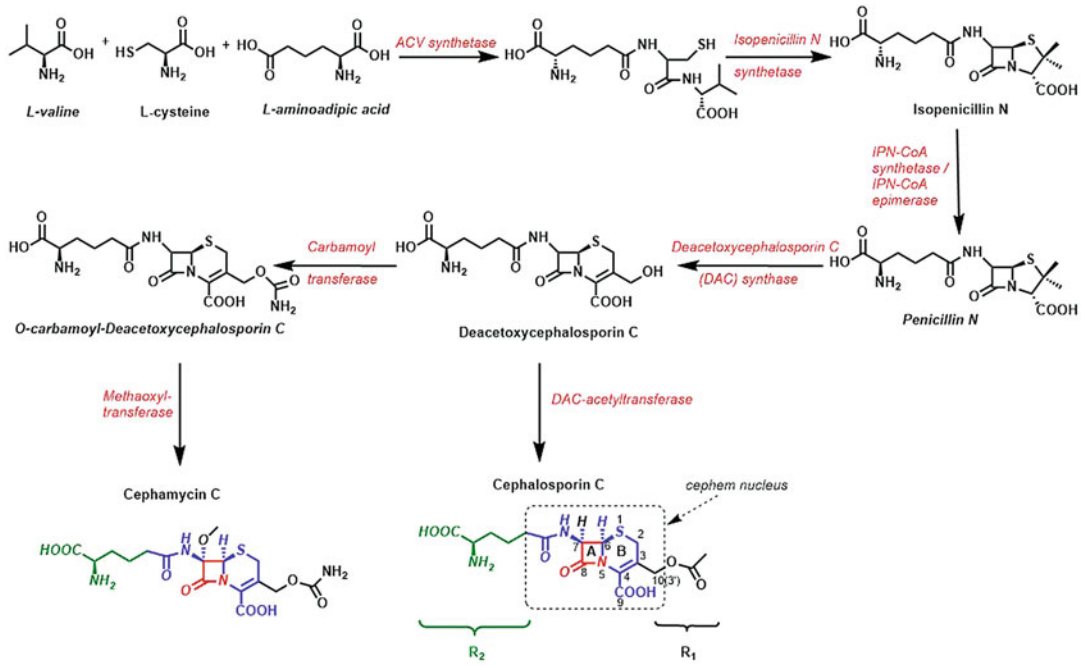
Definition

Cephalosporins are a group of β-lactam antibiotics similar to penicillin in their chemical structure and mechanism of action. Natural cephalosporins are produced as secondary metabolites by fungi of the genus *Acremonium* (formerly known as *Cephalosporium*) (Fig. 1).

The first evidence of their existence was obtained in 1945 by Giuseppe Brotzu, an Italian pharmacologist (Bo 2000), about 50 years after the first discovery of another "principle with bactericidal action" contained in molds, made by another Italian physician, Vincenzo Tiberio (Bucci and Galli 2011), and about 15 years after that such "principle" was serendipitously rediscovered and identified as the penicillin by the British Nobel laureate Alexander Fleming (1929).

Brotzu observed that crude filtrates of *Cephalosporium* cultures were able to inhibit the growth in vitro of several pathogens. It was only in 1953, however, that the founder of this new group of antibiotics, cephalosporin C (Fig. 1), was isolated from these crude extracts and its active nucleus, the 7-aminocephalosporanic acid (7-ACA), was chemically characterized (Abraham 1987).

Starting from this compound, many semisynthetic derivatives were obtained to modulate the



Cephalosporins, Fig. 1 Biosynthesis of cephalosporin C

spectrum of action, the bioavailability, the safety, and the pharmacological properties of this class of antibiotics.

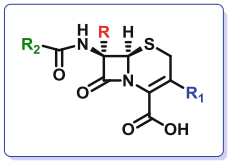
Nowadays, hundreds of cephalosporins are known, and they are usually classified into different "generations" according mainly to their spectrum of coverage against Gram-positive and Gram-negative bacteria, and against bacteria that have developed resistance. First-generation cephalosporins have a fairly good activity against Gram-positive bacteria (in particular *Staphylococci* spp. and *Streptococci* spp.) and are moderately active against few Gram-negative bacteria. Second- and third-generation cephalosporins increase progressively the antimicrobial coverage against Gram-negative bacteria; fourth- and fifth-generation cephalosporins show enhanced coverage against bacteria that have developed resistance towards the previous generations of cephalosporins (Table 1).

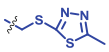
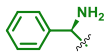
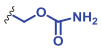
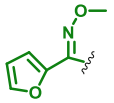
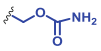
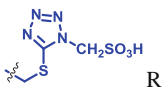
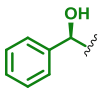
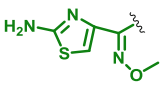
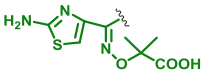
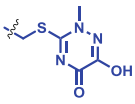
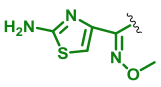
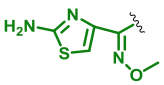
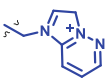
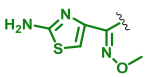
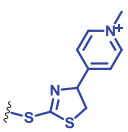
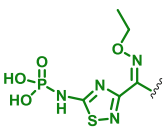
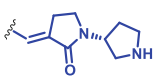
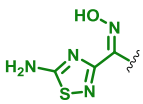
Chemistry

The basic chemical structure of cephalosporins contains a four-membered beta-lactam ring (A) fused to a six-membered dihydrothiazine ring (B). Other common structural characteristics include a carboxyl group on the six-membered ring on the carbon next to ring nitrogen and an amino group on the carbon of the four-membered ring opposite to nitrogen (Fig. 1).

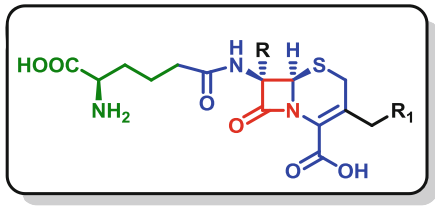
Regarding the chemical nomenclature, two different systems are generally reported in literature: (1) a systematic nomenclature (IUPAC) adopted by *Chemical Abstracts* where the fused system is named as 5-thia-1-azabicyclo[4.2.0]oct-2-ene; (2) a more simplified and common nomenclature with the fused ring system called cephem or Δ^3-cephem to indicate the position of the double bond, and the carbon atom bound to C-3 position numbered as C-10 or C-3′ (Fig. 1). The two nomenclature systems show a different structure numbering of the basic nucleus, even if the key C-7, C-6, and C-3 positions remain unchanged. The stereochemistry around the β-lactam ring of naturally occurring cephalosporins shows an absolute configuration *R* at C-7 and C-6 positions. For the substituents attached to the bicyclic system, the notation α/β is generally used depending on the orientation of the substituent below (α) or above (β) the imaginary plane of the

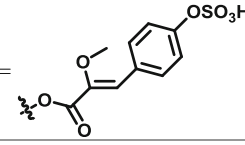
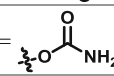
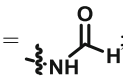
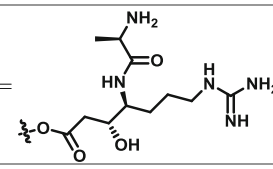
Cephalosporins, Table 1 Representative cephalosporins of different generations and variation of their spectrum of action (Bui and Preuss 2020). The general nucleus is shown at the top of the table



Generation	Representative compounds	Spectrum of action
First	Cefazolin: R= H R_1=  R_2= 	Gram-positives: +++ Gram-negatives: +
	Cephalotin: R= H R_1=  R_2= 	
	Cephalexin : R=H R_1= -CH_3 R_2= 	
Second	Cefuroxime: R= H R_1=  R_2= 	Gram-positives: ++ Gram-negatives: ++
	Cefoxitin: R= OCH_3 R_1=  R_2= 	
	Cefonicid R= H R_1=  R_2= 	
Third	Cefotaxime: R= H R_1=  R_2= 	Gram-positives: + Gram-negatives: +++
	Ceftadizime:R= H R_1=  R_2= 	
	Ceftriaxone: R= H R_1=  R_2= 	
Fourth	Cefepime: R= H R_1=  R_2= 	Gram-positives: ++ Gram-negatives: +++
	Cefzopran: R= H R_1=  R_2= 	
Fifth	Ceftaroline: R= H R_1=  R_2= 	Gram-positives: +++ Gram-negatives: +++
	Ceftobiprole: R= H R_1=  R_2= 	

Cephalosporins, Table 2 Representative chemical structures of selected naturally occurring compounds characterized by cephem nucleus. The general nucleus is shown at the top of the table



class	Representative compound	Structural characteristics
Cephalosporins	Cephalosporin C	R = H; R_1 = 
Cephamicins	Cephamycin A	R = OCH_3; R_1 = 
	Cephamycin C	R = OCH_3; R_1 = 
Cephabacins or Chitinovorins	Cephabacin F1 (Chitinovorin)	R =  ; R_1 = 

bicyclic system. Naturally occurring compounds containing a cephem nucleus are all characterized by the presence of a D-α-aminoadipic acid 7-acyl side chain on the cephem nucleus (Table 2) and, on the basis of further structural characteristics, they can be grouped in: (1) Cephalosporins with the aminoadipic side chain in C-7β, (2) cephamycins with an additional methoxy substituent at C-7α position, and (3) cephabacins or chitinovorins with a 7α-formamido group and different amino acids or oligopeptides at the C-3 position (Aharonowitz et al. 1992) (Table 2).

Most of compounds belonging to this class of β-lactam antibiotics are semisynthetic cephalosporins synthesized starting from 7-ACA or from 7-aminodeacetoxycephalosporanic acid (7-ADCA) (García-Estrada and Martín 2019). 7-ACA is derived from naturally occurring cephalosporin C by chemical or enzymatic removal of the amino adipoyl side chain at C-7 position. The chemical route is generally based on three steps: (1) protection of the amino and carboxylic groups, (2) treatment with phosphorous pentachloride, and (3) a hydrolysis reaction. The enzymatic approach has become an effective alternative to chemical one. There are two principal routes: (1) direct conversion of cephalosporin C to 7-ACA via an enzymolysis reaction by Cephalosporin C acylase, or (2) a no fully enzymatic procedure consisting in an oxidation reaction to keto acid by an amino acid oxidase (DAO), a nonenzymatic oxidative decarboxylation by H_2O_2 and a hydrolysis reaction by glutaryl acylase (GAC).

The other intermediate 7-ADCA is traditionally produced on an industrial scale from natural penicillin G by chemical expansion of the five-membered thiazolidine ring into the six-membered dihydrothiazine ring followed by enzymatic deacylation. However, over the years alternative processes have been developed in order to improve the cost-efficiency and environmental impact (García-Estrada and Martín 2019).

The cephem core offers many opportunities for chemical modifications at the two side chains in

C-7 and C-3 position, and it is possible to identify few structural features common to different cephalosporine generations. The first generation cephalosporins shows fairly simple structural modifications (Table 1). All compounds belonging to this generation have an α-amide group at C-7 position which makes them vulnerable to hydrolysis by β-lactamases. In some of them, there is a methyl at C-3 position, responsible for low affinity for common penicillin-binding proteins (PBPs) and consequently for their low level of antibacterial activity, or an aminobenzyl group at C-7 position, which is important for oral absorption (Neu 1986). The second generation cephalosporins are a heterogeneous group including also cephamycins, compounds characterized by the presence of a 7-alpha-methoxyl group on the cephem nucleus which confers more resistance to β-lactamases (Table 1). Their chemical structure is similar to the previous generation; an interesting modification is the introduction of a α-iminomethoxy group in the side chain at C-7 position, which enhances the stability of the β-lactam ring to β-lactamases.

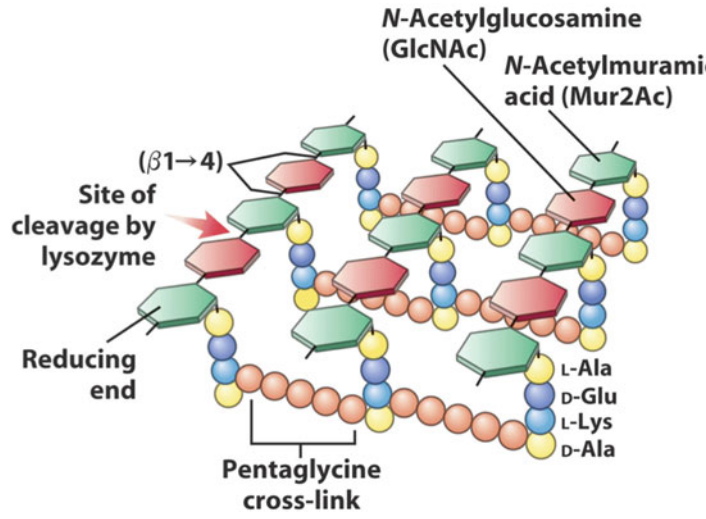
The third generation cephalosporins are generally characterized by the presence of an aminothiazoyl group and iminomethoxy group as substituents (Neu 1986) that improve the antibacterial activity against Gram-negative bacilli and the stability to β-lactamases (Table 1). Furthermore, the substitution at the C-3 position affects the half-life and the toxicity. For example, in ceftriaxone the heterocyclic moiety at the C-3 increases the plasma half-life, in ceftazidime the presence of a charged side chain in C-3 enhances penetration through the porins embedded in the outer membrane of Gram-negative bacteria. The fourth generation cephalosporins are structurally related to third ones but, in addition, they possess a quaternary ammonium group at the C-3 position, useful in facilitating the crossing of the Gram-negative bacterial membrane.

The fifth generation cephalosporins are not characterized by a general structure (Table 1); the molecules belonging to this class have been developed to specifically target against resistant strains of bacteria. For example, ceftobiprole and ceftaroline are the only β-lactam antibiotics that are effective against methicillin-resistant *Staphylococcus aureus* (MRSA) (Nisha et al. 2017). In ceftobiprole, the C-3 side chain is specifically planned to possess a strong binding affinity to PBP2a and PBP2x, and the aminothiazoylhydroxyimino side chain in C-7 to enable good resistance to β-lactamases from *S. aureus*. In ceftaroline, the alkoxyimino group at C-7 and a 2-thioazolythio spacer linkage in C-3 improve its stability to many β-lactamases and anti-MRSA activity, respectively. Furthermore, the introduction of a N-phosphonoamino group in ceftaroline fosamil acetate derivative allows to overcome its poor water solubility.

Mechanism of Action

Like penicillin, the target of cephalosporins are PBPs, a group of enzymes involved in the synthesis of peptidoglycan, an essential component of the bacterial cell wall (Fig. 2).

This structure allows bacteria to assume a well-defined shape and is of outmost importance for their survival, since it confers resistance towards the very high intracellular pressure. Peptidoglycan is a copolymer formed by linear glycan strands made by units of two different aminosugars, *N*-acetylglucosamine and *N*-acetylmuramic acid that are joint together by β-1 → 4 glycosidic links to form a carbohydrate backbone. Moreover, the *N*-acetylmuramic acid is linked to a pentapeptide made of different amino acid residues, the last two being usually D-Ala-D-Ala. This couple of unusual D-residues is essential for the formation of cross-links between two adjacent glycan strands, which confer mechanical resistance to the peptidoglycan. The most common type of cross-link is formed between the residue in position 3 of a pentapeptide of the first glycan strand and the D-Ala in position 4 of a pentapeptide of the flanking glycan strand, either directly or via an interpeptide bridge, with the loss of the final D-Ala (Vollmer et al. 2008). PBPs are involved mainly in the transpeptidation reaction that allows the formation of the cross-linking between the glycan strands, but they can also catalyze the transglycosylation reaction that allows the polymerization of the glycan



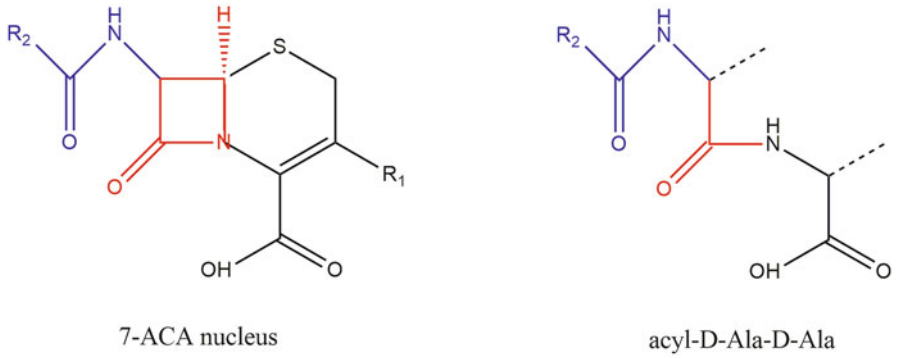
Cephalosporins, Fig. 2 Structure of peptidoglycan (Reproduced by permission by Creative Proteomics)

strand. Additionally, some PBPs can catalyze the hydrolysis of the last D-Ala of the pentapeptide, or the hydrolysis of the peptide bond connecting the two glycan strands (the inverse reaction of transpeptidation). The activity of these enzymes is higher when bacteria actively replicates, since the process of peptidoglycan synthesis is more relevant during these phases. However, the bacteria continuously remodel peptidoglycan during their life, therefore PBPs are active components of the fine balance between peptidoglycan synthesis and degradation. Thus, these enzymes represent an ideal pharmacological target, both because they are involved in a crucial process for the survival of the bacteria, and because there is no homologous enzyme in humans that could in turn be impaired by this class of antibiotics (Sauvage et al. 2008).

The key of the mechanism of action of cephalosporins (as well as of all β-lactam antibiotics) is the structural resemblance of the β-lactam ring with the natural target of PBPs, the D-Ala-D-Ala dipeptide involved in the transpeptidation reaction (Fig. 3).

In the presence of these antibiotics, PBPs are misled and identify them as the substrates for the transpeptidation reaction. The four-member ring binds covalently to the nucleophilic residue (usually, a Ser) in the active site of these enzymes and forms an acyl-enzyme that is unable to synthesize the cross-links between two glycan chains. Additionally, there are evidences, especially in cocci, that these antibiotics induce the release of endogenous substances that enhance the spontaneous process of peptidoglycan hydrolysis. This causes a weakening of the newly synthesized cell wall. Therefore, bacteria are no longer able to resist to the osmotic stress caused by the high pressure inside the cell and this results in their lysis (Murray and Moellering 1981).

Unfortunately, bacteria have evolved several mechanisms to neutralize the activity of β-lactam antibiotics, thus becoming resistant to these compounds. The main mechanism of resistance is the action of β-lactamases, a family of hydrolytic enzymes able to disrupt the β-lactam ring, thus inactivating these molecules (King et al. 2016). The first β-lactamase (penicillinase) was discovered soon after the introduction of penicillin in the clinics (Abraham and Chain 1940). In this regard, the discovery of cephalosporins was highly welcomed, because the 7-ACA nucleus was less susceptible of hydrolysis by penicillinase with respect to the β-lactam ring of penicillins (Abraham 1987). Regrettably, many β-lactamases progressively evolved by selective pressure, especially in Gram-negative bacteria, and were able to hydrolyze the β-lactam ring of cephalosporins, too. Therefore, the development of further generations of cephalosporins after the discovery of the founder molecules was made both to broaden the spectrum of action of these compounds, and to develop molecules resistant to β-lactamase hydrolysis.



Cephalosporins, Fig. 3 Structural resemblance of the 7-ACA nucleus with the D-Ala-D-Ala dipeptide

Other mechanisms of bacterial resistance include the down-regulation of membrane channels, such as porins, to limit the permeability of the cell wall and decrease the drug entry, or the active expulsion of the drug through efflux pumps. Moreover, several bacteria modified their PBPs with mutations in order to render them less susceptible to the irreversible acylation by the β-lactam ring (King et al. 2016). This is the case of the MRSA, identified in the early 1960s. Its resistance is caused by a specific PBP, PBP2a, whose expression by gene *mecA* is triggered by the presence of β-lactams. The kinetic comparison of the properties of PBP2a with respect to other β-lactam sensitive PBPs reveals that this enzyme is characterized by a slow acylation rate, because the active site cleft of this protein is distorted, causing a misalignment of the substrate with the catalytic Ser residue (King et al. 2016). Out of the five generations of cephalosporin, only the fifth generation has coverage against MRSA (Bui and Preuss 2020). In particular, ceftobiprole is a fifth-generation cephalosporin with in vitro activity towards a broad range of Gram-negative pathogens, including MRSA and other methicillino-resistant bacteria (King et al. 2016). Crystallographic studies showed that ceftobiprole irreversibly acylates the catalytic Ser403 of PBP2a with more favorable kinetic parameters, because this compound can perfectly fit into the narrow active site pocket of PBP2a forming interactions with the residues of the cavity. This facilitates the rearrangement required by the acylation reaction (King et al. 2016) (Fig. 4).

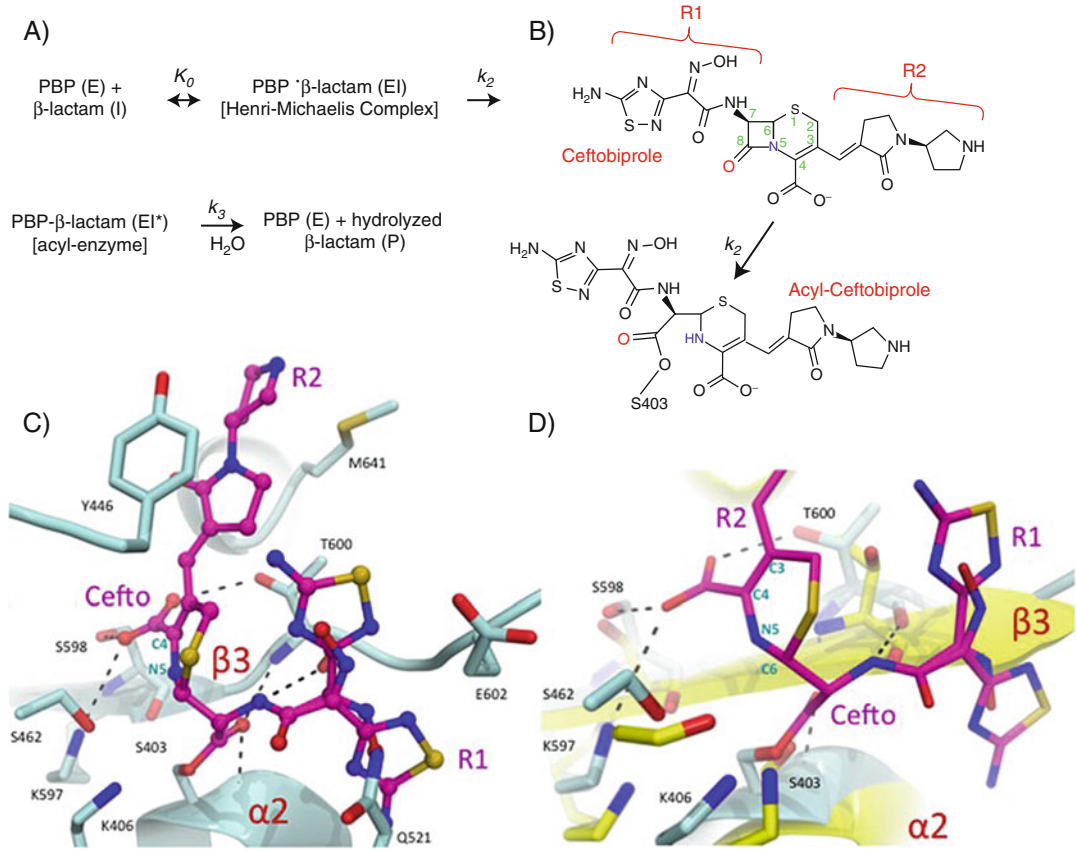
Another fifth-generation cephalosporin, ceftaroline, has up to 256-fold higher affinity for PBP2a than other cephalosporins (King et al. 2016). The mechanism of action of this compound seems different from that of ceftobiprole, since it involves the allosteric regulation of PBP2a in addition to the binding of this antibiotic in the active site of the enzyme (King et al. 2016) (Fig. 5).

Clinical Use (Including Side Effects)

All cephalosporins are bactericidal agents and are used to treat infections made by different bacteria sensitive to these antibiotics. Given their potent activity, general safety, low toxicity, and small cost, they are still very popular antibiotics, despite the development of bacterial resistance.

First-generation cephalosporins such as cefazolin and cephalexin are commonly used to treat infections of skin and soft tissue and of the upper respiratory tract caused by Gram-positive cocci such as *Staphylococci* spp., *S. pneumoniae, S. viridans*, and by Gram-negative bacteria sensitive to these compounds, such as *P. mirabilis, E. coli, K. pneumoniae.* Other indications include infections of bone, genitourinary tract, biliary tract, otitis, and surgical prophylaxis. Another possible indication is endocarditis prophylaxis for susceptible individuals (Deck and Winston 2012).

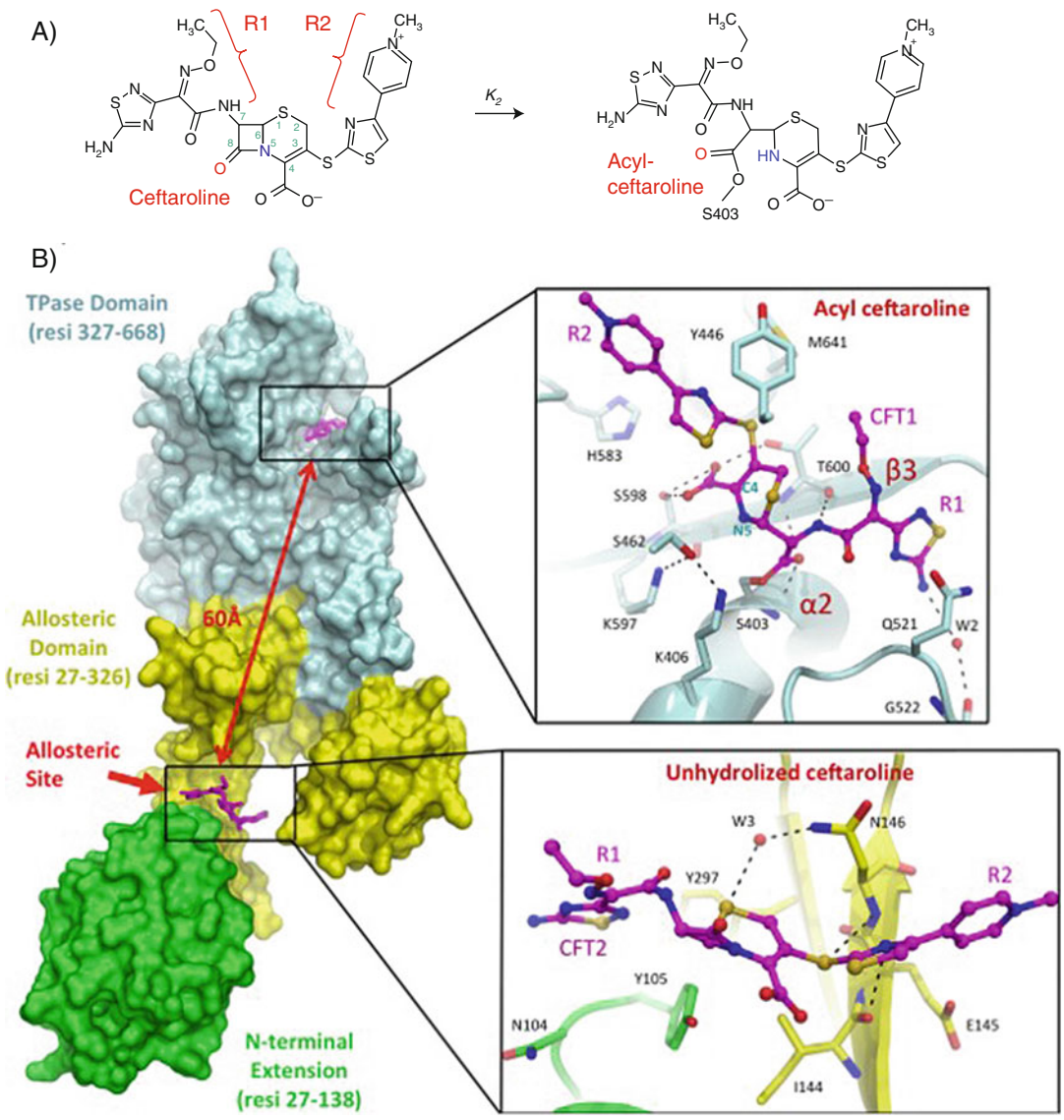
Second-generation cephalosporins show an increased activity towards Gram-negative bacteria such as *H. influenzae* and *Bacteroides* spp., but less activity against Gram-positive cocci, compared to the first generation compounds. Cefuroxime can be used to treat Lyme disease in



Cephalosporins, Fig. 4 Inhibition of the *S. aureus* PBP2a by Ceftobiprole. (**a**) Kinetic model for PBP inhibition by β-lactams. (**b**) General model for SBL mediated ceftobiprole acylation. (**c**) Active site close-up of acyl-ceftobiprole bound *S. aureus* PBP2a (PDB ID: 4DKI). The acyl-enzyme protein chain is displayed as a teal cartoon with key active site residues shown in stick representation with atoms colored by type. (**d**) Active site overlay of unbound (PDB ID: 1VQQ) and ceftobiprole bound *S. aureus* PBP2a. The bound and unbound protein backbones are shown as teal and yellow cartoons with key active site residues illustrated in stick representation with non-carbon atoms colored according to atom type. The bound ceftobiprole is shown in pink sticks with atoms colored by type and hydrogen-bonding interactions are depicted as black dashes. (Reprinted with permission by Wiley from King et al. (2016))

pregnant women, and can be used in combination with probenecid (a drug that increases uric acid excretion and can decrease the concentration of cephalosporins in urines) to treat noncomplicated gonorrhea. Because of its ability to cross the blood-brain barrier and to diffuse into cerebrospinal fluid, cefuroxime is used to treat meningitis in children. Cefoxitin is used as a preventive agent before pelvic or colorectal surgery, and can be administered to treat gonococcal infections caused by *N. gonorrhoeae* strains producing penicillinase. Cefonicid is the second-generation cephalosporin with the longest half-life and can be used as a single-dose administration to treat infections such as community-acquired pneumonia (Deck and Winston 2012).

Third-generation cephalosporins have an extended Gram-negative coverage and are often used to treat Gram-negative infections that show resistance to other β-lactam antibiotics. Ceftriaxone, one of the most popular third-generation cephalosporins, is able cross the blood-brain barrier and is a drug of choice to treat meningitis caused by *H. influenzae, N. meningitidis, and S. pneumoniae*, as well as to prevent the pharyngeal transmission of *N. meningitis*. It is also very active against *N. gonorrhoeae* and is frequently used for monotherapy against infections of the lower



Cephalosporins, Fig. 5 Inhibition of the *S. aureus* PBP2a by ceftaroline. (**a**) General model for ceftaroline mediated PBP acylation. (**b**) Structural details of ceftaroline mediated PBP2a inhibition. On the left, the overall ceftaroline bound PBP2a protein structure (PDB ID: 3ZG0) is shown in surface representation with the N-terminal extension, allosteric domain, and TPase domain colored green, yellow, and cyan. On the right side are close-up views of the ceftaroline bound allosteric and TPase domains with the protein chain depicted in cartoon representation and key residues shown as sticks with non-carbon atoms colored by atom type. Ceftaroline is shown as pink sticks. (Reprinted with permission by Wiley from King et al. (2016))

respiratory tract, skin, bone and serious urinary infections. Its long half-life allows a daily administration, making this drug excellent for outpatient antibiotic therapy. Ceftazidime is another third-generation cephalosporin that shows activity against *P. aeruginosa*, a Gram-negative bacterium often associated with serious infections, such as hospital-acquired pneumonia, sepsis, and meningitis. This drug has an excellent ability to cross the blood-brain barrier, therefore it is also employed to treat meningitis caused by other Gram-negative species (Deck and Winston 2012).

Fourth-generation cephalosporin cefepime is an anti-Pseudomonal agent, active also against

ceftadizime-resistant strains. Its quaternary ammonium group allows it to penetrate the outer membrane of Gram-negative bacteria. Moreover, this drug is active against Gram-negative bacilli producing β-lactamase (but not MRSA). In order to reduce the spreading of resistance against this compound, it is reserved for systemic infections caused by multi-resistant bacteria (Deck and Winston 2012).

Finally, fifth-generation cephalosporins include the two compounds, ceftaroline and ceftobiprole, active against MRSA in addition to the broad spectrum of action against Gram-positive and Gram-negative bacteria typical of fourth-generation cephalosporins. Ceftaroline was originally approved for the treatment of community-acquired pneumonia and acute bacterial skin infections, but it has proved effective to treat infections caused by MRSA, with rapid bloodstream eradication even in patients non-responding to other therapies. Given the dramatic spreading of resistance (also due to the senseless and indiscriminate use of antibiotics outside the clinical environment), ceftaroline is considered a salvage treatment against MRSA and it should not be used as a first-line therapy for other pathologies (Zhanel et al. 2009). Ceftobiprole is another fifth-generation cephalosporin active against MRSA, *P. aeruginosa* and resistant strains of *S. pneumoniae*. It is administered intravenously as a prodrug, but it is almost immediately converted to the active form. It is well tolerated, safe, and effective, and in contrast to other cephalosporins, it presents a low risk of superinfection by *C. difficilis* leading to pseudomembranous colitis (Barbour et al. 2009).

Cephalosporins belonging to the first generation are mainly administered parenterally, but the following manipulation of the side chains of the 7-ACA allowed to produce semisynthetic derivatives in which pharmacokinetic properties could be modulated. Therefore, from the second generation, many cephalosporins can be administered orally, reaching adequate levels in the whole body. However, fourth- and fifth-generation cephalosporins must be administer parenterally. Cephalosporin derivatives are able to reach many different tissues, including the pleural, pericardial and synovial fluids. They can cross the placenta and be excreted in breast milk. As described before, several cephalosporins such as ceftriaxone, ceftadizime, cefotaxime, and cefuroxime can cross the blood-brain barrier, even in the presence of infected meninges.

Usually, these compounds do not undergo metabolic modification and are excreted unmodified in the urines, but some cephalosporins with an acetyl group in R′ position (Fig. 2) loose this acetyl group before being excreted. The half-life of the parenterally available compounds is often short, but some compounds such as cefuroxime, cefonicid, cefazolin, ceftazidime, and ceftriaxone have a longer half-life. Since the main route of excretion is renal, people with impaired renal function are at higher risk of accumulating cephalosporins and should reduce the dose.

Cephalosporins are generally well tolerated, being gastrointestinal manifestations, such as nausea, vomiting, and abdominal pain, the most common adverse effects especially after oral administration. Like penicillin, cephalosporins can induce a hypersensitivity reaction in about 2% of people, with skin rash, fever, hives, and swelling, but rarely this leads to anaphylaxis. First- and second-generation cephalosporins are more likely to induce hypersensitivity reactions, and to show cross-reactivity with penicillin. Some evidences of a potential increase in nephrotoxicity caused by cephaloridine and cephalotin, both alone and in combination with other classes of antibiotics such as aminoglycosides, were reported in literature in the past, but these reports have been questioned (Murray and Moellering 1981). Although few other cephalosporins (e.g., cefazolin and cefamandole) can produce nephrotoxic effects in animals, they are less likely to increase the risk of nephrotoxic events in humans. Ceftriaxone can frequently cause cholecystitis because of the precipitation of the calcium salt in the gallbladder; therefore, it must not be administered together with calcium additives or calcium-containing solutions. It can also displace bilirubin from albumin, increasing the risk of jaundice in newborns. Ceftriaxone and cefotetan are also associated to the development of drug-induced immune hemolytic anemia, caused by IgG against these drugs, which bind to the red blood cells that carry the drugs on their cell membrane, provoking hemolysis (Bui and Preuss 2020).

In conclusion, despite nearly 75 years of use, cephalosporins appear to be still a valid weapon against bacteria. Nevertheless, clinicians must keep the guard up and researchers must pursue the development of new generations of innovative representatives of this class of compounds, to be always ready to the fight against the threat of resistance against β-lactam antibiotics.

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C

Cerebral Microvessels

► Blood-Brain Barrier

CGRP Receptor

► Calcitonin Family Receptors

Chaperones

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Synonyms

Heat shock proteins (**Hsp**); Molecular chaperones (note: not all molecular chaperones are stress proteins); Polypeptide chain-binding proteins; Stress proteins (note: not all stress proteins are molecular chaperones, heat shock is one form of cellular stress, and glucose deprivation is another one)

Definition

Chaperones bind to exposed hydrophobic surfaces of polypeptide substrates and, through either ATP-dependent or ATP-independent mechanism, facilitate the folding/assembly, intracellular transport, and activity of polypeptides.

Basic Mechanisms

Molecular Chaperones

The term *molecular chaperone* was coined by Ron A. Laskey and coworkers in 1978 to describe the properties of the nuclear protein nucleoplasmin in assisting the in vitro assembly of nucleosomes from isolated histones and DNA (Laskey et al. 1978). Laskey introduced the term *molecular chaperone* to describe the function of nucleoplasmin because of the analogy with the human chaperone. The role of a human chaperone was to prevent incorrect interactions between pairs of human beings, without either providing a blueprint (or steric information) necessary for their correct interaction or the necessity to be present during their continued (functional) interaction. This concept was extended by Hugh R. B. Pelham in 1986 (Pelham 1986). Pelham proposed that members of the Hsp70 and Hsp90 protein families are involved in the assembly and disassembly of proteins in the cytosol, nucleus, and **endoplasmic reticulum** under non-stress conditions but are required in increased amounts during cellular stress – such as heat shock – conditions, both to unscramble protein aggregates and to prevent aggregation by binding to hydrophobic surfaces that are exposed as a result of the stress. In addition, Pelham proposed that all cells contain a variety of proteins that act as molecular chaperones, in preventing incorrect interactions during the operation of several basic cellular processes under normal growth conditions. These cellular processes are protein folding plus assembly and protein topogenesis in the course of protein biogenesis. More recently, it was appreciated that certain molecular chaperones can also act as modulators/regulators of the activity of folded proteins. Furthermore, these proteins have also been shown to facilitate protein degradation, thereby making them important integration points or sites of "triage" of the cellular pathways of protein folding, assembly, topogenesis, and degradation. Therefore, at a broad cellular level, molecular chaperones are some of the key quality control components of the cell.

Today, molecular chaperones are divided into three principle classes, according to their mode of action (Table 1). The first class comprises the chaperonins or Hsp60 protein family members. Chaperonins are oligomeric proteins, composed of two rings placed back to back, each one enclosing a cavity that allows protein folding in a secluded environment. The term "Anfinsen cage" was coined by R. John Ellis in 1994 to illustrate this mechanistic principle (Ellis 1994). They are divided in two groups, those found in eubacteria or endosymbiotic organelles and those found in archaea and the eukaryotic cytosol. The second class includes all the other Hsp protein families (sHsp, Hsp70, Hsp90) and their respective co-chaperones (such as Hsp40 and nucleotide exchange factors, NEF). These chaperones have highly diverse three-dimensional structures but share as a mechanistic principle that they aid protein folding plus assembly and protein transport by cycles of binding and release of exposed hydrophobic surfaces of polypeptides that, otherwise, would be prone to aggregation. This mode of action is described in detail below in the case of Hsp70. The third class, termed non-Hsp, represents polypeptide-binding proteins that appear to be more specialized for certain classes of substrate polypeptides, such as nascent polypeptides (NAC, nascent polypeptide-associated complex; SRP, signal recognition particle; TRC40, transmembrane recognition complex 40) or glycoproteins (calnexin, calreticulin) or even single-substrate proteins (nucleoplasmin, Hsp47, NDUFAF1, B172L).

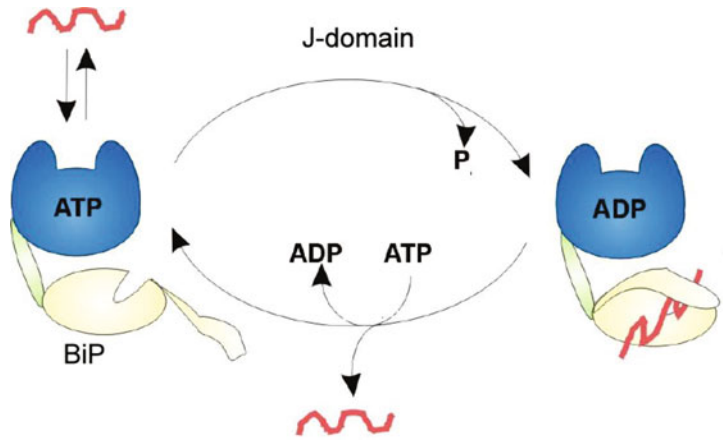
The Hsp 70 is the most widely distributed chaperone system in the human cell (Fig. 1). The major molecular chaperone, Hsp70, consists of an N-terminal nucleotide-binding and ATPase domain (NBD) and a C-terminal substrate-binding domain (SBD). The affinity of Hsp70 for protein client is modulated by ATP binding and hydrolysis. In the ATP-bound state, affinity of the substrate-binding domain for the client protein is low, and exchange rates are high. Hydrolysis of ATP to ADP results in high affinity for the substrate and low exchange rates, effectively locking the substrate into the binding pocket. This integral step in assisted protein folding is directly modulated by the binding of Hsp40 proteins. Hsp40s are defined by the presence of an approximately

Chaperones, Table 1 Molecular chaperones in humans. Synonyms are given in parentheses. For a more comprehensive list, see SnapShot: Molecular Chaperones, Part 1 (https://doi.org/10.1016/j.cell.2007.01.001)

Protein type	Cytosol and nucleus	Endoplasmic reticulum	Mitochondria
sHsps	Hsp27	–	–
	α-Crystallin	–	–
Hsp60	TRIC (CCT)	–	Cpn60 (mtHsp60)
Co-chaperones	Prefoldin	–	Cpn10 (mtHsp10)
Hsp70	Hsc70 (HSPA8)	Grp78 (BiP, HSPA5)	Grp75 (mtHsp75)
	Hsp70.1-3	Grp170	
	Hsp70L1		
Hsp40	Hdj1 (Hsp40)	ERj1 (DNAJC1)	mtHsp40
	Hdj2	ERj2 (Sec63)	Tim14
	Auxilin	ERj3 (DNAJB11)	Tim16
	P58IPK	ERj4 (DNAJB9)	Tim44
	MPP11	ERj5 (DNAJC10)	
	CSP	ERj6 (DNAJC3)	
	Hsj1	ERj7 (DNAJC25)	
		ERj8 (DNAJC16)	
NEF	Hsp110	Grp170 (HYOU1)	mtGrpE
	Hspbp1	Sil1 (BAP)	
Co-chaperones	Hip		
	Hop		
	CHIP		
	Bag1 through 5		
	TPR1		
	Tom70		
Hsp90	Hsp90	Grp94	–
Non-Hsp	NAC	Hsp47	
	Nucleoplasmin	Calreticulin	
	SRP	Calnexin	
	TRC40 (Get3)		

70-amino acid region known as the J-domain, which is essential for interaction with Hsp70. The J-domain is a highly conserved α-helical structure that interacts with the Hsp70 ATPase domain and possibly also with the Hsp70 substrate-binding domain. Hsp40s are divided into three groups based on their possession of domains in addition to the J-domain. Type I Hsp40s contain four domains: an N-terminal J-domain, a glycine/phenylalanine (GF)-rich region, a zinc finger domain, and a C-terminal domain. Type I Hsp40s have been shown to bind protein substrates at their C-terminal domain and to have independent chaperone activity by inhibiting denaturation and aggregation. Type II Hsp40s contain an N-terminal J-domain, a GF-rich region, and a C-terminal domain. Type III Hsp40s contain the J-domain, and this may occur at any position within the protein. Other than the J-domain, the type III Hsp40s are highly divergent in size, sequence, and structure and tend to serve highly specialized functions.

The various roles of a certain Hsp70 shall be described with respect to glucose-regulated protein 78 (Grp78, also called immunoglobulin heavy-chain-binding protein or BiP) (Fig. 2). BiP is the Hsp70 protein family member resident in the endoplasmic reticulum (ER). It is involved (i) in the import of polypeptides into the ER, working both as an allosteric modulator of the folded polypeptide-conducting Sec61 channel and as a molecular ratchet on the polypeptide in



Chaperones, Fig. 1 The functional cycle of Hsp70

transit through the channel, (ii) in the efficient gating of the open Sec61 channel to the closed state, (iii) in folding and assembly of proteins in the ER, (iv) in the export of misfolded polypeptides from the ER to the cytosol for degradation by the proteasome (ER-associated degradation, **ERAD**), and (v) and in the so-called unfolded protein response (**UPR**).

RNA Chaperones

As it is the case in polypeptide folding, non-specific or promiscuous RNA-binding proteins can prevent RNA misfolding and resolve misfolded RNAs, thereby ensuring that RNA is accessible for its biological function (Herschlag 1995). Certain DEAD-box proteins as well as some proteins that are involved in the assembly of ribonucleoparticles were shown to act as RNA chaperones.

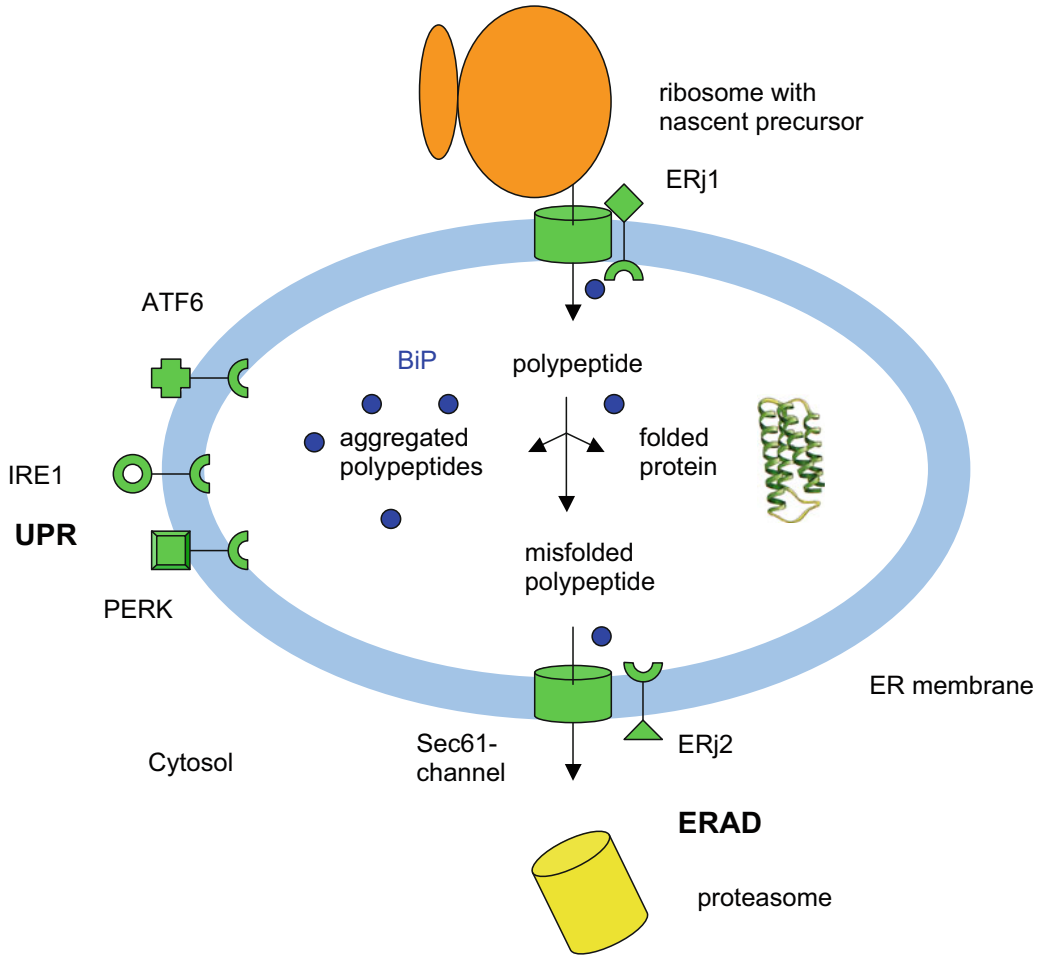
Chaperone Diseases

There are a number of human diseases associated with mutations in genes encoding chaperones, and the functional failure of certain molecular chaperones has been linked to human diseases (Macario and Conway de Macario 2005). In addition, disease-linked mutations have been found in proteins that regulate the activity of Hsp70 (i.e., co-chaperones and NEF). To name just a few examples and in staying with the Hsp70 chaperone network in the ER (Fig. 2), Marinesco-Sjögren syndrome (MSS; OMIM 248800), polycystic kidney disease (PKD; OMIM 173900), diabetes mellitus (OMIM 616192), and polycystic liver disease (PCLD; OMIM 174050) have been linked to Sil1, ERj3, ERj6, and ERj2 (Sec63), a nucleotide exchange factor, and various Hsp40s, respectively, for BiP (Dudek et al. 2009). Furthermore, overexpression of various molecular chaperones as well as mutations in certain chaperone genes have been observed in a wide range of tumors (Dudek et al. 2009). In some cases, the chaperone overexpression was linked to poor prognosis and/or resistance to therapy. More recent work linked a human infectious disease to inactivation of the ER-resident chaperone BiP (Dudek et al. 2009). The AB_5 subtilase cytotoxin of certain pathogenic bacteria causes morbidity and mortality among children in developing countries by inactivating BiP (i.e., hemolytic uremic syndrome, HUS).

Pharmacological Intervention

Chemical Chaperones

A number of different low molecular weight compounds are known to stabilize proteins in their native conformation and, therefore, may be effective in correcting protein folding abnormalities in vivo. Relevant compounds are N-acetyl-L-lysine, L-carnitine, L-proline, betaine, ectoine, and hydroxy-ectoine (Welch and Brown 1996). Some of these **chemical chaperones** are already in clinical use to combat protein folding diseases,



Chaperones, Fig. 2 The multiple roles of BiP in the biogenesis of the secretory pathway. *BiP* immunoglobulin heavy-chain-binding protein, *ER* endoplasmic reticulum, *ERAD* ER-associated degradation, *ERj* resident ER protein with J-domain, *Sec61* core subunit of the protein translocase, *UPR* unfolded protein response, which involves several signal transduction pathways that are activated in order to increase the biosynthetic capacity and decrease the biosynthetic burden of the ER

such as cystic fibrosis (i.e., the combinations of Lumacaftor® plus Ivacaftor® and Tezacaftor® plus Ivacaftor®).

Drugs That Target Molecular Chaperones

Several small compounds have been discovered that alter expression or function of Hsp70 and Hsp90, respectively (Brodsky and Chiosis 2006). The proteasome inhibitor bortezomib (Velcade®) leads to accumulation of misfolded proteins and, therefore, stress response and subsequent induction of stress proteins, such as Hsp70; the natural compound celastrol and geranylgeranylacetone (GGA) were also shown to induce a stress response. Thus, these compounds may also be used to combat protein folding diseases. Furthermore, small molecules with structural similarity to 15-deoxyspergualin or NSC 630668.R/1 were described to inhibit the ATPase activity of Hsp70. Thus, these compounds may be able to combat certain cancers. Last but not least, based on the discovery of the Hsp70 inhibitors, screening for positive modulators is pursued. The established anticancer drug geldanamycin and its less toxic analogue 17-AAG, on the other hand, target Hsp90.

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ChE

▶ Cholinesterases

ChE I

▶ Cholinesterases

ChE II

▶ Cholinesterases

Checkpoint Inhibitors (CPI)

▶ Immune Checkpoint Blockade

Chemical Biology

▶ Small-Molecule Screens

Chemical Probes

▶ High-Throughput Screening (HTS) Technology

Chemoattractant Receptors

▶ Chemokine Receptors

Chemokine Receptors

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Synonyms

Chemoattractant receptors

Definition

G-protein-coupled receptors (GPCRs) play a major role in regulating the overall homeostasis of complex organisms, like mammals, but are also found in primitive species like *Dictyostelium* (slime mold) and yeast (Fredriksson and Schioth 2005). The GPCR superfamily is quite diverse, and sequencing has revealed more than 850 genes comprising around 3% of the human genome. The diversity of the GPCRs is equally matched by the variety of ligands that activate them, which include odorants, taste ligands, light, metals, biogenic amines, fatty acids, amino acids, peptides, proteins, nucleotides, lipids, Krebs cycle intermediates, and steroids. Because of their central role

in regulating normal physiological responses, GPCRs have attracted considerable attention from the pharmaceutical industry as targets for disease. This large superfamily of proteins remains one of the most druggable targets accounting for more than 40% of all marketed therapeutics. Chemokine receptors are members of the GPCR superfamily, and seven CXC, ten CC, and one CX3C and XC chemokine receptors have been cloned so far (Pease and Horuk 2012). Receptor binding initiates a cascade of intracellular events mediated by the receptor-associated heterotrimeric G-proteins. These G-protein subunits trigger various effector enzymes that lead to the activation not only of chemotaxis but also to a wide range of functions in different leukocytes such as an increase in respiratory burst, degranulation, phagocytosis, and lipid mediator synthesis.

Basic Characteristics

Chemokines belong to a large family of small, chemotactic cytokines characterized by a distinctive pattern of four conserved cysteine residues (Baggiolini 1998). They are divided into two major (CXC and CC) and two minor (C and CX3C) groups dependent on the number and spacing of the first two conserved cysteine residues. Although originally identified on the basis of their ability to regulate the trafficking of immune cells, the biological role of chemokines goes well beyond this simple description of their function as chemoattractants; they have been shown to be involved in a number of biological processes, including growth regulation, hematopoiesis, embryologic development, angiogenesis, and HIV-1 infection (Fig. 1).

Chemokines have been shown to be associated with a number of autoinflammatory diseases including multiple sclerosis, rheumatoid arthritis, atherosclerosis, dermatitis, and organ transplant rejection. Evidence, reviewed below, is mounting that chemokines may play a major role in the pathophysiology of these diseases, and thus chemokine receptor antagonists could prove to be useful therapeutics in treating these and other proinflammatory diseases.

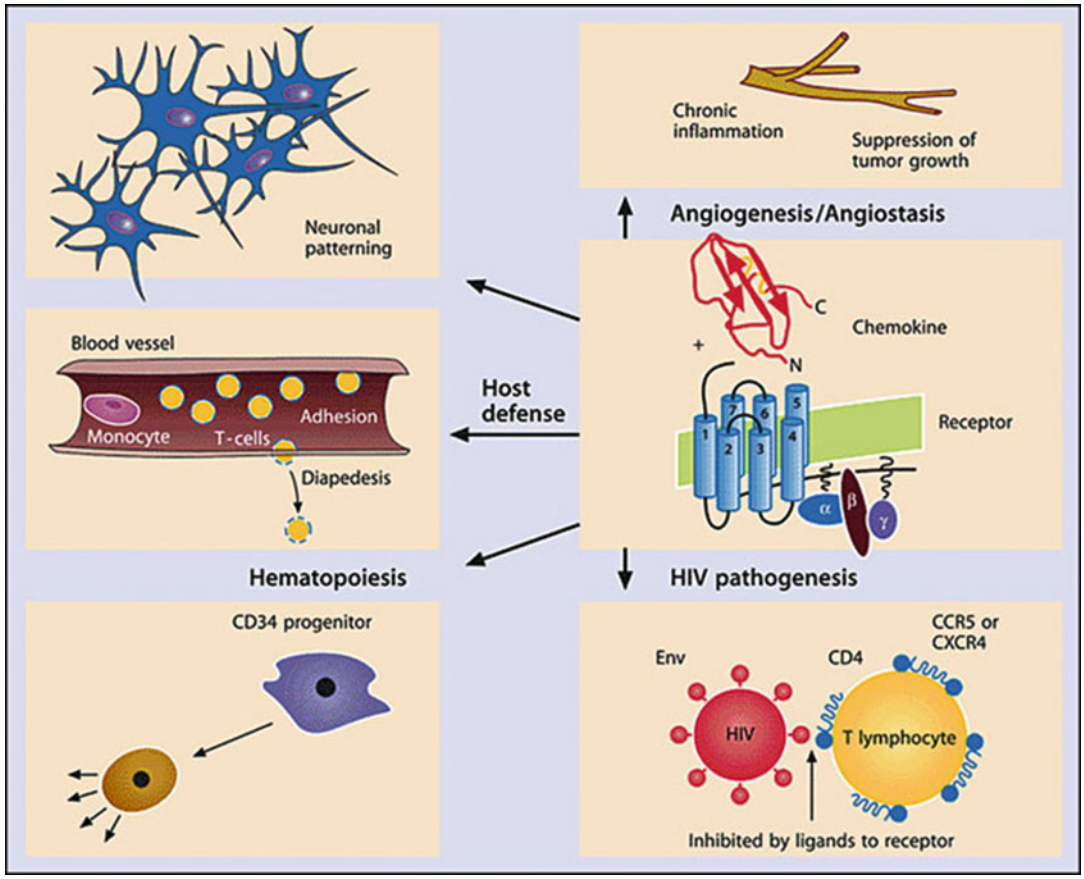
Chemokine Receptors

Although leukocytes continue to be the major site of expression of chemokine receptors, several studies have recently demonstrated chemokine receptor expression on neurons in the CNS.

A number of chemokine receptors including CXCR2, CXCR4, CCR1, CCR5, and DARC have been demonstrated in either adult or fetal brain. Not only were these receptors present on the cell surface but they were also functional. Clearly, the role of these receptors on CNS neurons must be very different from their role on immune cells. Given that human astrocytes can be stimulated with cytokines to upregulate the expression of chemokines, it is tempting to speculate that in vivo during CNS development, chemokines secreted by astrocytes might engage specific receptors expressed on neurons and may play a role in the directed migration of specific subsets of neurons to distinctive regions of the brain.

Genetic mutations of receptors, both natural and induced (by targeted gene disruption), can help to unravel their biological roles. Nature has been generous in this regard by providing us with two naturally occurring examples of gene inactivation for chemokine receptors. Humans homozygous for inherited inactivating mutations of the Duffy (*DARC*) gene and the *CCR5* gene have been identified and appear to be phenotypically normal and healthy. Indeed, these gene inactivations appear to be beneficial to their hosts, rendering them resistant to certain infectious diseases. For example, DARC-negative individuals are resistant to malaria induced by *Plasmodium vivax*, which utilizes DARC to attach to and enter erythrocytes. CCR5-negative individuals are resistant to HIV-1, which utilizes this chemokine receptor as a coreceptor for invasion (see section role of receptors in HIV infection).

The role of chemokine receptors on disease has been clarified not only by genetic mutations but also by receptor antagonists such as small molecules and neutralizing antibodies. For example, the pathophysiological role of CXCR4 has recently been expanded by the finding that it appears to be highly expressed in a number of human breast cancer cells, malignant breast



Chemokine Receptors, Fig. 1 Biological functions of chemokine receptors as exemplified by the chemokine receptor CXCR4

tumors, and metastases. This expression is complemented by the fact that the expression of the CXCR4 ligand CXCL12 is the highest in the lymph node, lung, liver, and bone marrow, distributions that exactly correspond to regions where one would expect to find breast cancer metastasis appearing. These findings point to an important role for CXCR4 in oncology. Data demonstrating that breast cancer cells signaling through CXCR4 can mediate activities such as actin polymerization and pseudopodia formation, functions that would support the ability of these cells to migrate and become invasive, further supports this. In support of these studies, a neutralizing antibody to CXCR4 was used in an animal model of carcinogenesis and was able to significantly impair the metastasis of breast cancer cells to regional lymph nodes and lung. These studies demonstrate that the chemoattractant properties of the CXCR4/CXCL12 interaction, which are so important during morphogenesis in helping to keep cells together to form blood vessels, can also have a darker side in helping to propagate and promote tumor growth. Finally, they highlight another therapeutic opportunity in which CXCR4 small molecule antagonists could be useful.

Role in Immune Response

Chemokines are potent chemoattractants that provide directional cues to summon leukocytes. Leukocyte recruitment is a three-step process that involves the formation of solid-phase chemokine gradients generated by the binding of chemokines to extracellular matrix proteins like

glycosaminoglycans, which decorate the cell surface of endothelial cells. These gradients then attract immune cells that first undergo selectin-mediated rolling along the endothelial cells. Chemokine-mediated upregulation of CD11/18 complexes then results in a much firmer adherence of immune cells to the endothelium, and this culminates in diapedesis of leukocytes across the endothelial space into tissues.

By regulating the movement of different subsets of leukocytes from the peripheral blood to extravascular sites such as organs, skin, or connective tissue, chemokines play a critical role in the maintenance of host defense as well as in the development of immune response. However, sometimes these molecules can inappropriately target immune cells to attack their own tissues and organs leading to inflammation and cellular destruction. Indeed, strong evidence supports the idea that chemokines play an important role in the pathogenesis of a number of autoimmune diseases such as multiple sclerosis and rheumatoid arthritis.

Rheumatoid arthritis is a chronic inflammatory disease characterized in part by a memory T lymphocyte and monocyte infiltrate. Recent studies using neutralizing antibodies have provided strong in vivo concept validation for the role of chemokines in animal models of rheumatoid arthritis. For example, a neutralizing monoclonal antibody against rat CCL2 was efficacious in a collagen-induced arthritis model. In addition, the CCR2 ligands CCL2 and CCL5 are elevated in the joints of patients with rheumatoid arthritis which promotes the recruitment of monocytes and T cells into synovial tissues. Furthermore CCR2 is strongly expressed on monocytes/macrophages in patients with rheumatoid arthritis compared to control patients. These and other studies strongly suggested that CCR2 plays an important role in monocyte recruitment and in Th1-type inflammatory diseases such as rheumatoid arthritis. Based on these findings, a number of companies have embarked on CCR2 antagonist programs, and some of these are still in active human clinical trials (see Table 1).

In contrast to the proinflammatory role of chemokine receptors discussed above, some appear to play a key role in noninflammatory settings. For example, CCR3 has been proposed as a target for asthma given its potential role in activating eosinophils, but a huge number of clinical trials targeting CCR3 in this indication have all failed. In contrast CCR3 may play a key role in a non-allergic setting, namely, choroidal neovascularization, and has been proposed as a target for "wet" age-related macular degeneration (AMD), a leading cause of blindness in the elderly. In "wet" AMD, abnormal development of blood vessels within the choroid (choroidal neovascularization or CNV) results in infiltration of the retina and potential for retinal detachment. Choroidal expression of CCR3 has been observed in both human tissue and also in animal models of AMD, which correlates with CNV. In line with these observations, blockade of CCR3 appears to be beneficial in some animal models of AMD and is being pursued as a drug target in this disease with recent positive phase II clinical trials (Table 1).

Based on the demonstrated role of chemokines in disease, the generation of small molecule chemokine receptor antagonists has received great interest from pharmaceutical companies as attractive therapeutic approaches. GPCRs like chemokine receptors have in the past been an extremely fertile source of biological targets in the pharmaceutical industry, and compound library screening has proven successful in the discovery of antagonists for a number of these receptors, i.e., CCK and neurotensin antagonists. Using similar approaches several drug companies have now identified potent small molecule antagonists of a number of chemokine receptors, which should find broad utility in a variety of acute and chronic inflammatory diseases.

Drugs

Insight into the physiological and pathophysiological roles of chemokine receptors have been provided by studies with potent antagonists for a number of receptors (Pease and Horuk 2012). However, despite multi-million dollar efforts by many academic groups and pharmaceutical

Chemokine Receptors, Table 1 Chemokine receptor antagonists in clinical trials. (Text in red indicates terminated programs, those in orange ongoing programs, and those in green registered drugs)

Receptor	Company	Compound	Affinity (nM)	Indication	Clinical phase	Status
CCR1	Schering AG (Berlex)	BX 471	1	MS, Psoriasis endometriosis	II	No efficacy
CCR1	Millennium	MLN 3701		MS, MM	II	No longer reported
CCR1	Millennium	MLN 3897	2.3	RA, MM	II	No efficacy in RA
CCR1	Pfizer	CP-481,715	64	RA	II	No efficacy
CCR1	BMS	BMS-817399	1	RA	II	No efficacy in RA
CCR1	BI	BI-639667	5.4	?	I	Terminated
CCR1	GSK	CCX354	1.5	RA, MM	II	Ongoing
CCR2	CCX	CCX915		MS	I	Terminated
CCR2	AstraZeneca	AZD2423	2.6	Diabetic neuropathy	II	No efficacy
CCR2	Merck	MK-0812	5	RA, MS	II	No efficacy
CCR2	Incyte	INCB8696		MS, lupus	I	No longer reported
CCR2	Incyte	INCB3284	3.7	RA, type II diabetes	II	No longer reported
CCR2	Pfizer/Incyte	PF-4136309/ INCB8761	5.2	Oncology	I	No longer reported
CCR2	J & J	JNJ-17166864	20	Allergic rhinitis	II	No efficacy
CCR2	J & J	JNJ-41443532		Type II diabetes	II	Terminated
CCR2	Millennium	MLN 1202* Plozalizumab		RA and MS Atherosclerosis	II II	Discontinued Ongoing
CCR2	CCX in collaboration with Vifor	CCX140	2.3	Focal segmental glomerular sclerosis	II	Ongoing
CCR2	CCX	CCX872	3	Pancreatic cancer	Ib	Ongoing
CCR2	BMS	BMS-741672	2.7	Diabetic neuropathy	II	Ongoing
CCR3	Pharmaxis	ASM8**		Asthma	II	No longer reported
CCR3	GSK	GSK766994	10	Asthma and allergic rhinitis	II	Terminated no efficacy
CCR3	GSK	GSK766904		Asthma	II	No longer reported
CCR3	GSK	GW824575		Asthma	I	Terminated
CCR3	Dupont	DPC168	2	Asthma	I	Terminated
CCR3	BMS	BMS-639623	0.3	Asthma	I	No longer reported
CCR3	Novartis	QAP-642		Allergic rhinitis	I	Terminated no efficacy
CCR3	AstraZeneca	AZD3778	8.1	Allergic rhinitis	II	Terminated no efficacy
CCR3	Axikin	AXP1275		Asthma	II	No efficacy
CCR3	Alkahest	AKST4290		AMD	II	Ongoing
CCR4	GSK	GSK2239633	10	Asthma	I	Terminated
CCR4	RAPT therpt.	FLX475		Cancer	I/II	Ongoing
CCR4	BMS	BMS-936564*		MM	I	Ongoing
CCR4	**Amgen**	**Mogamulizumab* (KW-0761)**		**T cell lymphoma**	**Approved**	**Registered drug**
CCR5	Pfizer	PF-232798	2	AIDS	II	No longer reported
CCR5	SP	SCH-C	2	AIDS RA	I II	Terminated No efficacy
CCR5	SP	Vicriviroc/SCH-D	0.45	AIDS	III	Terminated
CCR5	GSK	Aplaviroc GW2239633	3	AIDS	III	Terminated
CCR5	Incyte	INCB9471	3.1	AIDS	II	Terminated

(continued)

Chemokine Receptors, Table 1 (continued)

CCR5	AstraZeneca	AZD5672	0.26	RA	II	No efficacy
CCR5	HGS	HGS004*		AIDS	I	No longer reported
CCR5	Sangamo	SB-728***		AIDS	II	Ongoing
CCR5	CytoDyn (Progenics)	Leronlimab[a] (Pro 140		AIDS GvHD	III II	Ongoing
CCR5	Tobira	TBR652/ Cenicriviroc	3.1	AIDS Fatty liver	II III	Ongoing
CCR5	**Pfizer**	**Maraviroc (UK-427,857)**	**3**	RA **AIDS**	II **Approved**	No efficacy **Registered drug**
CCR5/ CCR2	Pfizer	PF-04634817		Diabetic nephropathy	II	No efficacy
CCR5/ CCR2	Novartis	NIBR-6465	1 (CCR2) 0.8 (CCR5)	AIDS	I	No longer reported
CCR5/ CCR2	BMS	BMS-813160	6.2 (CCR2) 3.6 (CCR5)	Diabetes pancreatic cancer	II Ib/II	Discontinued Ongoing
CCR9	ChemoCentryx	Vercirnon CCX282	6	IBD, Crohn's	III	Failed to meet clinical endpoint
CCR9	ChemoCentryx	CCX507		IBD, Crohn's	I	Ongoing
CXCR1/ CXCR2	Dompé	Reparixin	1 (CXCR1) 100(CXCR2)	Breast cancer	II	Ongoing
CXCR1/ CXCR2	Dompé	Ladarixin		Type 1 diabetes	II	Ongoing
CXCR2	GSK	Elubrixin SB-656933	5.1	COPD, cystic fibrosis	I	No efficacy
CXCR2	GSK	Danirixin GSK-1325756	12.5nM	COPD	I	No efficacy
CXCR2	AstraZeneca	AZD8309		COPD	I	Terminated
CXCR2	AstraZeneca	AZD5069	0.8nM	Bronchiectasis Breast cancer	II II	Terminated Ongoing
CXCR2	Merck	Navarixin	3.9 0.049	COPD Cancer	II II	No efficacy Ongoing
CXCR3	Amgen	AMG487	8	Psoriasis	II	No efficacy
CXCR4	TaiGen	Burixafor		SCM	II	Terminated
CXCR4	Ablynx	ALX-0651[d]		SCM	I	Terminated
CXCR4	BioLineRx	BL8040[c]		SCM/pancreatic cancer	II/III	Ongoing
CXCR4	BMS	Ulocuplumab[a] BMS-936564	2nM	MM	II	Ongoing
CXCR4	Lilly	LY2510924[e]	0.08nM	Oncology	II	Ongoing
CXCR4	Polyphor	Balixafortide POL6326	10nM	SCM Breast cancer	II	Ongoing
CXCR4	**Genzyme/ Aventis**	**Plerixafor (AMD3100)**	**74**	**SCM, MM, NHL**	**Approved**	**Registered drug**
CX3CR1	Ablynx/BI	BI665088		CKD	I	Ongoing

Abbreviations: BI, Boehringer Ingelheim; BMS, Bristol-Myers Squibb; CCX, ChemoCentryx; SP, Schering-Plough; COPD, chronic obstructive pulmonary disease; GSK, GlaxoSmithKline; IBD, inflammatory bowel disease; MM, multiple myeloma; MS, multiple sclerosis; RA, rheumatoid arthritis; SCM, stem cell mobilization; NHL, non-Hodgkin's lymphoma; GvHD, graft-versus-host disease

[a]Neutralizing monoclonal antibodies

[b]Antisense oligonucleotide

[c]Zinc finger nuclease

[d]Nanobodies (antibody fragments)

[e]Peptide

companies, only three chemokine receptor antagonists have been licensed for clinical use: Pfizer's small molecule CCR5 inhibitor, Maraviroc; Genzyme/Aventis small molecule CXCR4 inhibitor, Plerixafor; and Amgen's CCR4 neutralizing antibody, Mogamulizumab (Pease and Horuk

2012) (Table 1). All three antagonists show efficacy in the noninflammatory setting: inhibition of HIV-1 entry (Maraviroc), stem cell mobilization (Plerixafor), and treatment of T cell lymphomas (Mogamulizumab). In contrast, the inflammatory setting has been a graveyard for chemokine receptor drug discovery efforts, with multiple antagonists failing in a range of disorders, including multiple sclerosis, rheumatoid arthritis, asthma, and psoriasis. The reasons for such failures are likely to be many and varied. Recent studies have questioned whether some antagonists achieved sufficient target coverage in vivo, with the suggestion that over 90% of the target needs to be blocked to achieve efficacy in vivo. Alternatively, the target selection may not have been appropriate for treatment of the disorder; for example, despite numerous animal studies demonstrating that CCR3 plays a role in eosinophil recruitment and activation, blockade of this receptor in human clinical trials of asthma and allergy has failed miserably (Table 1) demonstrating that this receptor might not be a major driver for the human disease. Alternatively, given the potential redundancy of the chemokine/chemokine receptor system, it may be necessary to block more than one receptor in certain inflammatory settings (Pease and Horuk 2010). For example, the failures of human clinical trials targeting CCR3 in asthma may have failed because eosinophils can also be activated by CCL3 which is a CCR1 ligand. Thus targeting CCR1 and CCR3 with a dual antagonist might show benefit in asthma.

Chemokine Receptors in Pathogen Infection

A number of viruses, including those in the herpes and pox families, express chemokine-like or chemokine receptor-like molecules that presumably help them to survive immune attack and proliferate. In addition to these strategies, chemoattractant receptors have themselves been targeted as vehicles of cellular invasion by a wide variety of microbes. These range from the Duffy blood group antigen, a promiscuous chemokine receptor on human erythrocytes which serves as a binding protein for the malarial parasite *Plasmodium vivax*, to the fractalkine receptor, CX3CR1, which is a portal of entry for the respiratory syncytial virus, and the HIV-1 virus which utilizes the chemokine receptors CXCR4 and CCR5 as coreceptors for cellular entry. CCR5 is an entry cofactor for M-tropic isolates of HIV-1 and is important in the early proliferative part of the disease, while CXCR4 is a coreceptor for T-tropic isolates of HIV-1 whose emergence in infected individuals usually correlates with accelerated disease progression.

HIV-1 resistance exhibited by some exposed but uninfected individuals is due, in part, to a 32-base pair deletion in the *CCR5* gene (CCR5D32) which results in a truncated protein that is not expressed on the cell surface. About 1% of Caucasians are homozygous for the CCR5D32 allele and appear to be healthy with no untoward signs of disease. In fact, recent findings suggest that homozygosity for the CCR5D32 alleles confers other selective advantages to these individuals, rendering them less susceptible to rheumatoid arthritis and asthma and prolonging survival of transplanted solid organs.

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Chemokines

▶ Cytokines

Chloride Channels

▶ Cl^- Channels and Cl^-/H^+ Exchangers

Cholesterol Oxidation Products

► Oxysterols

Cholesteryl Ester Transfer Protein Inhibitors

► Lipid-Lowering Drugs

Cholinesterases

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Synonyms

Acetylcholine acetylhydrolase; Acetylcholine hydrolase; Acetylcholinesterase (EC 3.1.1.7) (AChE); Acetylthiocholinesterase; Acetyl-β-methylcholinesterase; Acylcholine acylhydrolase; Benzoylcholinesterase; Butyrylcholinesterase (EC 3.1.1.8) (BChE or BuChE): ChE; ChE; ChE I; ChE II; Erythrocyte ChE; Nonspecific ChE; Plasma ChE; Propionylcholinesterase; Pseudocholinesterase; True ChE

Definition

Cholinesterases (ChEs), polymorphic carboxylesterases of broad substrate specificity, terminate neurotransmission at cholinergic synapses and neuromuscular junctions (NMJs). Being sensitive to inhibition by organophosphate (OP) poisons, ChEs belong to the serine hydrolases (B type). ChEs share 65% amino acid sequence homology and have similar molecular forms and active center structures (Soreq and Seidman 2001). Substrate and inhibitor specificities classify ChEs into two subtypes:

1. Acetylcholinesterase (EC 3.1.1.7) (AChE)
   Primarily hydrolyses esters with short acyl moiety, such as acetylcholine (ACh). It is the major ChE in human red blood cells, muscle cells, and brain cells. In the mammalian brain, AChE mRNA is 20-fold more abundant than BChE mRNA.
2. Butyrylcholinesterase (EC 3.1.1.8) (BChE or BuChE)
   Primarily hydrolyses esters with longer aliphatic (compared to AChE) or aromatic acyl moiety, such as butyrylcholine (BCh) and benzoylcholine (BzCh). BChE is the primary circulating ChE. It is threefold more abundant than AChE in human blood and is found in the liver, lungs, muscles, brain, and heart.

Basic Characteristics

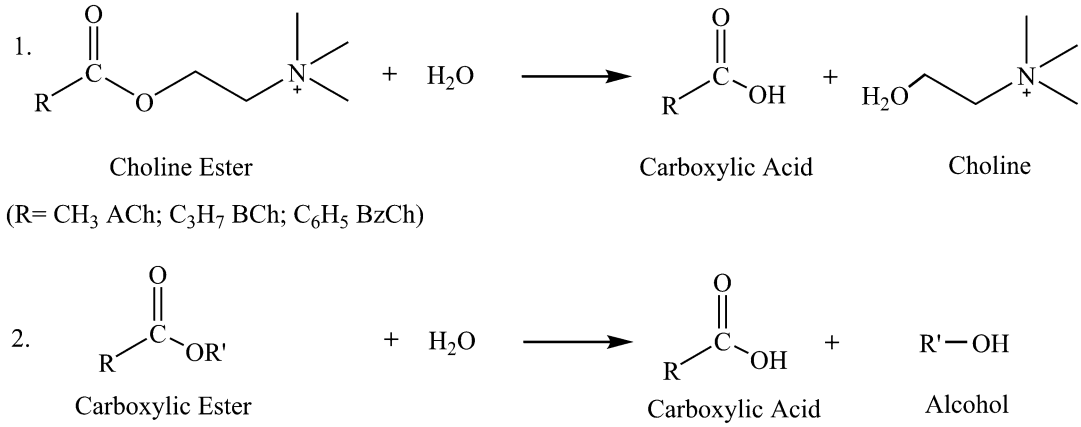
Cellular Processes

ChEs control the duration of ACh-mediated action on postsynaptic receptors in cholinergic synapses and have non-hydrolytic roles in nervous systems' development and plasticity.

Catalytic Activity

ChEs hydrolyze choline esters and carboxylic esters (1, 2, respectively, in Fig. 1).

Hydrolysis involves a Glu-His-Ser catalytic triad, similar to other serine hydrolases, through a "charge relay" process. The imidazole ring of His can relay electrons from Glu to Ser, and the hydroxyl oxygen of Ser becomes a nucleophile and attacks the ester bond of the substrate, yielding an acyl-enzyme intermediate releasing a choline moiety. A water molecule then hydrolyzes the acetylated Ser by nucleophilic attack. Carbamates and OPs chemically modify the active site Ser, slowing down the last hydrolysis step and irreversibly inhibiting hydrolysis.



Cholinesterases, Fig. 1 Hydrolysis of choline and carboxylic esters by cholinesterases

Cholinergic neurotransmission: ChEs terminate cholinergic transmission in the central nervous system (CNS), in NMJs, and in the autonomic system (the parasympathetic system, somatic motor nerves, and preganglionic sympathetic nerves) as well as in circulating blood cells expressing cholinergic proteins. A few sensory cells and the NMJ in nematodes also include ChEs.

Physiological roles: Both enzymes can catalyze ACh hydrolysis into acetic acid and choline, thus maintaining proper transmission of impulses between nerve cells or from neurons to muscles and gland or immune cells. The catalytic mechanism of AChE is extremely efficient, approaching diffusion-controlled rates (kcat/Km = 1.47×10^8 $M^{-1} \cdot s^{-1}$), considerably faster than BChE's hydrolysis rate of ACh. BChE deficiency, an uncommon genetic disorder, causes no pathology but increases carriers' susceptibility to prolonged apnea under muscle relaxant agents, such as succinylcholine or mivacurium, which are naturally destroyed by BChE. When administered under anesthesia to a susceptible person, these compounds are ineffectively removed, which prolongs muscle relaxation. Likewise, subjects with BChE deficiency are susceptible for adverse reactions to other anti-ChEs including commonly used pesticides.

Both ChEs show aryl-acylamidase activity, but BChE has a wider range of substrates; it hydrolyzes several choline esters from acetyl to heptanoylcholine, as well as other aliphatic esters. High BChE activity in many first contact tissues (e.g., lungs, skin, blood, and placenta) as well as high affinity toward a wide range of orally ingested toxic compounds and xenobiotics (e.g., cocaine, procainamide, acetylsalicylic acid) supports the hypothesis that BChE functions as a natural bio-scavenger by absorbing and degrading organophosphate (OP) poisons (e.g., nerve agents) before these lead to neurological damage.

Non-catalytic Activity

ChEs exist throughout the entire animal kingdom, and they also appear in locations where no ACh is released, suggesting that they possess additional non-hydrolytic functional properties, designated "nonclassical." These may depend on protein-protein interactions, compatible with ChE's structural homology with synaptic proteins such as neuroligins, which interact with other synaptic proteins – the neurexins. Also, AChE contains two helix-loop-helix motifs, each forming a calcium-binding site (EF-hand). These do not play a role in its catalytic activity but may participate in protein-protein interactions which are affected in mutated BChE variants.

Within the nervous system, ChEs are involved in membrane conductance and transmission of excitatory amino acids, learning and memory, neurite growth, neuritic translocation,

apoptosome formation, and acute stress reactions (Soreq 2015).

Reports of altered ChE levels in tumors, e.g., meningioma, glioma, acoustic neurinomas and lung cancers, megakaryocytopoietic disorders and leukemias, ovarian tumors, and neuroblastomas, suggest cell proliferation and differentiation activities. Inversely, altered BChE levels in neurodegenerative diseases may reflect systemic cholinergic impairments.

Structure

ChEs possess the α-/β-fold structure, which is shared with other esterases and non-catalytic proteins such as thyroglobulin, glutactin, neurotactin, gliotactin, and neuroligins, all of which include a single ChE domain. Both ChEs are ellipsoidal molecules of ~45·60·65 $Å^3$. Their structure consists of a central, highly twisted, 8–12-stranded β-sheet, in which most strands are parallel, flanked on both sides by α-helices. Studies have indicated three major domains within the protein: the active site, the aromatic gorge, and the peripheral active site.

The active site is composed of two subsites:

The esteratic subsite contains the catalytic machinery of the enzyme. The catalytic triad residues – Ser 200, His 440, and Glu 327 (the residue numbering in this section refers to *Torpedo californica acetylcholinesterase*, *Tc*AChE) – are identical in both enzymes and are basically located in the same positions.

The anionic subsite (Trp 84 and Phe 330) lies between the peripheral and acylation sites halfway down the gorge and accommodates the positively charged quaternary ammonium of the choline moiety. Trp 84 orients the charged part of the substrate to the active center. This subsite is involved in a "cross-talk" mechanism with the peripheral anionic site (PAS) (Silman and Sussman 2005).

The aromatic gorge composed of 14 highly conserved aromatic residues is placed ~20 Å deep, penetrating halfway into the gorge, harboring the active site. In BChE, 6 of the 14 aromatic amino acid residues are replaced by aliphatic ones. Therefore, BChE's gorge is larger in volume and shows a distinct inhibitor sensitivity profile. Other crucial residue changes exist in the acyl-binding pocket, with the replacements of Phe 288 and Phe 290 of *Tc*AChE by Leu 286 and Val 288, respectively; these changes enable the binding of the bulkier butyrate substrate moiety in BChE.

The PAS is located at the rim of the aromatic gorge, on the protein's surface. It spans six AChE residues: Tyr 72, Tyr 124, Glu 285, and Trp 286, on one side of the gorge entrance, and Asp 74 and Tyr 341, on its opposite side. Its core is comprised of Trp 286 and Asp 74 and accommodates many distinct ligands. BChE also has a PAS, but its relatively aromatic content and the response upon ligand binding differ significantly from those of AChE.

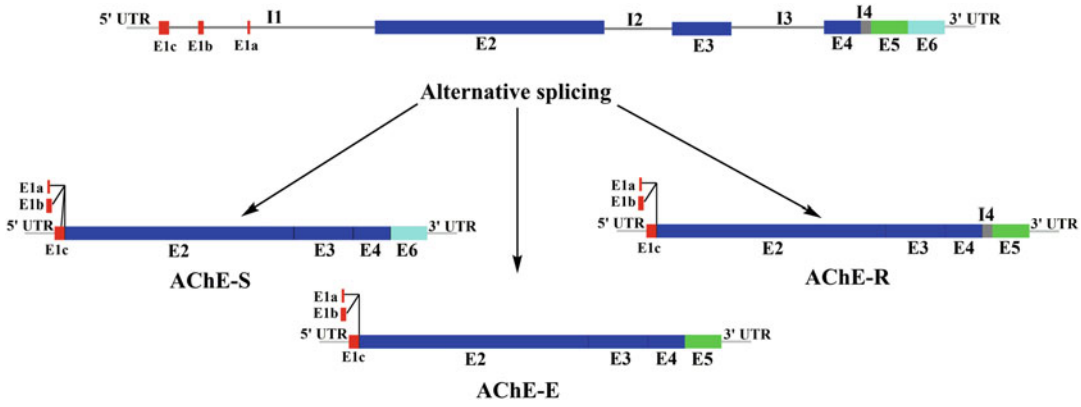
Molecular Mechanisms of Assembly

ChEs present a wide molecular diversity that modulates their function in cholinergic synapses and nonsynaptic contexts. This diversity arises at the genetic, posttranscriptional, and posttranslational levels.

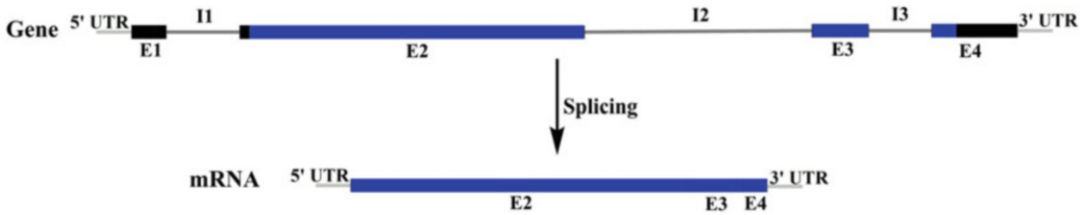
Genetics and Evolution

Genomic comparisons suggest evolutionary gene duplications creating new ChE encoding genes, which initiated before the divergence of nematodes and insects. Subsequent independent duplications lead, for example, to four *ACHE* genes in nematodes. Some families or species may in turn have lost copies of the duplicated genes (e.g., *Drosophila melanogaster*). The duplication that formed *ACHE* and *BCHE* occurred in the tetrapod lineage; thus, amphibians and reptiles but not fish have a *BCHE* homologue resembling *BCHE* of mammals or birds.

The human ACHE gene (7q22) is 7 kb long, and it encodes all variants of AChE (Fig. 2), which share a core domain, spanning the 543 amino acids encoded by exons E2, E3, and E4 of this gene (E1 is a noncoding exon). Posttranscriptional events modify the N′- and the C′-terminus, adding an extended N-terminal domain, exchanging the C-terminal domain, and causing differences in assembly and localization of the AChE multiple forms (Soreq 2015). A single nucleotide



Cholinesterases, Fig. 2 The human ACHE gene and its alternative mRNAs. Structure of the human ACHE gene. Exons are depicted as rectangles and introns as horizontal lines. Alternative splicing options of AChE mRNAs are shown with arrows. E1a, E1b, and E1c are alternative versions of human ACHE exon 1



Cholinesterases, Fig. 3 The human BCHE gene structure and its mRNA. BCHE exons are indicated in blue (coding) and black rectangles. Introns are marked as gray horizontal lines. The spliced form of BChE mRNA is shown by the arrow

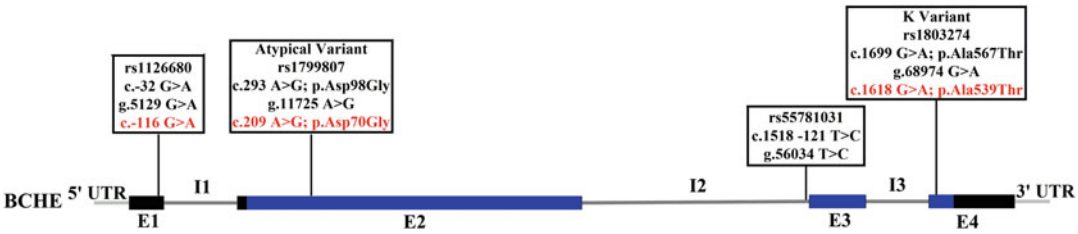
polymorphism (SNP) in position 353 (His353Asn) is responsible for the Yt blood group system; His-353 AChE corresponds to the Yt(a) and the rare Asn-353 AChE variant to Yt(b) blood group antigen.

The human BCHE gene (3q26.1-q26.2) includes 4 exons and 3 introns, yielding a somewhat longer protein, 602 amino acids long, also with an option for an N-terminal extension (Fig. 3). Human BChE has no any other alternatively spliced forms.

More than 70 natural mutations have been described for human BChE (Fig. 4). "Atypical" BChE (the dibucaine-resistant Asp70Gly) shows reduced activity, because Asp70 facilitates the initial binding of positively charged substrates to the active site gorge. The K (Ala539Thr), J (Glu497Val), and fluoride-resistant (Thr247Met or Gly390Val) variants also show reduced BChE activities. In Caucasian populations, the atypical variant and the K variant are the most common BChE variants. The atypical variant is carried in 1 out of 25 subjects, and the K variant allele is found in 1 out of 5 people. Also, 20 different "silent" genotypes have been recognized, with 0–2% of normal activity. Inversely, the Cynthiana and Johannesburg variants show higher activity than "usual" BChE.

Posttranscriptional Modifications

The *ACHE* gene includes sites for alternative splicing of its pre-mRNA product both at the 5′ and the 3′ ends. Three different carboxy termini exist: the "synaptic" or S variant also called "tailed," the "erythrocytic" or E variant, and the "readthrough" or R variant. These join the short and long N-termini to yield variants with the common or the "extended" N-terminus. In comparison, the *BCHE* gene mainly produces a transcript similar to the "synaptic" or S variant of AChE, yet



Cholinesterases, Fig. 4 Some of the important genetic variants in the human BCHE gene. BCHE exons (E) are indicated in blue (coding) and black (noncoding) rectangulars. Introns (I) are marked as gray horizontal lines. Residue numbers indicated in red follow the numbers in the mature BChE protein as present in the PDB database. In contradistinction, residue numbers indicated in black follow the genetics databases (gDNA NCBI Reference Sequence: NG_009031.1; cDNA NCBI Reference Sequence: NM_000055.2, protein NP_000046.1). Red-marked residues imply modified protein sequences

it as well may yield two different N-termini: a variant with the common or the "extended" N-terminus which may be produced due to the coding-altering SNP (c.-32G > A (rs1126680), Fig. 4).

Posttranslational Modifications

Both ChEs undergo several posttranslational modifications, including glycosylation and glycosylphosphatidylinositolation (GPI), phosphorylation, and carbamylation.

Glycosylation: AChE and BChE carry three and nine, respectively, N-glycosylation consensus sequences attaching carbohydrate residues to the core protein via asparagines. Different molecular forms of these enzymes in various tissues show different numbers and compositions of carbohydrate residues. N-glycosylation at all sites was shown to be important for effective biosynthesis, secretion, and clearance of ChEs from the circulation. Altered patterns of AChE glycosylation have been observed in the brain and the cerebrospinal fluid of Alzheimer's disease (AD) patients, with potential diagnostic value.

Glycosylphosphatidylinositolation: The glycophosphatidylinositol moiety anchor of AChE consists exclusively of diacyl molecular species. Over 85% of the molecular species are composed of palmitoyl, stearoyl, and oleoyl. The posttranslational process of glypiation takes place in the endoplasmic reticulum, after completion of the polypeptide chain; the newly synthesized protein interacts with a transamidase complex, which cleaves the chain upstream of the hydrophobic region and adds a preformed GPI anchor to the resulting C-terminal residue.

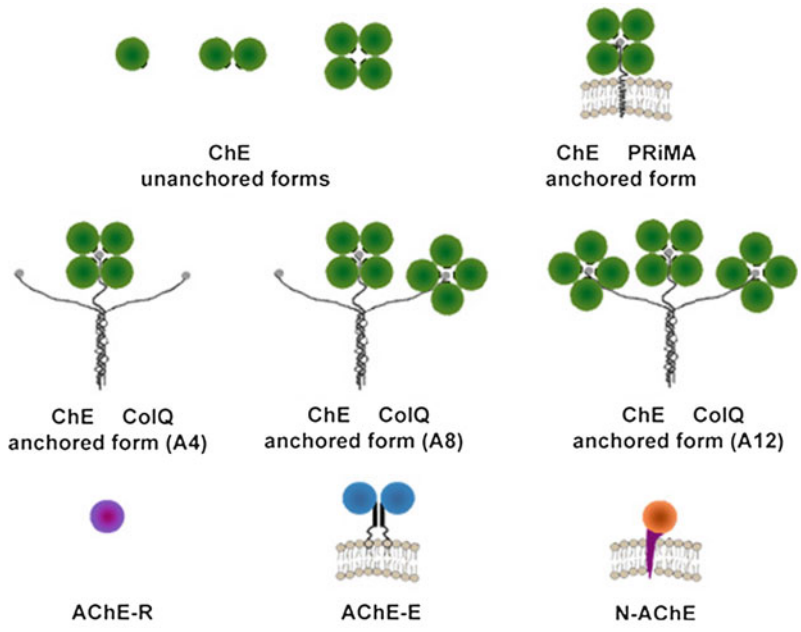
Disulfide bridges formation: ChEs contain eight to ten cysteines; six of these form three internal disulfide bridges. The cysteine that is located four amino acids upstream to the carboxyl terminus forms a disulfide bridge with a cysteine of an identical subunit, creating an interchain disulfide bridge, which stabilizes the dimeric structure.

Molecular Forms of ChEs

ChEs present several amphiphilic and soluble homo- and hetero-oligomeric molecular forms in tissues and body fluids, with different tissue distributions (Fig. 5).

BChE and AChE-S (1–4, in green).

1. Amphiphilic monomers and dimers (G1, G2): Abundant in mammalian brain, muscles, and intestine for both ChEs.
2. Soluble tetrameric form (G4): Composed of four identical monomers and stabilized by hydrophobic interactions of hydrophobic amino acids at the C-terminus of monomers. Abundant for brain AChE and BChE in mammalian body fluids and in the soluble fraction of tissue homogenates.
3. Hydrophobic-tailed tetramers: Abundant form in the mammalian CNS. Anchored to plasma membranes by a hydrophobic, 20 k dalton length polypeptide subunit named PRiMA (proline-rich membrane anchor).



Cholinesterases, Fig. 5 Shown are the seven subgroups of ChEs' molecular forms: top, in green – monomeric, dimeric, and tetrameric AChE-S forms (G1, G2, G4, and PRiMA). Middle, in green – ColQ tailed AChE-S forms (A4, A8, and A12). Parallel forms exist for BChE. Down – other AChE splicing variants (AChE-R, AChE-E, N-AChE) and N-BChE

4. Collagen-like (ColQ) tailed forms or asymmetric multimers: Characterized by triple helical structure of three collagen subunits Q, each associated with one (A4), two (A8), or three (A12) tetramers of ChEs, anchoring to the basal lamina. It is more abundant for AChE than BChE in NMJ.
5. AChE-R (in purple): Naturally rare, stress-induced variant, which lacks a hydrophobic domain and is incapable of binding to ColQ or PRiMA. Therefore, it remains soluble, and its secreted form shows greater mobility than AChE-S. AChE-R can intracellularly interact through its C-terminal tail with the protein kinase C receptor RACK1, a scaffold protein which modifies multiple cellular processes.
6. AChE-E (in blue): GPI-anchored dimers to plasma membranes in mammalian muscles, erythrocytes, and lymphocytes.
7. N-AChE (in purple): The N-terminus extension may serve as a transmembrane domain, enabling AChE-R anchorage to the membrane without ColQ/PRiMA. In principle, N-AChE-S, N-AChE-E, and N-AChE-R may exist. N-AChE (AY389977 is accession number within the GeneBank) shows a high predicted pI value (11.76), matching that of histones and other nucleic acid-binding proteins.
8. N-BChE (in turquoise): The N-terminal extension of BChE is likewise positively charged and may also enable membrane insertion of this protein. The corresponding SNP may change this value, indicating potential differences between the common and the modified variants. Predicted N-BChE amino acid sequence is 41 aa longer than wild-type (wt) BChE (Jasiecki et al. 2019). Based on this predicted sequence, computed pI value (https://web.expasy.org/compute_pi/) for N-BChE was found to be 8.64. To calculate the pI value for N-extended BChE, the following sequence was used: **MSVQSNLQAGAAAASCISPKYYMIFTPCKLCHLCCRESEIN**MHSKVTIICIRFL (extended aa sequence bolded, underlined sequence corresponds to the coding sequence of wt BChE).

Drugs

Alzheimer's disease (AD) is an age-related neurodegenerative disorder characterized by progressive cognitive impairment, a variety of neuropsychiatric and behavioral disturbances, and restrictions in activities of daily living. Both AChE and BChE are found in human neurons, glia, and in AD's plaques and tangles. AChE activity decreases, while BChE activity increases with age (>60 years), in a sex-related manner which is exacerbated in the AD brain. The cholinergic hypothesis attributes to decreased cholinergic transmission, a major role in the expression of cognitive, functional, and possibly behavioral symptoms in AD. Correspondingly, cholinesterase inhibitors (ChEIs) became a common treatment for dementia symptoms. Male carriers of both *BCHE-K* and the debilitated apolipoprotein E4 variant, when older than 75 years, show high risk for late-onset AD in some, but not all, studies.

Another pathway of influence in AD is the facilitation of amyloid-β (Aβ) aggregation through an interaction with the PAS of AChE but not of BChE. Inversely, the "usual" BChE (and more specifically its C-terminus) was shown to attenuate in vitro the formation of amyloid fibrils. Also, carriers of single nucleotide polymorphisms (SNPs) interrupting the interactions of cholinesterase mRNA transcripts with inhibiting microRNAs in noncoding regions of these transcripts show modified cholinesterase levels (Simchovitz et al. 2017).

Gulf War syndrome (GWS) is the name given to a variety of psychological and physical symptoms suffered by veterans of the 1991 Persian Gulf War. The symptoms have been remarkably wide-ranging, sometimes somewhat ill-defined. These symptoms were interpreted to reflect exposure to centrally acting anti-AChEs (Soreq and Seidman 2001).

Cholinesterase Inhibitors

Organophosphates (OPs) or carbamate esters are potent inhibitors of both AChE and BChE. These include drugs (e.g., for AD or myasthenia gravis, MG), pesticides, insecticides, and chemical warfare agents. Phosphorylation or carbamylation of the serine hydroxyl group in the substrate-binding domain inhibits the enzyme, causing ACh accumulation in synaptic clefts and overstimulation of cholinergic receptors. This induces various symptoms, including tension, anxiety, headaches, slurred speech, tremor, convulsions, muscle paralysis, seizure, and even death by asphyxiation.

Drugs

ChEIs represent the treatment of choice for AD therapy, currently including donepezil, rivastigmine, and galantamine. ChEIs vary widely in their pharmacological profiles and affinities for AChE and BChE. Donepezil and galantamine inhibit AChE by 1000- and 50-fold more than BChE, respectively, whereas rivastigmine inhibits both enzymes with similar affinity and in a semi-irreversible manner. ChEI treatments have been expanded also to include other dementias and CNS disorders, e.g., delirium, traumatic brain injuries, and memory impairments, as well as myasthenia gravis, glaucoma, and parasite infections.

Dementia with Lewy bodies (DLB) is considered the second most common cause of dementia after AD. The disorder is characterized by progressive fluctuating cognitive impairment, visual hallucinations, and motor features of Parkinsonism. Neocortical cholinergic activity is more severely depleted in DLB than in AD, and DLB also affects the caudate nucleus, the thalamus, and the brain stem. Tolerability of ChEI in DLB appears similar to AD, with some gastrointestinal effects and muscle cramps.

Parkinson's disease (PD) patients may suffer cognitive and behavioral impairments including apathy, personality changes, and visual hallucinations, with no currently recommended treatment. Their significant cholinergic deficits led to recommendation of ChEI therapeutics, but sex and genotype differences were not yet considered.

Vascular dementia (VD) accounts for ~20–30% of dementia cases, with clinical and pathological overlap with AD. Reductions in cholinergic biomarkers suggest cholinergic deficits in VD, and ChEIs increase ACh availability and improve their cerebral blood flow.

Down's syndrome (DS) carriers develop early-onset AD, likely due to the chromosomal origin of the amyloid gene on their triple chromosome 21, motivating the use of cholinergic therapy in this disorder.

Traumatic brain injury, *including concussion*, is the most common cause of death in subjects under the age of 40 and an important risk factor for AD. Loss of hippocampal cells and depletion of ACh and of muscarinic receptors can be attenuated in injured experimental animals, improve blood perfusion in ischemic areas, and increase cholinergic transmission in the cortex and hippocampus; the same mechanism invoked for treatment of vascular dementia.

Delirium, e.g., post-narcotic delirium, somnolence, or coma, is a common complication involving dementia, with fluctuating attention and consciousness and considerable morbidity. It is not always reversible, and there is no specific treatment. Some of the accompanying central cholinergic syndromes can be reversed by ChEIs.

Myasthenia gravis (*MG*) is an autoimmune disorder characterized by weakness of the face, tongue, and/or neck, which result in double vision or drooping eyelids, along with difficulty in chewing, swallowing, and talking. The most commonly used ChEIs in MG are the cholinesterase inhibitors pyridostigmine, neostigmine, and huperzine A. An AChE mRNA-targeted oligonucleotide agent, Monarsen, is under clinical trials for ameliorating MG symptoms, with significant advantages over ChEIs.

Chemical Warfare Agents, Pesticides, and Insecticides

Discovered in the late 1930s in Germany as improved poisonous insecticides, organophosphorus ChEIs were developed as chemical warfare agents (e.g., sarin, soman, and tabun) and were more recently employed in the 1995 terrorist attack in the Tokyo subway system and in the internal Syrian war (Taylor 2011).

Therapy against acute nerve gas toxicity includes pretreatment with pyridostigmine, a reversible carbamate ChEI, capable of inducing AChE overexpression. Postexposure treatment is continuous with administration of cholinolytic agents such as atropine, the oxime reactivator, pralidoxime chloride, and diazepam. Although multidrug combination therapy is effective in increasing survival, it must be administered immediately and cannot prevent the occurrence of postexposure toxic symptoms. A recently developing alternative involves the use of highly purified ChEs as therapeutic agents. Recombinant production systems for such proteins include transgenic goats producing human BChE in their milk or plant production of human AChE.

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Chromatin Remodeling

► Transcriptional Regulation

Chronic Bronchitis

► Chronic Obstructive Pulmonary Disease

Chronic Obstructive Pulmonary Disease

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Synonyms

Chronic bronchitis; Pulmonary emphysema

Definition

Chronic obstructive pulmonary disease (COPD) affects over 5% of the adult population, is the fourth leading cause of death worldwide, and is the only major cause of mortality that is increasing worldwide. It is a complex and heterogeneous inflammatory disorder of the lungs, caused mainly, but not exclusively, by cigarette smoking. Fifteen to twenty percent of smokers develop COPD.

The Global Initiative for Chronic Obstructive Lung Disease (GOLD; (http://www.goldcopd.com) has defined COPD as follows: "COPD is a common, preventable, and treatable disease that is characterized by persistent respiratory symptoms and airflow limitation that is due to airway and/or alveolar abnormalities usually caused by significant exposure to noxious particles or gases. The chronic airflow limitation that characterizes COPD is caused by a mixture of small airways disease (e.g., obstructive bronchiolitis) and parenchymal destruction (emphysema), the relative contributions of which vary from person to person. Chronic inflammation causes structural changes, small airways narrowing, and destruction of lung parenchyma. A loss of small airways may contribute to airflow limitation and mucociliary dysfunction, a characteristic feature of the disease." The severity of COPD is classified on the basis of chronic symptoms (cough, sputum production), spirometric lung function tests, and the history of exacerbations.

Basic Mechanisms

During COPD the following symptoms occur, usually in the order mucus hypersecretion (associated with MUC5AC and MUC5B overexpression), ciliary dysfunction, airflow limitation, pulmonary hyperinflation, gas exchange abnormalities, pulmonary hypertension, and cor pulmonale. Acute exacerbations appear to be mainly triggered by bacteria, viruses, or environmental pollutants. They lead to a worsening of lung functions and reduced quality of life and mainly impact on long-term outcome. COPD is considered a systemic disease because cardiovascular disease, sarcopenia, osteoporosis, and diabetes are frequent comorbidities.

COPD is a chronic inflammatory disease that results from prolonged and repeated inhalation of particles and gases, chronic (or latent) infection, or an interaction of these factors. In many cases the inflammation persists even when the exposure (in most cases smoking) is stopped. The inflammation is found predominantly in the smaller airways and in the lung parenchyma. Prominent among the infiltrating leukocytes are neutrophils, **$CD8^+$** lymphocytes and **$CD68^+$** monocytic cells (Table 1). The chronic inflammation leads to small airway fibrosis causing airway narrowing and to lung parenchyma stiffening that promotes small airway collapse. Both mechanisms are irreversible and lead to air trapping. Superimposed are reversible cholinergic responses such as small airway contraction and mucus hyperproduction (Barnes 2017).

Oxidative stress is considered an important pathogenic factor. It may promote COPD by many factors such as induction of pro-inflammatory genes in many cells including epithelial and endothelial cells, inactivation of antiproteases, and key repair molecules such as sirtuin-1, which may contribute to the accelerated aging responses seen in COPD. **Reactive oxygen species** and other factors such as ceramide or blockade of vascular endothelial growth factor (VEGF) receptors may cause

Chronic Obstructive Pulmonary Disease, Table 1 Pathological changes in COPD

	Large airways	Small airways (<2 mm Ø)	Parenchyma	Products/consequences
Goblet cell hyperplasia/ metaplasia	+[a]	+	–	Mucus
Neutrophil infiltration	+[b]	+[b]	(+)[b]	ROS, elastase, MMP-8, MMP-9
$CD8^+$ T cells[c]	+	+	+	TNF, perforins, granzymes, activation of Fas-dependent apoptosis
Macrophages ($CD68^+$)	–	+	+	TNF, IL-8 cathepsins B, L, and S MMP-2, MMP-9, MMP-12
Airway wall fibrosis, thickening	–	+	–	Flow limitation
Smooth muscle	–	+	–	AHR?
Emphysema	–	–	+	Flow limitation

ROS reactive oxygen species, *AHR* airway hyperresponsiveness, *MMP* matrix metalloprotease
[a]Correlates with cough and chronic bronchitis
[b]Number increases with disease severity
[c]Correlates with amount of alveolar destruction and severity of airflow obstruction

emphysema by induction of apoptosis (in the case of reactive oxygen species also necrosis) in endothelial or epithelial cells. Furthermore, inefficient apoptotic cell clearance (efferocytosis) may also contribute to a probably genetically modulated progressive lung destruction with fibrosis.

The inflammation in COPD appears to be complex and heterogeneous. In general the immune response appears to be skewed – albeit not in a clear-cut manner – toward pro-inflammatory M1-like macrophages and Th1 as well as Th17 responses. Among the typical mediators and effectors found in the disease are TNF, IL-1, IL-8, IP10, LTB_4, and elastolytic enzymes such as matrix metalloproteases and cathepsins. The majority of drugs in development for COPD are based on anti-inflammatory concepts, e.g., antagonists for IL-33, CXCR2, IFNβ, IL-1β, IL-5, IL-13, IL-17A, TNF, or FGF and inhibitors for LOX5, MMP12, PI3Kδ, p38, IKK2, or iNOS (Barnes 2017).

Both elastase and MMPs have physiological antagonists, named α1AT (α1-antitrypsin) (the primary inhibitor of neutrophil elastase) and TIMP (tissue inhibitor of matrix metalloproteases), respectively. Smoke, presumably through oxidative stress, may inactivate these antiproteases. Consequently, it has been suggested that emphysema results from an imbalance of the protease and antiprotease ratio and indeed, hereditary α1AT deficiency is a rare but well-known cause of emphysema. In experimental animals, TIMP-3 deficiency leads to a combination of developmental airspace enlargement combined with progressive destructive emphysema in adults. However, almost certainly, no single protease/antiprotease alone is responsible for the development of COPD.

Today, COPD is regarded as a systemic disease. The different pathomechanisms do not only cause local lung injury and remodelling but also alter systemic structures and function. Relatively little is known about the low-grade systemic inflammation present in COPD that is considered the main cause for the systemic effects. These systemic effects of COPD include involuntary weight loss, muscle atrophy, impaired bone metabolism, reduced functional capacity and health status, and increased cardiovascular morbidity as an important resource-consuming comorbidity. Thus, COPD is multidimensional disease (Singh et al. 2019). To account for the multiple changes in COPD, the BODE index was introduced, a 10-point scale combining measures of BMI (B), the degree of airflow

obstruction (O) and dyspnea (D), and exercise capacity (E) (Celli et al. 2004).

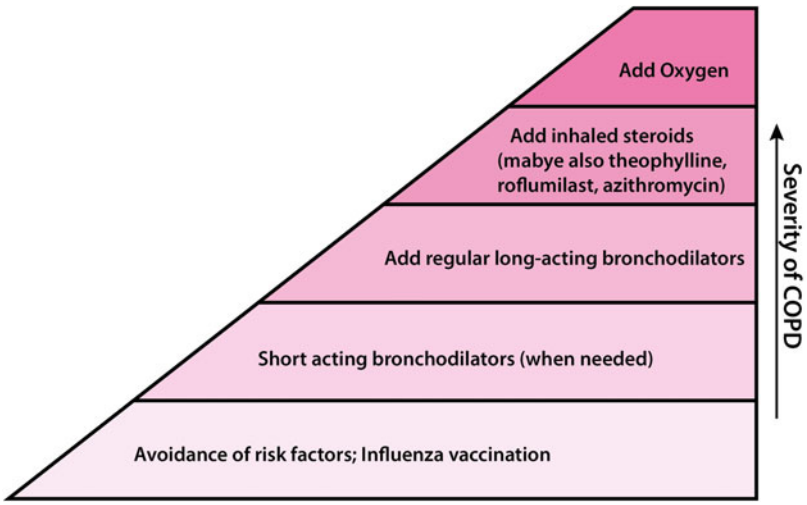
Pharmacological Interventions

Usually it takes years of toxin exposure to cause the pathological alterations seen in COPD. In most cases the disease is already well progressed when COPD is diagnosed. Reversal of established chronic inflammatory disease is always extremely difficult to achieve, and at present healing of COPD is impossible. Most of the pharmacological agents that are used in COPD (Fig. 1) have been developed for the treatment of asthma, where their benefit is clearly greater. The comparatively small effect of inhaled β-agonists on airway resistance is even used as a diagnostic criterion to distinguish COPD from asthma. The management of COPD is largely symptom-driven, and there is only an imperfect relationship between the degree of airflow limitation and the presence of symptoms. Currently, there is no effective therapy for the irreversible airflow obstruction that results from airway remodelling, fibrosis, and emphysema. Available pharmacotherapy can reduce or abolish symptoms, increase exercise capacity, reduce the number and severity of exacerbations, and improve health status. The inhaled route is preferred. The mainstay of therapy are anticholinergics, β2-agonists, and steroids, and much effort has been put into evaluating the benefits of single, double, and triple therapies of these three classes. In addition to these drugs, roflumilast or azithromycin may be used to treat exacerbations (van Haarst et al. 2019; Singh et al. 2016, 2019; Woodruff et al. 2015).

Bronchodilators

The primary aim of the current COPD therapy is to reduce airway resistance by reducing bronchial smooth muscle constriction and mucous plugging. β2-adrenoreceptor agonists and anticholinergics are the mainstay of therapy for symptomatic management. These bronchodilators improve symptoms and exercise tolerance and may reduce exacerbations but have little effect on inflammation and on the long-term decline in lung functions. β2-agonists and to some extent also anticholinergics may increase the risk of adverse cardiovascular events, although the cardiovascular risks of the latest drugs are quite low.

β2-adrenoreceptor agonists: β-agonists increase intracellular cAMP, which in turn leads to bronchodilation and improved lung emptying during breathing. Short-acting β-agonists (SABA, half-life <6 h: salbutamol, terbutaline, orciprenaline, fenoterol, albuterol), long-acting β-agonists (LABA, ~12 h: formoterol, salmeterol), and ultra-long-acting β-agonists



Chronic Obstructive Pulmonary Disease, Fig. 1 Pharmacotherapy of COPD

(uLABA, ~24 h: indacaterol, olodaterol) are used. Regular treatment with long-acting bronchodilators is more effective and convenient than treatment with short-acting bronchodilators. There is a relatively small and flat dose-response relationship with all β-agonists. Possible side effects are palpitations and premature ventricular contraction (resulting from stimulation of β_1 receptors in the heart), tremor, sleep disturbances, and hypokalemia (see also "► Asthma" entry).

Anticholinergics: Modern long-acting muscarinic receptor antagonists (LAMA) are tiotropium, aclidinium, umeclidinium, and glycopyrrolate. All show higher selectivity for M3-receptors compared to M2-receptors and in addition remain bound to M3-receptors longer than to M2-receptors. They have to be given once or twice daily. Older anticholinergics (e.g., ipratropium, oxytropium) that have to be given up to four times daily are often used as maintenance treatment. Possible side effects are dry mouth, metallic taste after inhalation, and very rarely close angle glaucoma. The effects of anticholinergics and β-agonists show some additive effects (combination therapy).

Anti-inflammatory Therapy

Inhaled corticosteroids. Inhaled steroids (commonly used are beclomethasone, budesonide, triamcinolone, fluticasone and its derivatives, flunisolide) are typically used in conjunction with LABAs and appear to attenuate the inflammatory response, to reduce bronchial hyperreactivity, to decrease exacerbations and to improve health status. Many patients appear to be resistant to steroids, and large, long-term trials have shown only limited effectiveness of inhaled corticosteroid therapy. They appear to work best in patients with frequent exacerbations and with eosinophilia; of note, the IL-5 antibody mepolizumab is currently under debate for use in eosinophilic COPD. Certainly, the benefit from steroids is smaller in COPD than in asthma. Topical side effects of inhaled steroids are oropharyngeal candidiasis and hoarse voice; in addition systemic side effects such as osteoporosis may develop.

Systemic steroids. Systemic steroids are used to treat acute exacerbations. Here, short-term use of 3–5 days is standard of care. Long-term use of systemic steroids should be avoided in order to prevent steroid-associated side effects. In addition, it has been shown that long-term use of systemic steroids is associated with negative long-term outcome.

Phosphodiesterase inhibitors: The phosphodiesterase-4 (PDE-4) inhibitor roflumilast is approved to reduce the risk of COPD exacerbations in patients with frequent COPD exacerbations despite bronchodilators and inhaled steroids. Its mechanism of action is considered to be largely based on its anti-inflammatory effects; as an example, it decreases TNF expression. Another albeit unspecific phosphodiesterase inhibitor that also hits other targets such as A1-receptors is theophylline; theophylline is a third-line bronchodilator drug in chronic COPD behind inhaled anticholinergics and β2-agonists, with slow-release forms of oral theophylline preferred. Methylxanthines have a narrow therapeutic margin; major side effects are ventricular and atrial dysrhythmias and convulsions. Possible side effects of PDE-4 inhibitors include headache, nausea, vomiting, diarrhea, and heartburn.

Anti-microbial Therapy

The use of antibiotics is not recommended, except for the treatment of infectious exacerbations of COPD and other bacterial infections. An exception are macrolides, in particular azithromycin, that do also have immunomodulatory effects. Azithromycin may be beneficial for older patients with milder disease and for ex-smokers. Known risks are hearing loss and prolonged QTc interval (Woodruff et al. 2015).

Anti-trypsin Augmentation Therapy

Approximately 2% of all COPD patients suffer from homozygous α1-AT deficiency. Intravenous infusion of replacement protein twice weekly in patients with established α_1-antitrypsin deficiency has been approved in both in the USA and in Europe.

Oxygen Therapy

Long-term oxygen therapy (>15 h per day) is introduced in very severe hypoxemic COPD and improves survival, exercise capacity, and cognitive performance. Oxygen therapy is also temporarily used for hospital treatment of hypoxemic COPD exacerbations. In addition to improving oxygenation, oxygen therapy is thought to be effective because it reduces pulmonary hypertension by opposing hypoxic pulmonary vasoconstriction.

Psychopharmacological Therapy

Up to 30% of COPD patients suffer from anxiety disorder or depression and should be treated with conventional pharmacotherapy (Woodruff et al. 2015).

Non-pharmacological Interventions (Singh et al. 2019)

Reduction of Risk Factors

Smoking is the single most important single-risk factor for COPD, and smoking cessation is the single most effective – and cost-effective – intervention to reduce the risk of developing COPD and to stop its progression. Other risk factors include open fire cooking.

Vaccination

Because infections are a common cause of exacerbations, age-appropriate vaccination against influenza and pneumococci is recommended.

Noninvasive Ventilation

Noninvasive ventilation is used to treat acute hypercapnic respiratory failure during an acute exacerbation. This treatment is administered in hospital. Nevertheless, current data have shown that NIV is able to treat chronic hypercapnic respiratory failure as well. Here, NIV is used mainly at home during nighttime for long-term treatment.

Pulmonary Rehabilitation

Pulmonary rehabilitation as add-on to medication and vaccination has proven to be much more efficient in motivated patients than medication alone. It is aiming at self-management and training but also includes nutritional, pharmacological, and psychosocial support. Training increases the proportion of type I (minimal fatiguable aerobic low-velocity fibers) versus type II fibers (vs. the high-velocity anaerobic), stimulates the release of endogenous opioids, provides psychological reinforcement during exercise, and improves self-confidence.

Lung Volume Reduction (Surgery and Endoscopic)

Lung volume reduction surgery is a treatment option for patients with severe emphysema. Endoscopic lung volume reduction using valves, coils, and other approaches are newly developed treatment options that can alternatively be used in COPD patients with severe emphysema.

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Circadian Rhythms

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Synonyms

Daily rhythms; 24h-rhythms

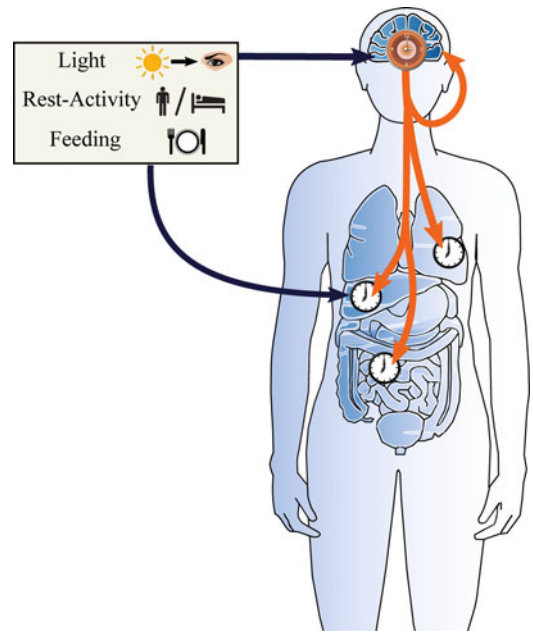
Definition

Most mammals display 24 h-rhythms in their activity and internal physiological functions also called circadian rhythms. The word "circadian" comes from the Latin words "circa" – "dies" which means around – a day, thus referring to phenomenon with a period comprised between 20 and 28 h. The 24 h-rhythms of the organism are governed by a hierarchical internal system called the circadian timing system (CTS) which aims to optimize the energy use and supply over the day and night cycles though the anticipation of the body needs. More than 30 years of research in the field of circadian rhythms have recently been acknowledged by the 2017 Nobel Prize of physiology and medicine awarded to Prof. Jeffrey C. Hall, Michael Rosbash, and Michael W. Young for their discoveries of the molecular mechanisms controlling the circadian clock. The daily oscillations are endogenous, meaning that they are created by the CTS itself and are sustainable in the absence of external synchronizers. However, the inner period of the rhythms is not exactly equal to 24 h and varies from one individual to another. Environmental cues such as light or social interactions entrain the body to a period of exactly 24 h, equal to that of the rotation of the Earth.

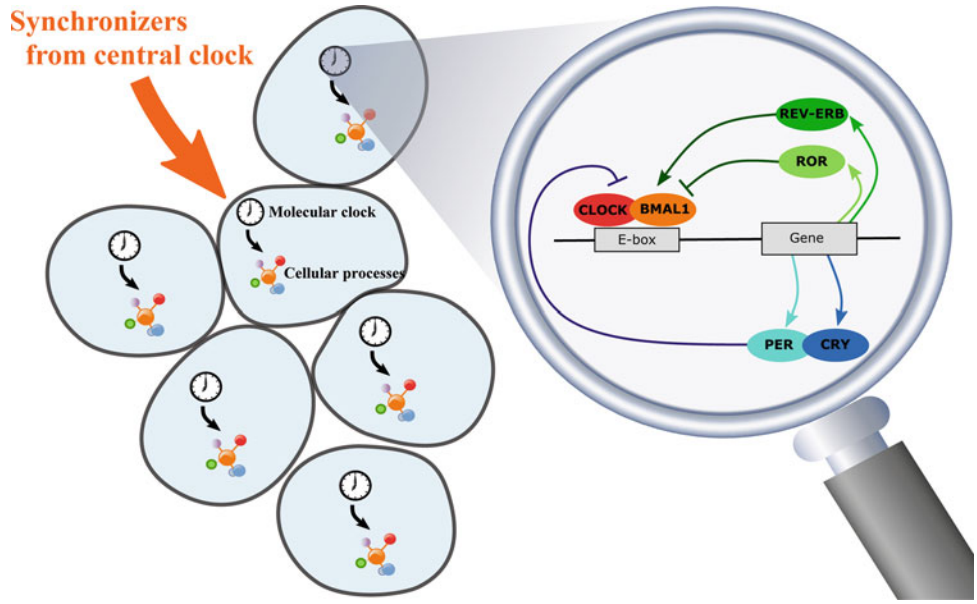
Basic Mechanisms

The Circadian Timing System

The mammalian Circadian Timing System (CTS) is constituted of a central pacemaker located in the hypothalamus – the suprachiasmatic nuclei – that spontaneously oscillates at its own period of nearly 24 h and is daily synchronized to an exactly 24 h-period by external time cues such as light-dark cycles, food intake, or socio-professional interactions (Levi and Schibler 2007). This central pacemaker transmits rhythmic physiological signals to molecular circadian clocks present in all nucleated cells of the peripheral organs, in various forms including hormone variations or temperature cycles (Fig. 1). Indeed, each nucleated cell is endowed with a molecular clock which is composed of approximately 15 genes involved in at least two interconnected feedback loops that spontaneously generate circadian oscillations (Fig. 2). More precisely, the central node of the clock is the complex formed by two clock genes: Clock and Bmal1. The CLOCK/BMAL1 complex attaches to Enhancer–boxes (E-boxes) and promotes the transcription of Period homolog 1, 2, and 3 (Per1, Per2, Per3), Cryptochromes (Cry1, Cry2), retinoic acid-related orphan receptors



Circadian Rhythms, Fig. 1 The circadian timing system. A central pacemaker located in the hypothalamus display rhythms of a period close to 24 h and is entrained by external cues such as light or feeding patterns to exactly 24 h. This central clock exerts a control on the cellular clocks of the peripheral organs in various forms including rhythmic hormone secretion or temperature cycles



Circadian Rhythms, Fig. 2 The molecular clocks in peripheral organs. In mammals, each nucleated cell is endowed with a molecular clock spontaneously oscillating with a circadian period as a result of interconnected transcriptional and translational feedback loops. The clock genes Clock and Bmal1 form dimers that attach to E-boxes to promote the transcription of Pers, Crys, Rors, and Rev-erbs genes. Per and Cry undergo post-translational modifications and associate to repress the transcriptional activity of Clock/Bmal1, thus decreasing their own transcription. After few hours, Per/Cry complexes are degraded which starts a new cycle of the oscillator. Another feedback loop involves the respective positive and negative action of REV-ERB and ROR proteins on Bmal1 transcription which increases the robustness of the oscillator. At the level of a tissue, cellular clocks are synchronized with each other through rhythmic physiological signals coordinated by the central pacemaker in the brain

(Rora, Rorb, Rorc), and Rev-Erb nuclear orphan receptors (Rev-Erbα, Rev-Erbβ). PER and CRY proteins undergo post-translational modifications in the cytoplasm followed by nuclear translocation probably under the form of a PER/CRY complex, and inhibit CLOCK/BMAL1 positive action on transcription, including their own. As a consequence, PER and CRY protein levels decrease, thus allowing CLOCK/BMAL complexes to be active again, thus starting a new cycle of the oscillator. A second feedback loop involves ROR and REV-ERB proteins which regulate BMAL1 transcription by respectively activating and inhibiting it (Levi and Schibler 2007). In vitro experiments have demonstrated that individual cells may oscillate with different periods, amplitudes, and phases in the absence of external synchronizers. The central pacemaker sends physiological signals to the peripheral organs to synchronize the cells. External synchronizers may also act independently from the central pacemaker, as for instance, meal timing modifies the systemic metabolic cues such as insulin or glucagon levels and impacts on local energy metabolism.

Circadian Rhythms of Key Intracellular Pathways

The CTS exerts multilevel controls on peripheral organs inducing 24 h-rhythms in major intracellular pathways governing cell fate. Recent genome wide studies have uncovered circadian regulation of general mechanisms such as chromatin remodeling, transcription, or translation (Takahashi 2017). As a result, more than 40% of the 20,000 protein-coding genes display circadian expression in at least 1 out of 12 mouse organs (Zhang et al. 2014; Robles et al. 2017). In baboons, approximately 80% of the detected protein-coding genes exhibited 24 h-rhythms in their

mRNA expression in at least one of the 64 studied tissues (Mure et al. 2018). These 24 h-rhythms in gene and protein expression translate into circadian variations of key intracellular pathways driving cellular processes such as energy metabolism, proliferation, or cell death (Fig. 2). In particular, extensive experimental and mathematical works have highlighted that the circadian clock and the cell cycle machinery are dynamically coupled through a robust phase locking mechanism in dividing cells involving both gene transcriptional control and direct protein–protein interactions (Ballesta et al. 2017). The 2015 Nobel Prize winner Aziz Sancar also evidenced daily variations in the Nucleotide Excision Repair pathway which is involved in repairing DNA damage caused by the exposure to irradiation or to various anticancer agents (Sancar et al. 2015).

Circadian Rhythms of Physiological Functions Governing Drug Absorption, Distribution, Metabolism, and Excretion (ADME)

The circadian organization of the body has great implications for drug response. Indeed, most physiological functions of the organism – including processes of drug Absorption, Distribution, Metabolism, and Elimination (ADME) – display 24 h-rhythms with differences of several folds between minimum and maximum activities. Daily variations have been demonstrated in laboratory animals and in humans for: gastric pH, gastric and small intestinal motility; plasma proteins levels, circulating blood cells and their membrane properties, blood flows though the main organs; liver metabolism, bile volume, and salt excretion; renal functions and urinary output (Levi and Schibler 2007). At the molecular level, numerous enzymes display circadian rhythms in their protein level and activity including phase I and phase II metabolism enzymes and phase III transporters (Bicker et al. 2020). In particular, the efflux pump P-glycoprotein (Abcb1), which is involved in the transport of many xenobiotics, display circadian rhythms in its hepatic and intestinal activity that vary according to sex and to feeding patterns (Bicker et al. 2020).

Inter-Individual Variabilities in Circadian Rhythms

Efforts have been made to monitor the CTS in individuals to decipher intersubject variabilities. Historically, questionnaires assessing diurnal preferences have been used to identify chronotypes and have demonstrated large interindividual variabilities. For instance, the time of midsleep on free days was equal to 4 a.m. local time on average and ranged from 11 p.m. to 11 a.m. in a population of 221,480 individuals from the Munich Chronotype Questionnaire database (Roenneberg et al. 2019). The mean value of the distribution of midsleep time is different with age, shifting later during adolescence, showing a peak around 19 years, and shifting earlier thereafter. Sex-related differences are also known as, on average, women go to bed earlier and get more total sleep than men (Bailey and Silver 2014). A large recent study in a US-based population revealed that men are typically later chronotypes than women before 40, but earlier types after 40 (Roenneberg et al. 2019).

However, such chronotype classification based on questionnaires may not precisely correlate with the underlying physiology and more molecular biomarkers have been investigated. The study of clock gene polymorphisms has been useful yet with limited results. For instance, PER3 polymorphisms have been associated with diurnal preferences (Hida et al. 2014). Next, melatonin is a hormone that is produced by the pineal gland with a strong circadian rhythms peaking at night. It can easily be measured either in the saliva or in the plasma of individuals. The time of Dim Light Melatonin Onset (DLMO) is considered as a relevant biomarker of the central clock. To measure it, the individual is subjected to dim light (approx. 10 lux) for few hours and blood samples are then collected over at least 12 h to assess melatonin plasma level and determine its onset time. This physiological measurement evidenced natural intra- and interindividual variability of internal time. In particular, sex differences in melatonin rhythms have been reported as women have a larger melatonin amplitude than men as well as an earlier onset (Bailey and Silver 2014). However, DMLO assessment requires dedicated

facilities and patient hospitalization which may not be amenable for everyday clinical practice.

Lately, the use of wearables has allowed to continuously monitor circadian biomarkers in individuals over several consecutive days in daily routine. When combined with suitable computer analysis techniques, wearable devices constitute promising tools for extracting insight from real-time monitoring of body rhythms (e.g., rest-activity, heart rate, skin temperature, . . .) and of external cues such as light. Rest-activity and skin surface temperature assessment has revealed up to 12 h intersubject differences regarding the circadian maxima of physical activity or skin temperature (Ballesta et al. 2017). The recording of rest-activity, position, and thoracic skin temperature of 55 healthy volunteers and 12 cancer patients of the InCasa study – named by the EU Commission as "one of the Top 25 influential projects in Information and Communication Technologies for Active and Healthy Ageing" – revealed that all participants displayed 24 h-rhythm in their rest-activity with maxima occurring from 12:09 to 20:25 (Komarzynski et al. 2018). The period of the temperature rhythm was around 24 h for only 51 out of 67 subjects, and around 12 h for the 13 others with maximum located at night for the majority of people, but during daytime for 18 out of 67 participants. Differences according to sex have also been found in between participants.

Efforts have also been made to assess peripheral clocks from patients' samples. In vitro analysis of skin fibroblasts or hair keratocytes isolated from individuals and subsequently cultured in vitro provided insights into the intersubject variability of the molecular clock although the relevance of such ex vivo experiments to predict the in vivo timing remains unclear (Levi and Schibler 2007).

Clock Disruption and Pathologies

Dysfunction of the CTS due to genetic alterations may be associated to various pathologies as, for instance, mutations in Per2 or Per3 clock genes are linked to sleep disorders. On the other hand, circadian disruption due to the exposure of mistimed environmental cues can be detrimental for health. Disruption of the CTS may occur for various reasons including sedentary lifestyle; mistimed eating patterns or unbalanced diet; suboptimal light-dark cycles including exposure to light at night; or shift work which implies work schedule changes on a week basis (Hatori et al. 2017; Sulli et al. 2018a). A well-known disruption of the CTS is caused by changing of time zones which induces jet lag usually associated to minor symptoms such as fatigue or dizziness. However, CTS disruption may be linked to the appearance of more severe pathologies as it is associated to a higher incidence of cancer, cardiovascular diseases, or metabolic disorders such as obesity or diabetes (Maury 2019; Ballesta et al. 2017). In particular, shift workers such as flying attendants or hospital nurses experience higher risk of sleep disorders, heart disease, respiratory disorders, and cancers (Sulli et al. 2018a).

Next, pathologies themselves may disrupt the internal timing system. Indeed, approximately 50% of cancer patients present disrupted circadian rhythms which are correlated to poor quality of life and overall survival (Ballesta et al. 2017). Furthermore, tumor cells often present disrupted circadian rhythms, including mutations of core clock genes, which has become a hallmark of cancer (Levi and Schibler 2007; Ye et al. 2018). This escape from the circadian control allows for increased proliferation (Levi et al. 2010) and may be associated with a decreased sensitivity to anticancer drugs (Ballesta et al. 2017). Tumor clock gene mutations or deregulated expression are associated with poorer patient survival in several cancer types including breast and renal cancers (Ye et al. 2018).

Pharmacological Intervention

The circadian control of the organism physiological functions has great implications for pharmacotherapies. First, the CTS itself can be seen as a target to pharmacological intervention in order to restore the Clock. Indeed, fighting against CTS disruption may be an indirect way to treat other pathologies which are under its control. On the other hand, both the efficacy and side effects of many drugs critically vary according to

administration timing over the 24 h span, to an extent which may be comparable to dose modifications. The concepts of chronoefficacy and chronotoxicity then relate to circadian rhythms in drug activity on diseased or healthy tissues respectively. Drug pharmacokinetics-pharmacodynamics (PK-PD) may vary as a function of dosing time, as shown for several hundreds of medications in laboratory animals and in patients (Levi et al. 2010). This may partly explain why systemic drug exposure after classical drug administration which does not account for circadian timing can vary by more than ten-fold in individual patients, despite dose adjustment to body weight or surface area. Chronotherapy relies on the optimization of drug timing based on the patient circadian rhythms to increase treatment efficacy and decrease side effects. It has been successful for several pathologies, yet with the challenge of inter-patient variability (Ballesta et al. 2017). However, it is still associated with technological and methodological challenges so that less than 1% of all reported clinical trials currently stipulate the time of the day.

Intervention to Restore Normal CTS Functions

The CTS in itself can be considered as a target for pharmacotherapies to restore normal circadian rhythms as an indirect treatment for pathologies affecting clock-controlled functions (Sulli et al. 2018a). Current strategies to restore normal circadian rhythms usually combine behavioral and pharmacological interventions.

Light – and in particular blue light – is considered as one of the stronger external cues affecting the CTS. Thus, clinicians have designed behavioral interventions jointly aiming to decrease evening light exposure and increase daytime light exposure. This has led to recommendations to avoid mistimed blue light exposure by limiting access to screens (phones, computers, . . .) before the resting phase for instance. In addition, bright light therapies using dedicated lamps can be used to increase light exposure at particular hours of the daytime and thus restore normal rhythms. For instance, bright light therapy is used to manage Delayed Sleep Phase Syndrome, a condition which shifts the normal sleeping pattern outside of what is considered the social norm.

Regarding pharmacological intervention, melatonin administration is now recognized as a possible treatment for CTS disruption. Oral administration of slow-release formulation of melatonin at bed time mimics natural shortens sleep onset latency defined as the amount of time it takes to go from being fully awake to sleeping. Hence, such therapies primarily treat sleep problems, which may indirectly improve other aspects of the daily rhythms leading to a better quality of life.

Time-restricted eating limiting the duration and circadian interval of food intake has emerged as another behavioral intervention to strengthen circadian rhythms in peripheral organs, even in the absence of a functioning circadian clock. Animal studies suggest that meal timing may improve brain health in Huntington's disease and reduce tumor progression, although clinical trials are needed to confirm these findings in patients and design optimize eating cycles (Ballesta et al. 2017; Sulli et al. 2018a).

Chronotherapies of Selected Pathologies

Rheumatology

Rheumatology diseases involve the immune system which is under the control of the CTS. As a result, the intensity of symptoms of such conditions including rheumatoid arthritis and osteoarthritis display 24 h-variations. The symptoms of rheumatoid arthritis, which is an inflammatory disease associated with joint pain and stiffness, tend to be more severe in the morning. Such rhythms have been correlated with the night increase in circulating proinflammatory cytokines levels that achieve their maximum value in the early morning. This result led to the development of modified-release formulations of the glucocorticoid prednisone in order to achieve drug exposure starting at the middle of the night span, after an administration at bedtime. Clinical trials in rheumatoid arthritis patients confirmed a better pain and stiffness control of modified-release prednisone as compared with the conventional prednisone formulation taken in the morning.

Other disease-modifying drugs such as methotrexate were also shown to present fewer side effects and be more effective in rheumatoid arthritis patients, following evening dosing (Ballesta et al. 2017).

Cardiovascular Pathologies

The cardiovascular system presents robust circadian rhythms at multiple levels including heart rate, blood pressure as well as clock gene expression in the heart and the arteries (Portaluppi and Hermida 2007; Smolensky et al. 2016). As a consequence, the occurrence of many cardiac events, such as myocardial infarction, or sudden cardiac death displays a circadian rhythm with their occurrence being greater in the morning. Next, side effects of heart surgery were lower during the afternoon as compared to morning surgery (Montaigne et al. 2018). Various drugs against cardiovascular pathologies may also present chronoefficacy rhythms. Indeed, the beta-blocker talinolol is a very good substrate of the efflux transporter P-glycoprotein which presents a circadian activity thus inducing circadian rhythm in talinolol pharmacokinetics. Next, most drugs against hypertension achieved both greater improvement of blood pressure and lower risks of cardiovascular events, after their oral intake at bedtime as compared with morning administration (Smolensky et al. 2016).

Anticancer Chronotherapies

Among the therapeutics strategies which have proven a benefit for cancer patients in the past decades, **cancer chronotherapy** – that is adjusting antitumor drug delivery to the patient's 24-h biological rhythms – is considered as very promising. The temporal differences between tumor tissues and healthy organs constitute an important domain of host-tumor differences and offer possibilities to specifically target times of best tolerability and/or best efficacy which coincides in animal models (Levi et al. 2010). Clinical trials in colorectal cancer patients have shown that proper chemotherapy timing decreases the incidence of severe toxicities by up to five-fold and nearly doubles efficacy (Levi et al. 2010). However, a large impact of sex on optimal timing has been demonstrated both in mouse and human studies (Ballesta et al. 2017; Innominato et al. 2020), which highlighted the need for personalized chronotherapies.

Such precision medicine approach relies on a mechanistic understanding of circadian determinants of drug chronoefficacy, which requires the development of dedicated technological and mathematical tools. This work has been initiated for irinotecan, an anticancer drug active against digestive malignancies. The molecular basis of the chronotoxicity of irinotecan was deciphered though a systems pharmacology study which concluded that irinotecan chronotoxicity rhythm mainly originated from the daily variations of both the drug activation enzymes (carboxylesterases) and the detoxification enzymes (UGT1As). This provided a preclinical argument to further investigate those two protein families as possible clinical biomarkers for chronotherapy individualization (Ballesta et al. 2017).

Another clock-related anticancer strategy that has recently appeared consists in directly targeting the clock of tumor cells as the circadian clock is a natural regulator of key cellular processes. Two agonists of the clock genes REV-ERBs – SR9009 and SR9011 – have been proven to be efficient against cancer cells, even in the absence of functional p53 or under hypoxic conditions, while having no effect on normal tissues (Sulli et al. 2018b). These findings open the way to new anticancer strategies consisting of pharmacological modulation of circadian regulators.

Cross-References

▶ Cell Cycle Regulation
▶ Melatonin

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Cl^- Channels and Cl^-/H^+ Exchangers

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Synonyms

Anion channels; Chloride channels

Definition

Chloride channels are membrane proteins that allow for the passive flow of anions across

biological membranes. As chloride is the most abundant anion under physiological conditions, these channels are often called chloride channels instead of anion channels, even though other anions (such as iodide, nitrate, bicarbonate, or larger organic anions) may also permeate them. A major family of chloride channels, the CLC proteins, comprises Cl^- channels as well as Cl^-/H^+-exchangers. Both will be reviewed here.

Basic Characteristics

Chloride channels are membrane proteins with several transmembrane domains, which form a pore that allows for the passive flow of anions along their electrochemical gradient. Like other channels, chloride channels can be opened or closed by a process called gating. Gating can be influenced by several factors, e.g., by the transmembrane voltage in voltage-gated chloride channels, by intracellular Ca^{2+} in Ca^{2+}-activated chloride channels, by extracellular ligands such as glycine or GABA as in ligand-gated chloride channels, by cAMP-dependent phosphorylation, by cell swelling, or by extracellular acidification. Chloride channels may be present in the plasma membrane or in the membranes of intracellular organelles. Several intracellular CLC proteins that were previously thought to be Cl^- channels turned out to rather be electrogenic Cl^-/H^+ exchangers. They will be included in this entry.

Classification of Chloride Channels

Chloride channels can be classified by their biophysical characteristics (e.g., single-channel conductance), regulation (e.g., voltage-dependent, ligand-gated, swelling-activated, Ca^{2+}-activated), or by their sequence (gene families).

Gene Families of Chloride Channels

Chloride channels are highly heterogeneous, with several phylogenetically unrelated genes or gene families encoding anion-selective channel proteins with strikingly different functional properties and physiological roles. The following molecular classes of mammalian chloride channels are well established: CLC chloride channels and Cl^-/H^+ exchangers, ligand-gated chloride channels (GABA and glycine receptors), TMEM16/ANO Ca^{2+}-activated chloride channels and phospholipid scramblases, the cystic fibrosis transmembrane conductance regulator CFTR, bestrophin Ca^{2+}-activated chloride channels, LRRC8/VRAC volume-regulated anion channels, and TMEM206/ASOR acid-activated anion channels.

CLC chloride channels form a large gene family with members in bacteria, archaea, and eukaryotes. Many CLCs gate in a voltage-dependent manner. In mammals, there are nine different genes encoding CLC proteins. While bacterial CLC proteins appear to exclusively function as Cl^-/H^+ exchangers, roughly half of vertebrate CLCs are Cl^-/H^+ exchangers that reside mainly in intracellular membranes; the other half are plasma membrane Cl^- channels. They function as dimers, with each monomer having its own pore ("double-barreled" channels). Some CLC channels or transporters have accessory β-subunits.

$GABA_A$ and glycine receptors are ligand-gated chloride channels that belong to the superfamily of pentameric ligand-gated channels or Cys-loop receptors which also include cation-selective channels (e.g., nicotinic acetylcholine receptors, $5\text{-}HT_3$ receptors). Ligand-gated chloride channels are involved in synaptic transmission and mostly exert inhibitory signals in the adult nervous system due to the direction of the chloride concentration gradient. These channels are important pharmacological targets, but will not be discussed in detail here (refer to the following entries: ▶ GABAergic System, ▶ Glycine Receptors).

TMEM16/ANO is a large family of proteins that mediate Ca^{2+}-activated phospholipid scrambling and in some cases ionic currents in the plasma membrane or membranes of intracellular organelles. A subset of TMEM16 proteins comprises selective plasma membrane chloride channels activated by intracellular Ca^{2+} which play roles in a wide variety of physiological processes. Similar to the unrelated CLC channels, TMEM16 proteins form dimers with one ion permeation pathway or scrambling domain per monomer.

The cystic fibrosis transmembrane conductance regulator (CFTR) is the only member of the large ABC-transporter gene family known to function as a chloride channel. Mutations in CFTR underlie **cystic fibrosis**, a potentially lethal disease affecting the lungs and other tissues such as the intestine and kidney where CFTR-mediated chloride excretion or resorption is important. Reflecting its heritage as a transporter, the permeation pathway of CFTR is formed within a monomer.

Bestrophins are a comparatively understudied family of plasma membrane Ca^{2+}-activated chloride channels, comprising four members (Best1–4). Mutations in Best1 cause macular degeneration, but little is known about the other isoforms. Bestrophins form pentameric channels.

LRRC8 volume-regulated anion channels (*VRACs*) are encoded by a gene family encompassing five members (LRRC8A-E) found in vertebrates. These ubiquitously expressed channels mediate the efflux of chloride and small organic molecules upon cell swelling, thereby facilitating cell volume regulation. The finding that VRACs also transport other substrates including drugs and signaling molecules such as neurotransmitters suggests their involvement in other physiological and pathological processes beyond volume regulation. LRRC8 proteins assemble as hetero-hexamers around a central pore.

TMEM206/ASOR acid-activated anion channels are the most recent addition to the known chloride channels. They appear to be ubiquitously expressed in all vertebrate cells and play a role in acid-induced cell death. Further physiological functions of these enigmatic channels remain to be discovered. The architecture of TMEM206 channels is not yet clear, but they may assemble to trimers akin to the structurally similar ENaC/degenerin or ASIC cation channels.

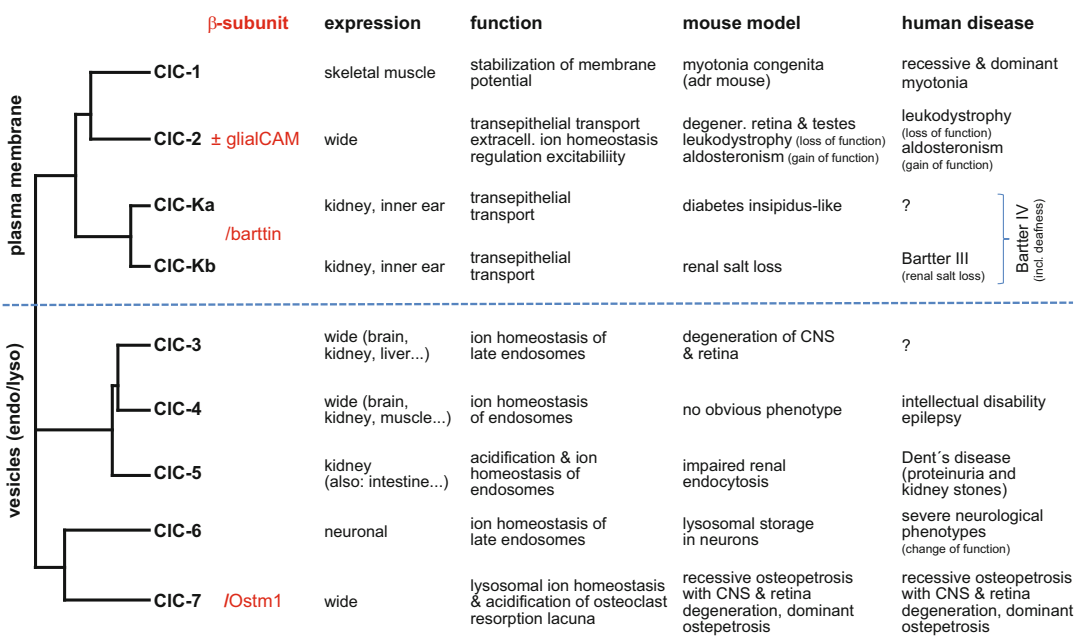
It has to be noted that several other transport proteins beyond those listed here have been shown to conduct chloride to some extent. For instance, pannexins which serve as a major pathway for cellular ATP release appear to be mostly anion-selective. Likewise, several members of the large SLC family of solute carriers can mediate passive chloride fluxes, including excitatory amino acid transporters (EAATs) and the prostaglandin transporter SLCO2A1, which has recently been reported to mediate the long-elusive Maxi-Cl currents.

The CLC Family of Chloride Channels and Transporters in Mammals

There are nine different CLC isoforms in mammals; the physiological roles of which are apparent from human diseases or mouse models in which these genes are disrupted (Jentsch and Pusch 2018). Based on homology, they can be classed into three branches (Fig. 1). The first branch includes the plasma membrane chloride channels ClC-1, ClC-2, ClC-Ka, and ClC-Kb.

ClC-1 is a skeletal muscle chloride channel, mutations in which lead to **myotonia**, a muscle stiffness that is associated with a hyperexcitability of the muscle plasma membrane. Thus, the high resting chloride conductance in muscle is necessary for its electrical stability. ClC-1 shows a distinct voltage-dependence and is activated by depolarization.

ClC-2 is a broadly expressed chloride channel that mediates an inwardly rectifying current slowly activated by hyperpolarization. It can also be activated by cell swelling and by moderate extracellular acidification. In mice, disruption of ClC-2 leads to leukodystrophy, infertility, and blindness. Likewise, ClC-2 loss-of-function mutations in humans cause leukodystrophy, a form of neurodegeneration restricted to the white matter of the brain, and were associated with infertility and visual impairments in few patients. The observed tissue degeneration may be caused by a defective regulation of extracellular ion homeostasis. In glial cells, ClC-2 associates with the cell adhesion molecule GlialCAM, which stabilizes and directs ClC-2 to cell-cell contacts and increases its current amplitudes by virtually abolishing its voltage-dependent gating. Loss of GlialCAM, disruption of its interaction with ClC-2, or loss of MLC1, another interaction partner, also led to leukodystrophy in patients and mouse models. Gain-of-function mutations in ClC-2 have recently been shown to cause primary aldosteronism in mice and man by causing a depolarization of adrenal glomerulosa cells.



Cl^- Channels and Cl^-/H^+ Exchangers, Fig. 1 The family of mammalian CLCs and associated proteins. In mammals, three homology branches can be identified (shown in dendrogram at left). Whereas members of the first branch function as plasma membrane voltage-gated anion channels, proteins of the two other branches are rather Cl^-/H^+ exchangers that reside predominantly on endosomes and lysosomes. ClC-1 and the two ClC-K isoforms show highly specific tissue expression patterns, whereas ClC-2 and the vesicular Cl^-/H^+ exchangers (with the exception of ClC-5/6) are widely and probably even ubiquitously expressed across tissues. Both ClC-K isoforms and ClC-7 need specific auxiliary proteins (β-subunits) for functional expression and protein stability. ClC-2 can function without a β-subunit but associates with the cell adhesion molecule GlialCAM in glia, which changes its localization, stability, and biophysical properties. These differential requirements for ancillary proteins are symbolized by "/" and "±", respectively. The main phenotypes of *Clcn* mouse models or human diseases caused by *CLCN* mutations are indicated. The loss of the common β-subunit barttin, or the loss of both ClC-K isoforms, leads to a combination of severe renal salt loss and congenital deafness, which in humans is known as Bartter syndrome type IV

ClC-Ka and ClC-Kb are highly homologous epithelial chloride channels that require the β-subunit barttin for proper function. Both channels are expressed specifically in different parts of the kidney and the inner ear, where they facilitate transepithelial chloride transport. Mutations in ClC-Kb lead to a form of **Bartter syndrome** (type III), a disease associated with severe renal salt loss. The disruption of the mouse ortholog of ClC-Ka causes a syndrome resembling nephrogenic diabetes insipidus. Mutations in the β-subunit barttin entail the loss-of-function of both, ClC-Ka and ClC-Kb, resulting in the more severe Bartter syndrome type IV with deafness. Considering the important role of the ClC-K/barttin channels in renal salt and fluid reabsorption, they are attractive candidate targets for the development of alternative diuretics that may also be useful in conditions of renal failure.

In contrast to the CLC channels, the two other branches of the CLC family including ClC-3 through ClC-7 are Cl^-/H^+ exchangers that reside in intracellular membranes of the endocytic pathway. Their function on those vesicles is presumably to facilitate H^+-ATPase-mediated acidification by providing an electrical shunt. Somewhat counterintuitively, this can be achieved more efficiently by a Cl^-/H^+ exchanger than by a pure chloride channel. However, alterations in endolysosomal pH were not consistently observed in knockout mouse models for intracellular CLCs and are absent in some (e.g., ClC-7). Cl^-/H^+

exchangers accumulate intravesicular chloride, resulting in an inside-negative electrical potential, both of which may be required for the biological function of endocytic organelles. The antiport stoichiometry of most CLC exchangers has been shown to be $2Cl^-:1H^+$.

The second CLC homology branch includes ClC-3 through ClC-5, which are ~80% identical (Fig. 1). The expression of ClC-3 and ClC-4 appears to be broad, while ClC-5 is predominantly expressed in the kidney.

ClC-3 mainly localizes to endosomes but can also be detected in a subset of synaptic vesicles. Surprisingly, the disruption of *Clcn3* in mice resulted in a drastic degeneration of the hippocampus and the retina, but the underlying mechanism remained obscure.

Even less is known about ClC-4, which also appears to be present in endosomal compartments. *Clcn4*$^{-/-}$ mice display no obvious phenotype, but several loss-of-function mutations in *CLCN4* have been linked to syndromic intellectual disability associated with behavior and seizure disorders in humans. As for ClC-3, the molecular mechanism remains enigmatic. A recent study suggests intimate functional interactions between ClC-4 and ClC-3, which form heteromers in which ClC-3 stabilizes ClC-4. ClC-4 can partially compensate for loss of ClC-3 activity, as illustrated by the more severe phenotype of double knockout animals.

ClC-5 is predominantly expressed in the kidney proximal tubule, where it localizes to endosomes. Disruption of ClC-5 leads to a defect in endocytosis in mouse models as well as in human Dent's disease, a disorder associated with proteinuria and kidney stones. The deficiency in endocytosis leads to secondary changes in calciotropic hormones, resulting in tertiary changes such as hyperphosphaturia, hypercalciuria, and the formation of kidney stones.

ClC-6 and ClC-7 define the third branch of the CLC family (Fig. 1). These proteins are only about 45% identical to each other. Whereas ClC-7 is very broadly expressed, the ClC-6 protein seems to be restricted to the nervous system.

ClC-6 is a late endosomal Cl^-/H^+ exchanger. Its disruption in mice led to mild neuronal lysosomal storage disease, whereas *CLCN6* missense mutations were found in patients with developmental delay and severe neurological symptoms.

ClC-7 is expressed in late endosomes and lysosomes. It needs Ostm1 as β-subunit. The disruption of either ClC-7 or Ostm1 in mice and man leads to severe osteopetrosis, retinal degeneration, and lysosomal storage disease. ClC-7/Ostm1 is highly expressed in osteoclasts, which degrade calcified bone in strongly acidic lysosome-derived structures called resorption lacunae. In mice lacking ClC-7, resorption lacunae fail to form adequately. Accordingly, it was assumed that ClC-7/Ostm1 is required for efficient acidification of lysosomes and the resorption lacuna. However, lysosomal pH was found to be unchanged in several ClC-7/Ostm1 mouse models. Lysosomal pathologies may instead be caused by an observed reduction in lysosomal $[Cl^-]$. Failure to acidify the resorption lacuna may be explained by perturbed formation of the acid-secreting ruffled border by lysosomal exocytosis. ClC-7 may be an interesting target for the treatment of osteoporosis as its partial inhibition might increase bone mass. This notion is indirectly supported by the observation that patients that are heterozygous for dominant negative ClC-7 mutations (a situation expected to lead only to a partial inhibition of ClC-7) present with a milder form of osteopetrosis in which retinal degeneration is absent.

TMEM16/ANO Ca^{2+}-activated Chloride Channels

The *TMEM16/ANO* gene family comprises ten members in humans (TMEM16A, B, C, D, E, F, G, H, J, and K), of which only two encode bona fide plasma membrane chloride channels activated by intracellular Ca^{2+} (TMEM16A and B). The other family members are thought to be Ca^{2+}-activated phospholipid scramblases, which may also mediate ionic currents of varying selectivity (Falzone et al. 2018). These will not be discussed here. TMEM16A and B differ in their expression profiles and biophysical properties, with TMEM16A being much more sensitive to Ca^{2+}. Whereas TMEM16A is expressed a variety of

cells types including epithelial, neuronal, and smooth muscle cells, TMEM16B expression appears to be more restricted to neurons.

TMEM16A has been shown to be essential for epithelial fluid secretion, e.g., in the salivary gland. In respiratory and digestive organs, it may support secretory functions in conjunction with CFTR, making it an interesting alternative drug target for cystic fibrosis. TMEM16A is also involved in contractibility of vascular smooth muscle cells and plays a role in blood pressure regulation. In interstitial cells of Cajal, TMEM16A is responsible for the generation of slow wave activity, which serves as pacemaker for stomach peristalsis. Curiously, TMEM16A is also upregulated in many cancer types and has been suggested as a diagnostic marker.

In neuronal cells, Ca^{2+}-activated chloride channels may serve excitatory or inhibitory roles, depending on the intracellular chloride concentration. TMEM16A, which is steeply temperature-dependent, has been suggested to be responsible for sensing heat in a subset of nociceptive neurons. Expression of both, TMEM16A or B, has been demonstrated in visual and olfactory systems. TMEM16A amplifies signal transduction in olfactory neurons, without, however, having a significant effect on olfaction in mice. Although expression of TMEM16B in brain is generally very low, some studies have reported neuromodulatory roles of TMEM16B in several brain regions including amygdala, lateral septum, and medulla oblongata.

CFTR

CFTR is considered a "broken" ABC-transporter as it is the only channel in this protein family, which comprises ATP-dependent pumps for a large variety of substrates. It is activated by the cAMP-dependent protein kinase A, which phosphorylates CFTR. Phosphorylated CFTR is gated by ATP binding. The channel is expressed in many epithelia, e.g., in apical membranes in the lung, pancreas, and intestine where it is essential for fluid secretion. Loss-of-function mutations in CFTR underlie cystic fibrosis, a potentially lethal and fairly common (~1 in 3,000) disease with impaired production of mucus, digestive fluids, and sweat. In addition to working as a chloride channel, several other functions such as the regulation of other ion channels or being a receptor for bacteria have been attributed to CFTR.

Bestrophins

Bestrophins represent another family of Ca^{2+}-activated chloride channels (Hartzell et al. 2008). There are four different bestrophin isoforms (Best1–4) in humans. Mutations in Best1 cause vitelliform macular dystrophy (**Best's disease**), but the underlying molecular mechanism is still not fully understood. A moderately wide expression pattern of Best1 may hint at physiological roles beyond those in the retina. Little is known about Best2–4 expression and function. All four human isoforms induce chloride currents when expressed heterologously. Although they show a dependence on intracellular calcium, their biophysical properties differ from Ca^{2+}-activated chloride channels of the unrelated TMEM16 family. Mutations in Best1 altered biophysical properties of currents induced by overexpression, lending support to the hypothesis that bestrophins themselves are Cl^- channels. This was confirmed by recent high-resolution structures of Best1.

LRRC8/VRACs: Volume-Regulated Anion Channels

VRACs (volume regulated anion channels) are a relatively recent addition to the known chloride channel proteins, having been identified as LRRC8 (leucine-rich repeat 8) heteromers in 2014 (Jentsch 2016). VRACs are found in all vertebrate cell types, appear mostly inactive under iso-osmotic conditions, and open upon cell swelling or a reduction in intracellular osmolarity. Extrusion of chloride and organic osmolytes through VRACs drives water loss and volume regulation in swollen cells. Functional VRACs are formed when the obligatory subunit LRRC8A heteromerizes in hexameric complexes with at least one of the other members of the protein family (LRRC8B-E), which determine permeation properties of the resulting channels. For instance, channels containing LRRC8D are permeable for organic compounds such as taurine, lysine or the neurotransmitters GABA, and

glutamate. The stoichiometry of LRRC8 heteromerization is poorly understood but may be quite flexible and thus enable the formation of a large variety of VRACs with different substrate specificities. Disruption of LRRC8A in mice leads to high pre- and postnatal lethality and tissue abnormalities in many organs. LRRC8A-dependent chloride currents have been shown to be involved in apoptosis induction, insulin release, adipocyte function, and cell volume regulation in developing spermatozoa. Besides their roles based on chloride and osmolyte extrusion upon cell swelling, the transport of signaling molecules and drugs by VRACs implicates these channels in several other (patho)physiological processes. For instance, commonly used platin-based anti-cancer drugs enter cells in part through LRRC8D-containing VRACs. By conducting the second messenger cGAMP (cyclic-GMP-AMP), LRRC8/VRAC channels also have a role in innate immunity. Astrocytic VRACs mediate tonic non-vesicular glutamate release, thereby tuning glutamatergic synaptic transmission. Mice lacking LRRC8A in astrocytes exhibited learning and memory deficiencies but were protected against excitotoxic cell death in a cerebral stroke model, corroborating earlier findings that (unspecific) VRAC inhibitors improved outcomes in such models. VRACs were reported to mediate pulsatile GABA secretion from pancreatic beta cells, an important autocrine and paracrine process regulating homeostasis in islets of Langerhans. However, it is unclear why release through VRACs should be pulsatile.

TMEM206/ASOR Acid Activated Anion Channels

A chloride current induced by strong extracellular acidification below pH 5.5 has been described to be universally present in mammalian cells. The corresponding channel was termed ASOR (acid-sensitive outward rectifier) or PAORAC (proton-activated outwardly rectifying anion channel), but its molecular correlate has only been identified in 2019 by two independent groups (Ullrich et al. 2019; Yang et al. 2019). TMEM206 (also named PAC for proton-activated chloride; or PACC1 for proton-activated chloride channel 1) is a ubiquitously expressed membrane protein that is necessary and sufficient to form functional ASOR channels. Disruption of TMEM206 protects cells against acid-induced cell death, which may occur under ischemic conditions. Accordingly, mice lacking TMEM206 were partially protected in a model for ischemic cerebral stroke. However, the physiological functions of TMEM206/ASOR remain unresolved to date.

Drugs

The pharmacology of chloride channels is still poorly developed. Specific and highly useful inhibitors or modulators (e.g., strychnine, picrotoxin, diazepams) are only available for ligand-gated chloride channels, but these are covered in a different chapter (see ▶ GABAergic System and ▶ Glycine Receptors). Another exception in this regard is CFTR, which has been the target of intense drug development to enable treatment of cystic fibrosis. Depending on the underlying mutation, drugs with different mechanisms of action may be used, including activators facilitating channel opening (e.g., ivacaftor), potentiators aiding correct folding and trafficking (e.g., lumacaftor, tezacaftor, elexacaftor), and compounds termed amplifiers which increase CFTR mRNA levels (e.g., nesolicaftor).

For other targets, there are several "chloride channel inhibitors" such as the stilbene disulfonates DIDS and SITS, 9-anthracene-carboxylic acid (9-AC), arylaminobenzoates such as DPC and NPPB, niflumic acid and derivatives, sulfonylureas, and zinc and cadmium. All of these, however, are not particularly specific. Several of them (e.g., DIDS) inhibit many chloride channels only partially even at millimolar concentrations and have effects on other types of transport proteins. CLC channels are quite insensitive to DIDS but can often be inhibited by Zn^{2+} or Cd^{2+} in the submillimolar range. 9-AC is a quite specific inhibitor for the muscle channel ClC-1, which can also be inhibited by clofibric acid derivatives. Inhibitors with affinities below 10 μM are

available for ClC-K channels, and inhibitors with nanomolar affinities are being developed for ClC-2.

Lubiprostone, a drug used for treating obstipation, has been claimed to be an activator of ClC-2. This was based on a paper showing activation by lubiprostone of currents thought to represent ClC-2. These currents, however, differ starkly from typical ClC-2 currents. Furthermore, ClC-2 is located in basolateral membranes of the intestine. This localization is incompatible with the hypothesis that its activation increases intestinal chloride and fluid secretion. Thus, the claim that lubiprostone is a chloride channel activator must be subject to considerable doubt. Being a prostone, it might rather affect ion transport by stimulating prostaglandin receptors.

TMEM16/ANO Ca^{2+}-activated chloride channels are sensitive to niflumic acid and derivatives, which also inhibit VRACs. More specific inhibitors of TMEM16A-mediated currents, such as T16Ainh-A01, CaCCinh-A01, and MONNA, have been developed. Activation of TMEM16A was proposed as an alternative strategy in cystic fibrosis therapy. Denufosol (INS37217) and duramycin (Moli-1901) have been employed to this end, but clinical trials have yielded disappointing results.

LRRC8/VRAC channels can be inhibited by tamoxifen, carbenoxolone, and DCPIB, all of which are quite unspecific. Likewise, no specific inhibitors of TMEM206/ASOR are available. However, these channels can be blocked by micromolar concentrations of pregnenolone sulfate. Development of potent and specific drugs targeting these channels may be beneficial for the acute treatment of ischemic stroke.

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Classic Hallucinogen

▶ Psychedelic Drugs

Clinical Chemistry

▶ Ca^{2+}-Binding Proteins
▶ S100 Proteins

CNP

▶ C-Type Natriuretic Peptide and Its Receptors

COMT

▶ Catechol-*O*-Methyltransferase

COMTase

▶ Catechol-*O*-Methyltransferase

Connexin Hemichannels

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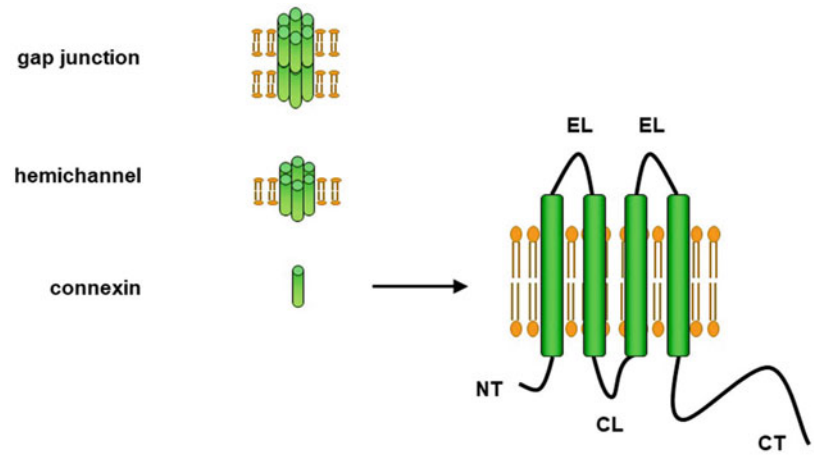
Synonyms

Gap junction hemichannels

Definition

Homeostasis is controlled by a myriad of extracellular, intracellular, and intercellular communication mechanisms. Typically, extracellular signals trigger intracellular signal transduction cascades. Several of these intracellular signals are subsequently propagated to adjacent cells. Such intercellular connections are formed by arrays of gap junctions. They are composed of two hemichannels of neighboring cells, which in turn are built up by six connexin (Cx) proteins (Fig. 1). More than 20 different connexin species have been identified. They are all designated based upon their molecular weight as predicted by cDNA sequencing and are expressed in a cell-type-specific way (Table 1). Among those, Cx43, with a molecular weight of 43 kDa, is the most widespread connexin species in vertebrate cells. Connexins share a similar structure consisting of four transmembrane domains, two extracellular loops, one cytosolic loop, one cytosolic carboxyl-terminal tail, and one cytosolic aminotail (Fig. 1) (Vinken 2015; Maes et al. 2015).

Gap junctions mediate the passive intercellular diffusion of small and hydrophilic molecules (Fig. 2). Numerous physiological processes are regulated by substances that are intercellularly exchanged via gap junctions, and hence gap junctional intercellular communication is considered as a key mechanism in the control of tissue homeostasis. In the last decade, it has become clear that connexin hemichannels also provide a pathway for communication independent of gap junctional intercellular communication, albeit between the intracellular compartment and the extracellular milieu. The messengers that are involved in this type of extracellular communication are, however, very similar to those exchanged through gap junctions (Fig. 2). Unlike gap junctions, connexin hemichannels are closed in physiological conditions. They only open up in stress conditions, and by doing so, they facilitate processes such as inflammation and cell death in plethora of diseases, including in the brain, spinal cord, heart, blood vessels, intestine, liver, pancreas, lungs, kidneys, skin, eyes, muscles, and bones (Willebrords et al. 2016; Decrock et al. 2009). This renders connexin hemichannels very



Connexin Hemichannels, Fig. 1 *Structure of connexin hemichannels and connexin proteins*. Gap junctions are composed of two hemichannels, which in turn are built up by six connexin proteins. Connexin proteins consist of four transmembrane domains (green bars), two extracellular loops (EL), one cytosolic loop (CL), one cytosolic carboxyl-terminal tail (CT), and one cytosolic aminotail (NT)

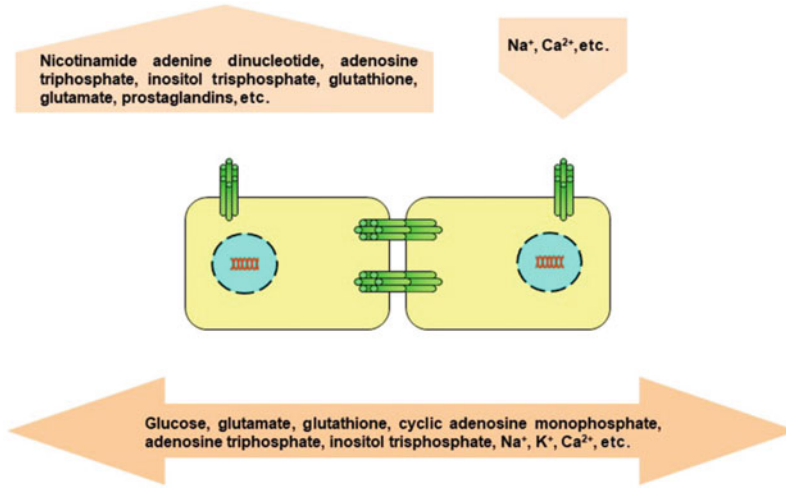
Connexin Hemichannels, Table 1 *Expression of connexins*. More than 20 different connexin (Cx) species are produced by vertebrate cells. They are all expressed in a cell-type-specific way

Organ	Cell type	Connexin species
Brain	Astrocytes	Cx30, Cx43
	Microglial cells	Cx26, Cx32
	Oligodendrocytes	Cx32, Cx47, Cx29
	Neurons	Cx36, Cx45
	Blood-brain barrier endothelial cells	Cx37, Cx40, Cx43
	Blood-brain barrier pericytes	Cx37, Cx40, Cx43
Heart	Smooth muscle cells	Cx40, Cx43, Cx45
	Cardiomyocytes	Cx40, Cx43, Cx45
Blood vessels	Endothelial cells	Cx43, Cx37, Cx40
	Smooth muscle cells	Cx43, Cx45, Cx40, Cx37
Liver	Sinusoidal endothelial cells	Cx26, Cx43
	Hepatic arteries and portal vein endothelial cells	Cx37, Cx40
	Hepatocytes	Cx26, Cx32
	Kupffer cells	Cx26, Cx43
	Stellate cells	Cx26, Cx43
Stomach and intestines	Stomach	Cx26, Cx32, Cx40, Cx43, Cx45
	Foveolar cells	Cx32
	Small intestine	Cx26, Cx31, Cx57
	Myenteric plexus cells	Cx36, Cx40, Cx43, Cx45
	Small intestine epithelial cells	Cx32, Cx37, Cx43
	Interstitial cells of Cajal	Cx43
	Colon	Cx31, Cx31.9, Cx43
	Musculus externa cells	Cx26, Cx40, Cx43
	Myenteric plexus cells	Cx36, Cx40, Cx43, Cx45
	Epithelial cells	Cx26, Cx32, Cx37, Cx43
	Muscularis mucosa cells	Cx43
Skin	Keratinocytes	Cx26, Cx30.3, Cx30, Cx31.1, Cx31, Cx40, Cx43, Cx45
	Fibroblasts	Cx43, Cx45, Cx40
	Melanocytes	Cx43
Kidney	Smooth muscle cells	Cx37, Cx45
	Podocytes	Cx43, Cx45
	Pericytes	Cx37
	Mesangial cells	Cx40, Cx45
Lung	Alveolar epithelium	Cx26, Cx32, Cx43, Cx46
Eye	Lens epithelial cells	Cx43, Cx46, Cx50
Immune cells	T cells	Cx40, Cx43
	B cells	Cx40, Cx43
	Monocytes	Cx43
	Macrophages	Cx37, Cx43
	Neutrophils	Cx43
	Dendritic cells	Cx43
Pancreas	B cells	Cx36, Cx43, Cx45
	Exocrine pancreas	Cx32
Skeletal muscle	Myoblasts	Cx39, Cx40, Cx43, Cx45

(continued)

Connexin Hemichannels, Table 1 (continued)

Organ	Cell type	Connexin species
Bone	Osteocytes	Cx37, Cx43
	Osteoblasts	Cx37, Cx43, Cx45, Cx46
	Osteoclasts	Cx37, Cx43
	Chondrocytes	Cx32, Cx43, Cx45, Cx46



Connexin Hemichannels, Fig. 2 *Function of gap junctions and connexin hemichannels*. Gap junctions and connexin hemichannels provide a pathway for the passive flux of small and hydrophilic substances between two neighboring cells and between an individual cell and its extracellular environment, respectively

attractive pharmacological targets, as their pharmacological closure may counteract the clinical manifestation of many pathologies. However, clinical exploration in this area is currently impeded by the lack of specific inhibitors of connexin hemichannels. Indeed, the vast majority of the connexin hemichannel inhibitors also suppress other channel types, including gap junctions. An exception in this respect are a number of peptides that reproduce specific amino acid sequences in the primary structure of connexin proteins (Willebrords et al. 2017) (Fig. 3).

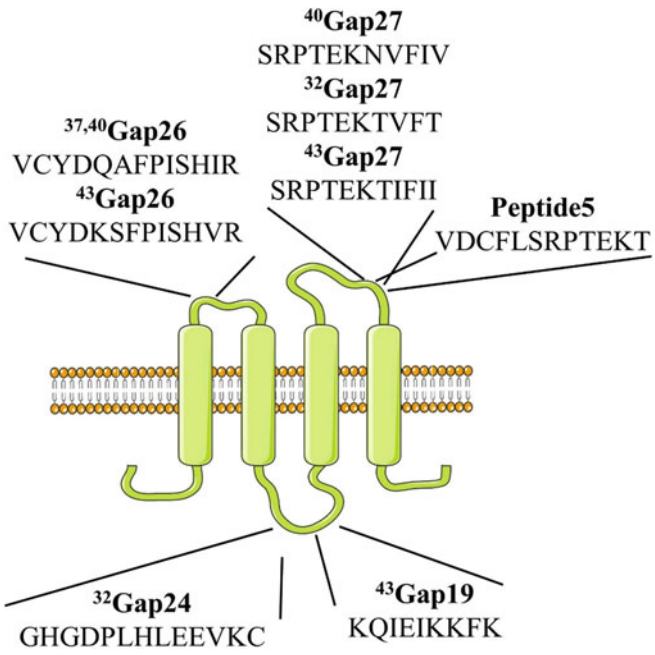
Basic Characteristics

Life Cycle

Most connexins are synthetized by membrane-bound ribosomes and co-translationally integrated into the endoplasmic reticulum, where six proteins oligomerize to form a connexin hemichannel. Connexin hemichannels packaged into vesicles are transported to the plasma membrane. They may remain uncoupled or dock with a connexin hemichannel of an adjacent cell, thereby creating a narrow extracellular space, a so-called 2–3 nm "gap." Connexin proteins usually display rapid turnover rates in comparison with other plasma membrane proteins. Both in vitro and in vivo, the half-lives of Cx26 and Cx32 have been found to be 2 and 3 h, respectively, whereas the turnover times of other integral membrane proteins generally range from 17 to 100 h. Upon degradation, gap junctions are internalized by one of the two opposing cells, resulting in the formation of "annular" gap junctions. These structures are further degraded by both lysosomes and proteasomes, although the precise degradation pathway depends on the identity of the connexin species and cell type (Vinken 2015; Maes et al. 2015).

Regulation

Short-term control of gap junction and connexin hemichannel opening is driven by a number of factors, including pH, transmembrane voltage,



Connexin Hemichannels, Fig. 3 *Connexin mimetic peptides*. Connexin mimetic peptides that inhibit connexin hemichannels reproduce amino acid sequences in the extracellular loop or cytoplasmic loop regions of connexin proteins

and calcium concentration. Posttranslational modifications, such as S-nitrosylation, sumoylation, and phosphorylation, also directly regulate their opening. Phosphorylation mainly occurs at the cytosolic carboxyl-terminal connexin tail. The regulation of gap junction and connexin hemichannel opening by phosphorylation is complex, as the outcome of this posttranslational modification depends on the nature of the kinase and connexin as well as on the cellular context. Furthermore, phosphorylation may have an opposing effect on gap junctions versus connexin hemichannels consisting of the same connexin species. Cx43 is a substrate for many kinases, including protein kinases A and C, members of the mitogen-activated protein kinase family, casein kinase 1, the cyclin-dependent kinase 1/cyclin B complex, and v-Src. Different from other connexins, shifts in electrophoretic mobility occur upon phosphorylation of Cx43. Usually, three bands appear during sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, representing the fast-migrating non-phosphorylated Cx43 isoform and two slow-migrating phosphorylated Cx43 isoforms.

Long-term control of gap junction and connexin hemichannel opening involves regulation at the transcriptional level of connexin expression. The architecture of most connexin genes is simple, consisting of a first exon that harbors the 5′-untranslated region, which is separated by an intron of varying length from a second exon, bearing the complete coding sequence, and the 3′-untranslated region. Connexin gene promoters show binding affinity for several general transcription factors, including activator protein 1, Yin Yang 1, and specificity protein 1. In parallel, a number of cell-type-specific transcription factors control connexin gene transcription. In the last decade, epigenetic mechanisms, including histone acetylation and DNA methylation, have also joined in as master regulators of connexin expression (Vinken 2015; Maes et al. 2015).

Drugs

Peptides Targeting the Connexin Extracellular Loop

Given its ubiquitous presence in a wide spectrum of cells, most efforts to produce connexin mimetic peptides have been focused on Cx43. Among the first peptides were ^{43}Gap26 and ^{43}Gap27, which reproduce amino acid sequences in the first and

second extracellular loops of Cx43 (Fig. 3). These peptides are thought to interact with complementary sites within the extracellular loop regions of Cx43 of opposing connexin hemichannels and thereby to prevent their docking and hence the formation of gap junctions. In course of time, it has been found that they also affect channels consisting of Cx32, Cx40, and Cx37. In vitro, both ^{43}Gap26 and ^{43}Gap27 inhibit connexin hemichannel-mediated adenosine triphosphate release and dye uptake upon short exposure, while longer exposures also result in the suppression of the corresponding gap junction activity. Additionally, exposure of cells to ^{43}Gap27 may have effects on Cx43 phosphorylation, especially occurring at serine368, and therefore could influence Cx43 channel activity. Both peptides have been used extensively in studies of the immune system, vascular system, heart, lung, brain, skin, and liver. In order to generate more specific inhibitors, a modified Gap27 peptide, namely, ^{40}Gap27, was developed. This peptide incorporates a sequence in the second extracellular loop of Cx40 and has also proven useful in vascular tissues. The same was performed for a part of the second extracellular loop of Cx32, generating ^{32}Gap27 (Fig. 3), which showed inhibition of adenosine triphosphate release in primary hepatocytes and no effect on gap junction activity.

^{43}Gap26M is a modified version of ^{43}Gap26 that has an acylated aminoterminus and shows greater solubility and stability. ^{43}Gap26M significantly increased migration rates across scrapes in keratinocytes and fibroblasts by blocking gap junction functionality.

Peptide5 is a peptide that shifted five amino acids in aminoterminal direction compared to Gap27 (Fig. 3). It inhibits connexin hemichannels at low (5–10 μM) concentration, while equally inhibiting gap junctions at higher concentration (100 μM). In vitro, peptide5 has been used to study a variety of cellular processes, such as dynamic calcium signals, release of neurotransmitters as well as propagation of cell death. In vivo, peptide5 reduces tissue damage secondary to spinal cord injury and attenuates vascular permeability increase following retinal ischemia/reperfusion insult (Willebrords et al. 2017).

Peptides Targeting the Connexin Cytoplasmic Loop

The L2 peptide, corresponding to a sequence in the cytoplasmic loop moiety of Cx43, inhibits its connexin hemichannel opening without affecting gap junctions independent of time of exposure. Based on this observation, the hypothesis was raised that prevention of the interaction between the carboxyl-terminal tail and the cytoplasmic loop areas of Cx43 leads to connexin hemichannel closure, which means that this interaction is necessary for connexin hemichannel opening. By contrast, carboxyl-terminal tail-cytoplasmic loop interaction results in the closure of gap junctions. Indeed, physical binding of the carboxyl-terminal tail and the L2 region of Cx43 in the cytoplasmic loop induces a residual state with very low conductance.

It has been shown that Gap19, a synthetic nonapeptide derived from the cytoplasmic loop region of Cx43, inhibits its connexin hemichannel activity without influencing the corresponding gap junction activity over time. Connexin hemichannel inhibition is due to the binding of Gap19 to the carboxyl-terminal tail area of Cx43, thereby preventing intramolecular carboxyl-terminal tail-cytoplasmic loop interactions. By doing so, Gap19 was found to inhibit Cx43 hemichannels in Cx43-overexpressing C6 rat glioma cells, cultured primary mouse astrocytes, and murine hippocampal slices. Blocking Cx43 hemichannels has been recently investigated as a therapeutic intervention for various diseases, including cardiac ischemia/reperfusion insults, myocardial infarction, cardiac arrhythmia, wound healing, Parkinson's disease, spinal cord injury, acute liver failure, liver fibrosis, and nonalcoholic steatohepatitis.

^{32}Gap24, which mimics a sequence in the cytoplasmic loop region of Cx32 (Fig. 3), specifically inhibits Cx32 hemichannels and not their full channel counterparts. ^{32}Gap24 completely blocked calcium-triggered adenosine triphosphate responses in ECV304 bladder cancer epithelial cells. It is believed that ^{32}Gap24 acts in a mechanistically similar way as Gap19 (Willebrords et al. 2017).

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Consumption

▶ Tuberculosis

Contraceptives

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Synonyms

Hormonal contraceptives; Oral contraceptives

Definition

Hormonal contraceptives belong to the most widely prescribed and most efficacious drugs that have a profound impact on western societies since their inauguration in the 1960s. In women, oral hormonal contraceptives are used to prevent fertilization or implantation in cases of unplanned pregnancies. Apart from these primary objectives, there are significant additional medical benefits contributing to a substantial improvement of reproductive health in women.

Mechanism of Action

The most frequently used oral contraceptives are composed of varying combinations of estrogens and progestins, which belong to the large family of steroid hormones. Steroids interact with intracellular receptors functioning as ligand-activated transcription factors to control the expression of a wide array of specific genes. The receptors for estrogens and progestins are members of a superfamily of approximately 50 structurally related nuclear receptors that bind ligands such as steroid hormones, retinoids, vitamin D_3, eicosanoids, and thyroid hormones (Fuentes and Silveyra 2019). Members of this receptor superfamily share a common architecture of mainly four conserved functional domains: The N-terminal transactivation domain (activation function, AF-1) is the most variable in the superfamily of receptors. The subsequent DNA-binding domain also participates in receptor dimerization, whereas nuclear localization is determined by the neighboring hinge region. The C-terminal hormone-binding domain comprises a ligand-dependent transcriptional activation function (AF-2) and provides the sites for the binding of chaperones like heat shock proteins that prevent dimerization and DNA binding of unliganded receptors.

Upon hormone binding, steroid hormone receptors undergo a conformational change, dissociate from heat shock proteins, and translocate into the nucleus where they interact as dimers with specific DNA regulatory sequences of target genes. The transcriptional regulation by steroid hormone receptors is mediated by co-regulatory proteins that either positively (co-activators) or negatively (co-repressors) influence steroid hormone-induced transcriptional activity. A complementary mode of steroid hormone receptor action relies on direct protein–protein interactions with other transcription factors such as AP-1, Sp-1, and NF-κB, thus providing an explanation for the well-known observation that steroid hormone receptors are able to regulate genes lacking consensus response elements in their noncoding 5′ region (Turgeon et al. 2006).

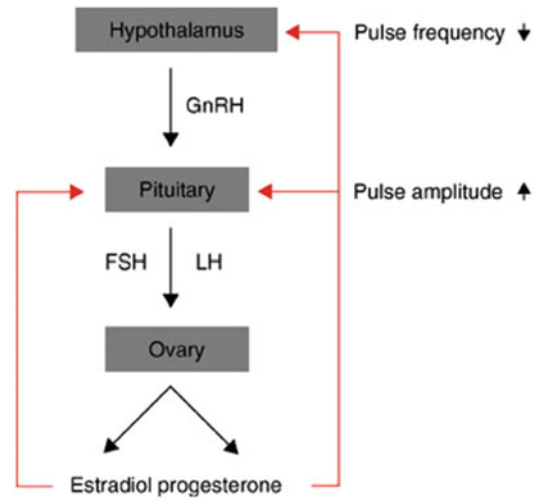
Most nuclear receptors are phosphoproteins, and their function can be influenced by

phosphorylation events that are initiated by membranous receptors like receptor tyrosine kinases or G-protein-coupled receptors. Thus, steroid hormone receptors are embedded in complex signaling networks that may give rise to ligand-independent activation of nuclear receptors. In addition to the well-understood nuclear events set in motion by steroid hormones, rapid nongenomic effects have been reported for estrogens and progesterone. These rapid effects of sex-steroids that are often initiated at the plasma membrane may result from steroid effects on membranous receptors other than classical steroid hormone receptors, for example, the G protein-coupled estrogen receptor (Prossnitz and Arterburn 2015). In particular, the rapid engagement of the MAP kinase cascade by estrogen has been studied in great detail, and it is quite likely that cardinal estrogenic effects like cell proliferation and survival are not primarily brought about by genomic actions of classical nuclear receptors but by rapid, nongenomic mechanisms.

At present, two distinct nuclear estrogen receptors, ERα and ERβ, are known. The receptors differ in their tissue distribution, ligand binding profile, and transcription activation functions in that ERβ is devoid of AF-1. In cells, that express both estrogen receptors, ERβ appears to oppose the transcriptional activity of ERα. Due to two distinct estrogen-dependent promoters in the single progesterone receptor (PR) gene, two isoforms of the progesterone receptor, PR-B and the N-terminally shortened PR-A, are generated. The biological activities of PR-A and PR-B are distinct and depend on the target tissue in question. In the reproductive system, progesterone acting through its respective nuclear receptors, PR-A and PR-B, serves the role of a physiological negative regulator of estrogen action by causing depletion of estrogen receptors. In addition, the major role of PR-A may be to inhibit transcriptional activity of other steroid receptors like ERα. Although one aspect of the biological relationship between estrogen and progesterone may be called functional antagonism, a concerted, sequential action of estrogen and progesterone is required in reproductive tissues like the endometrium and the breast to yield the desired complex biological response.

Estrogen and progesterone play a central role in the neuroendocrine control of the female menstrual cycle. In the early follicular phase, estrogen exerts an inhibitory effect on the pulsatile secretion of gonadotropins from the pituitary (Fig. 1). Thus, the gradual increase in the peripheral estradiol concentration during the follicular phase is accompanied by a reduced release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the gonadotropes. At midcycle, a different set of regulatory interactions becomes dominant. A sustained elevation of estradiol (150–200 pg/ml for approximately 36 h) induces a positive feedback on the anterior pituitary to trigger the ovulatory surge of LH. The underlying mechanism is a sensitization of the pituitary gonadotropes towards hypothalamic gonadotropin-releasing hormone (GnRH). Progesterone decreases the frequency of GnRH release from the hypothalamus resulting in a marked decrease of the frequency of gonadotropin pulses in the luteal phase (Fig. 1). At the same time, progesterone increases the amount of LH released per pulse (i.e., the pulse amplitude).

Estrogen exerts a negative feedback on the pituitary and decreases the secretion of FSH and LH during most of the menstrual cycle. Yet, it triggers the LH surge at midcycle by sensitizing the pituitary to hypothalamic GnRH. Progesterone increases the amplitude of gonadotropins



Contraceptives, Fig. 1 Regulation of hypothalamic and pituitary function by ovarian steroid hormones

released from the pituitary and at the same time decreases the GnRH pulse frequency at the level of the hypothalamus. These feedback controls give rise to fairly frequent LH pulses of low amplitude during the follicular phase of the cycle and less frequent pulses with a higher amplitude in the luteal phase.

The main mechanism of action of a combination oral contraceptive (a combination oral contraceptive contains both an estrogenic and a progestational component to achieve contraception) is to prevent ovulation by inhibiting gonadotropin secretion via an effect on both pituitary and hypothalamic centers. The progestational component primarily suppresses the surge-like LH release required to induce ovulation, while the estrogenic agent suppresses FSH secretion and thus prevents selection of a dominant follicle. Therefore, both estrogenic and progestational components of an oral contraceptive synergistically contribute to the contraceptive efficacy. However, even if follicular growth were not sufficiently inhibited, the progestational agent alone would suffice to abrogate the ovulatory LH surge. The estrogenic component, however, serves at least two other important purposes. It is responsible for the stability of the endometrium, thus minimizing events of irregular and unwanted breakthrough bleeding. In addition, estrogen action provides for a sufficient concentration of progesterone receptors. In aggregate, a small pharmacologic estrogen level is necessary to maintain the efficacy of the combination oral contraceptive.

As under most circumstances progesterone action will hold primacy over estrogenic effects, the cervical mucus, endometrium, and probably the fallopian tubes reflect progestational stimulation. The cervical mucus becomes thick and viscous and thus impervious to spermatozoa. The endometrium is in a state that is not receptive for implantation of a fertilized egg. Probably, the progestational impact on the secretory activity and peristalsis in the fallopian tubes also assists the general contraceptive effect. It is difficult, however, to assess the relative contribution of the various effects to the contraceptive efficacy, because combination oral contraceptives suppress ovulation very effectively.

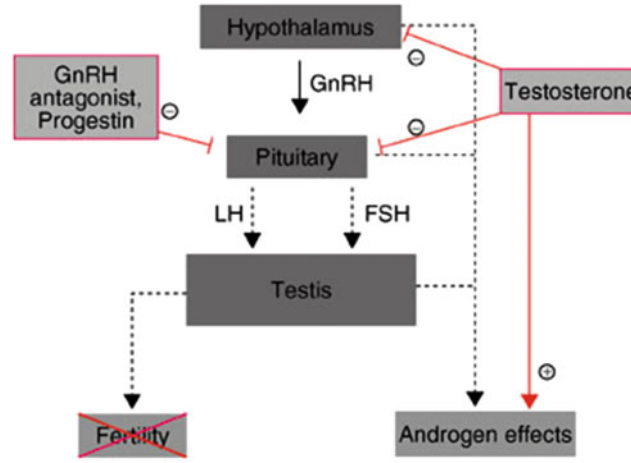
The progestin-only minipill contains a low-dose of a progestational agent which is sufficient to block ovulation in only 60–80% of cycles. The contraceptive effect is largely dependent on endometrial and cervical mucus effects, as gonadotropins are not reliably suppressed. Because of the low-dose of progestins, the minipill must be taken every day at the same time with great accuracy.

Long-acting methods of hormonal contraception are even more effective than oral methods. Two effective systems are available: a sustained-release method of levonorgestrel or etonogestrel using implanted steroid-permeable silastic tubing and depot injections of medroxyprogesterone acetate. The mechanism of action is similar to the progestin-only minipill. However, in addition, the long-acting methods yield progestin plasma levels high enough to prevent ovulation in basically all patients.

High doses of progestins are used for emergency postcoital contraception. A high dose of the progestin levonorgestrel (1,500 μg) can be used for postcoital emergency contraception. Administration of a total dose should be started within 12 h of unprotected sexual intercourse to achieve contraceptive protection of approximately 95%. Nausea and lower abdominal and breast pain as well as retarded menstruation have been reported as untoward effects. It is important to note that emergency contraceptives are not used as medical abortifacients to interrupt an established pregnancy defined to begin with implantation.

Ulipristal acetate is a new selective progesterone receptor modulator that has been approved for emergency contraception within 5 days of unprotected sexual intercourse. Thus, ulipristal acetate is longer acting compared to levonorgestrel providing reliable protection for only 3 days. The main action of 30 mg ulipristal acetate is to prevent ovulation similar to levonorgestrel. Main side effects are gastrointestinal and menstrual disturbances.

In recent years, strategies for hormonal male contraception have also been devised. Quantitatively and qualitatively normal spermatogenesis critically depends on neatly orchestrated LH and FSH release from the pituitary. In hormonal approaches to male contraception, gonadotropin secretion and intratesticular testosterone



Contraceptives, Fig. 2 Principles of hormonal male contraception

production need to be suppressed as efficiently as possible. Because androgens are key determinants of virilization and thus the male phenotype, but also strongly suppress gonadotropin release when applied exogenously, androgen preparations are essential components of all experimental approaches to male contraception (Fig. 2). Notably, the use of long-acting testosterone esters such as testosterone undecanoate causes azoospermia in only 30% of Caucasian men, while androgen-only regimens are highly effective for East Asian men. As additional agents, various combinations of androgens with progestins or GnRH antagonists have been evaluated in clinical trials. Testosterone administered in combination with various progestins such as depot medroxyprogesterone acetate, norethisterone enanthate, desogestrel or etonogestrel has yielded promising efficacy.

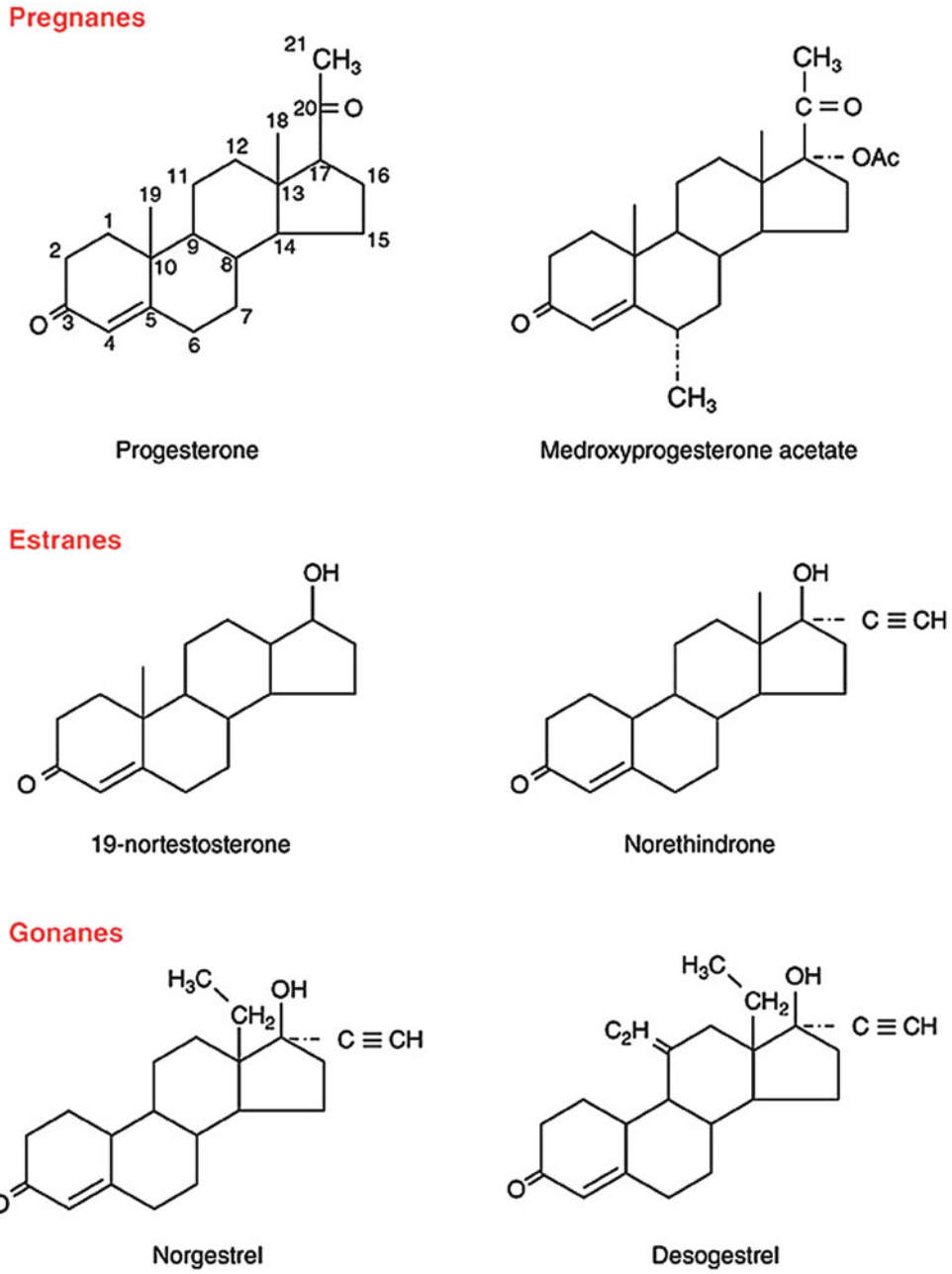
Testis function is regulated by hypothalamic and pituitary feedback circuits. The application of exogenous testosterone in conjunction with progestins or GnRH antagonists suppresses gonadotropin secretion from the pituitary and intratesticular testosterone production while maintaining extratesticular androgen effects. Such regimen reliably induced azoospermia and infertility.

Clinical Use (Including Side Effects)

After the seminal observation by Gregory Pincus and colleagues in the 1950s that progestins prevented ovulation in women, initial trials on humans were conducted using progestins like norethynodrel that were contaminated with about 1% mestranol. When subsequent efforts to provide a more pure progestin lowered the estrogenic component but provoked breakthrough bleeding, it was decided to keep the estrogen. Thus, the principles of combined estrogen–progestin oral contraception were established.

A major obstacle to the use of naturally occurring estrogens for the purpose of contraception was extensive first-pass hepatic metabolism and hence inactivation of the compounds when given orally. The addition of an ethinyl group at the 17 position made estradiol orally active. Ethinyl estradiol is a potent oral estrogen and represents one of the two forms of estrogens used in oral contraceptive pills. The other estrogenic compound is the 3-methyl ether of ethinyl estradiol, mestranol which is converted to ethinyl estradiol in the body.

The progestins used in oral contraceptives are 19-nor compounds of the estrane and gonane series (Fig. 3). Each compound possesses various degrees of androgenic, estrogenic, and antiandrogenic activities, thereby determining the scope of side effects. Animal and human studies showed, however, that only norethindrone, norethynodrel, and ethynodiol diacetate have estrogenic activity. Replacement of the 13-methyl group of norethindrone with a 13-ethyl moiety gives rise to the gonane norgestrel, which is a potent progestin with reduced androgenic activity (Fig. 3). More

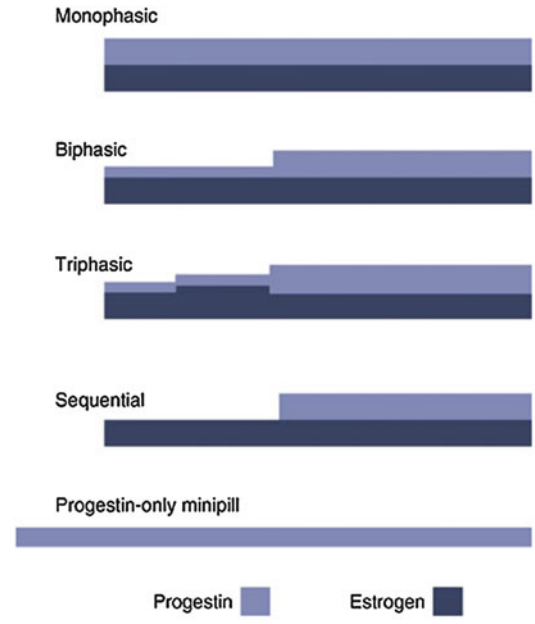


Contraceptives, Fig. 3 Examples of progestins derived from progesterone (pregnanes), 19-nortestosterone (estranes), and norgestrel (gonanes)

recently developed compounds like desogestrel (Fig. 3), norgestimate, gestoden, and etonogestrel display the least androgenic characteristics when compared with other 19-nor substances. Although norgestimate is a "newer" progestin, its activity is believed to be largely mediated by levonorgestrel or related metabolites. Therefore, epidemiologists do not generally include combination contraceptives containing norgestimate in the group of third generation compounds.

In epidemiologic studies, all products containing less than 50 μg ethinyl estradiol per pill are summarized as low-dose oral contraceptives. The first generation of oral contraceptives includes products with 50 μg or more of ethinyl estradiol. The second generation of oral contraceptives comprises formulations of norgestrel (0.3–0.5 mg), levonorgestrel (0.1–0.15 mg), norgestimate (0.25 mg), ethynodiol diacetate (1 mg), and other members of the norethindrone family in conjunction with 30 or 35 μg ethinyl estradiol. Desogestrel (0.15 mg) or gestodene (0.075 mg) are progestins in third generation contraceptives that are combined with 20 or 30 μg ethinyl estradiol. Most notably, the first oral contraceptive available contained 150 μg mestranol and 10 mg norethynodrel. It is nowadays commonly believed that the formulations of third generation oral contraceptives are very close to the lowest hormone levels which can be used without sacrificing contraceptive efficacy.

Combination oral contraceptives are the most frequently used agents and are characterized by a high therapeutic efficacy. Carefully controlled clinical studies with highly motivated subjects achieve an annual failure rate of 0.1%. The typical use effectiveness, however, amounts to 97–98%. Combination oral contraceptives are used as monophasic, biphasic, triphasic, and sequential preparations. In monophasic preparations (Fig. 4), a fixed estrogen/progestin combination is present in each pill which is administered daily for 21 consecutive days followed by a 7-day hormone-free period (usually the pills for the last 7 days of a 28-day pack contain only inert ingredients). In the bi- and triphasic preparations (Fig. 4), varying amounts and ratios of estrogen to progestin are present in order to mimic most closely the sex-steroid levels throughout a normal menstrual cycle. In addition, the total amount of steroids administered can be reduced when taking multiphasic oral contraceptives. Sequential preparations (Fig. 4) contain only estrogens for the first 7–11 days followed by a fixed estrogen/progestin combination in the remainder of the 21-day hormone application period. Phasic- and sequential-preparations were developed mainly to reduce the amount of progestins due to their untoward effects on the cardiovascular system.



Contraceptives, Fig. 4 Principal composition of various forms of oral contraceptives

Since a few years, a transdermal system received worldwide approval for hormonal contraception. In addition to ethinyl estradiol it contains norelgestromin as a progestational component. Norelgestromin is the active metabolite of norgestimate and is structurally related to 19-nortestosterone. Norelgestromin has negligible androgenic activity and may thus be suitable for women suffering from symptoms caused by androgen excess. The contraceptive efficacy of the transdermal patch is similar to that of oral contraceptives, but the transdermal system has the additional benefit of a once-weekly administration. In addition, transdermal delivery of hormones abolishes variability in gastrointestinal absorption and circumvents hepatic first-pass metabolism. It should be noted, however, that under steady-state conditions the area under the curve (AUC) describing plasma estrogen concentrations is 60% higher than corresponding levels resulting from a daily

oral application of 35 μg ethinyl estradiol. Two independent cohort studies recently demonstrated an increased risk of venous thromboembolism when using transdermal systems or vaginal rings (see below). Compared to levonorgestrel containing oral preparations the risk of venous thromboembolism nearly doubles.

Another fairly recent development in the field of hormonal contraception are combined contraceptive vaginal rings (NuvaRing) releasing 120 μg etonogestrel and 15 μg ethinyl estradiol daily. The small silastic ring is replaced weekly for 3 weeks followed by a ring-free period of 7 days. NuvaRing is well-tolerated and accepted by women, allows for a good control of the menstrual cycle, and has a therapeutic efficacy comparable to oral contraceptives.

Progestin-only contraceptives (Fig. 4) contain low-doses of progestins (e.g., 350 μg norethindrone or 75 μg norgestrel) that have to be administered daily without interruption. The lowest expected failure rate during the first year of use is 0.5%, while the typical failure rate amounts to 3%. Subdermal implants of norgestrel (216 mg) for sustained release provides for long-term (for up to 5 years) contraceptive effects characterized by failure rates of only 0.05%. Reliable contraception for 3 months can be achieved by an intramuscular injection of a crystalline suspension of 150 mg medroxyprogesterone acetate (Fig. 3) (failure rate 0.3%).

The main purpose of healthy women taking oral contraceptives is to prevent unwanted pregnancies. "Primum non nocere" applies particularly to preventive health care measures and therefore, untoward effects of oral contraceptives have to be monitored and assessed with great scrutiny. Shortly after the introduction of oral contraceptives approximately 40 years ago they soon became one of the most widely used drugs throughout the world. Hence, it is not surprising that reports on adverse effects began to appear rather quickly. Most of the untoward effects appeared to be dose-dependent, thus spurring on researchers to develop the current low-dose preparations. The most worrying adverse effects can be summarized in two main categories: the cardiovascular system and cancer.

Synthetic estrogens like ethinyl estradiol have a profound effect on the production of fibrinogen and clotting factors VII, VIII, X, and XII in the liver. In parallel, the concentrations of anticoagulation factors like protein C, S, and antithrombin III are diminished. This pro-coagulatory effect leads to an increased risk of thromboembolism in healthy women taking oral estrogens (Gialeraki et al. 2018). More than 20 years ago, several studies reported on a twofold increase in the risk of venous thromboembolism when third generation oral contraceptives containing desogestrel and gestoden were compared with older preparations mostly containing levonorgestrel. A number of independent recent studies clearly showed that drospirenone containing oral contraceptives are characterized by a three-fold increased risk of thromboembolism when compared to preparations containing levonorgestrel as a progestin. This does not come as a surprise, because drospirenone is characterized by partial agonism at the mineralocorticoid receptor in addition to its progestational activity. In summary, third generation oral contraceptives containing desogestrel, gestoden, or drospirenone as well as those with cyproterone acetate as a progestin have a 70% higher risk of thromboembolism compared to levonorgestrel based preparations. Because of the fairly low absolute risk in young women, a systematic search for cardiovascular risk factors is mandatory before prescribing a hormonal contraceptive.

The effects of oral contraceptives on thrombosis can be surmised as follows: All low-dose oral contraceptives, regardless of the type of progestin, have an increased risk of venous thromboembolism. Smoking has no effect on the risk of venous thrombosis. However, smoking and estrogen administration have an additive effect on the risk of arterial thrombosis.

Oral contraceptive-induced hypertension was previously observed in users of higher dose pills. An increased risk of clinically significant hypertension, however, has not been reported for low-dose oral contraceptives, including those containing the third generation progestins. Preexisting hypertension

is an important additive risk factor for stroke in oral contraceptive users. Most notably, recent clinical studies fail to find any substantial risk of myocardial infarction or stroke in healthy, nonsmoking women, regardless of age, who take low-dose oral contraceptives. The vast majority of myocardial infections and strokes in oral contraceptive users occur when women over the age of 35 and cardiovascular risk factors take high-dose products (more than 50 μg ethinyl estradiol per pill).

In 1996 already, a meta-analysis of 54 epidemiologic studies indicated that women had a slightly increased risk of breast cancer while taking oral contraceptives when compared to nonusers (relative risk = 1.24). The increased risk diminished steadily after cessation of medication and was not found elevated 10 years after discontinuation. A more recent population-based case-control study of more than 4,500 women with breast cancer and nearly 4,700 controls showed no association between past and present use of oral contraceptives (Marchbanks et al. 2002). Due to the large study, subgroups of women, for example, those taking a formulation with a high estrogen content, duration of oral contraceptive use, initiation of use during adolescence, history of breast cancer in a first-degree relative, could be analyzed. None of these subgroups had a significantly increased risk of breast cancer. More broadly speaking, a large cohort study in the UK did not observe any association between oral contraception and an increased overall risk of cancer (Hannaford et al. 2007). On the contrary, a recent population-based study in Denmark following 1.8 million women for more than 10 years corroborated the 1996 figures and showed that the risk of breast cancer was higher among women who currently or recently used hormonal contraceptives (relative risk between 1.20 and 1.38). After discontinuation of contraception, the risk of breast cancer still remained higher among women who had used hormonal contraceptives for 5 years or more than among never users. However, it should be noted that absolute increases in risk were small (13 per 100,000 person years) (Morch et al. 2017).

The flip side of the coin is that hormonal contraceptives indisputably have significant health benefits. The use of oral contraceptives for at least 12 months reduces the risk of developing endometrial cancer by 50%. Furthermore, the risk of epithelial ovarian cancer in users of oral contraceptives is reduced by 40% compared with that on nonusers. This kind of protection is already seen after as little as 3–6 months of use. Oral contraceptives also decrease the incidence of ovarian cysts and fibrocystic breast disease. They reduce menstrual blood loss and thus the incidence of iron-deficiency anemia. A decreased incidence of pelvic inflammatory disease and ectopic pregnancies has been reported as well as an ameliorating effect on the clinical course of endometriosis.

Future efforts should be directed at optimizing current formulations to finally come up with an ideal oral contraceptive which would reduce the risk of breast, ovarian, and endometrial cancer without any cardiovascular complications.

Cross-References

▶ Sex Steroid Receptors: Androgen Receptor, Estrogen Receptors, Progesterone Receptor

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Control of Food Intake

► Appetite Control

Control of Hypertension

► Blood Pressure Control

Corticosteroids

► Glucocorticoids

Corticotropin-Releasing Factor (CRF) System

► Corticotropin-Releasing Hormone System

Corticotropin-Releasing Hormone System

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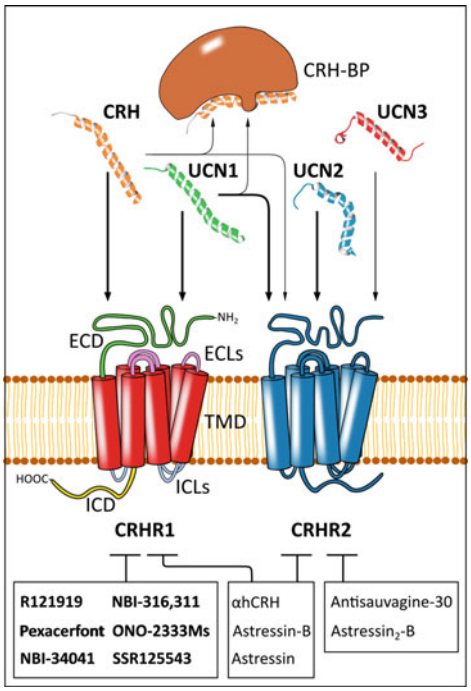
Synonyms

Corticotropin-releasing factor (CRF) system

Definition

The corticotropin-releasing hormone (CRH) system plays a prominent role in shaping the stress response. It acts through two receptors: the corticotropin-releasing hormone receptor type 1 (CRHR1) and 2 (CRHR2), which are differentially activated by four peptide ligands: CRH, urocortin 1 (UCN1), urocortin 2 (UCN2), and urocortin 3 (UCN3) (Fig. 1). CRH was originally discovered as the initiator of the hypothalamic-pituitary-adrenal (HPA) axis – the neuroendocrine limb of the stress response. In addition, the central CRH system is well-known for its capacity to modulate adaptive behavioral and autonomic responses to stress. Since its discovery, a dysregulation of the CRH system has been associated with different stress-related diseases including major depressive disorder (MDD) and anxiety disorders. Accordingly, the CRH system holds great expectations as a potential target for the treatment of stress-related disorders.



Corticotropin-Releasing Hormone System, Fig. 1 Ligand-receptor interaction within the corticotropin-releasing hormone (CRH) system: CRH binds to the CRH receptor 1 (CRHR1) and with lower affinity to the CRH receptor 2 (CRHR2). Urocortin 1 (UCN1) is also bound by both receptors, but with comparable affinity. CRH and UCN1 both bind to the CRH-binding protein (CRH-BP). Urocortin 2 (UCN2) and urocortin 3 (UCN3) are exclusive ligands of the CRHR2. Structural domains of CRHRs are illustrated for CRHR1: extracellular domain (ECD, green), transmembrane domain (TMD, red), extracellular loops (ECLs, pink), intracellular loops (ICLs, blue), and intracellular domain (ICD, yellow). Small molecule (bold) and peptide antagonists for CRHR1 and CRHR2 inhibition

Basic Characteristics

CRH and Its Related Neuropeptides

Members of the CRH-Related Neuropeptide Family

In 1981, the 41 amino acid (AA) neuropeptide corticotropin-releasing hormone (CRH) was isolated from the ovine hypothalamus by Wylie Vale and his team (Vale et al. 1981). In humans the *CRH* gene is located on chromosome 8 and is made up of two exons separated by an 800 bp intron. Exon 2 encodes the entire prepropeptide of 196 AAs. The N-terminal 24 AA signal sequence and the proregion are proteolytically processed by prohormone convertases. Its maturation is completed by C-terminal amidation. In mammals, the CRH-related neuropeptide family consists of three additional members, UCN1, UCN2, and UCN3, which were identified between 1995 and 2001. CRH shares 43% sequence identity with UCN1, 34% with UCN2, and 26% with UCN3. All of the CRH family members show a similar gene structure comprising two exons, with exon two encoding the prepropeptide. All mature peptides share a similar structure containing an N-terminal receptor activation domain and a C-terminal receptor-binding domain. In the case of CRH and UCN1, a sequence that conveys binding to the CRH-binding protein (CRH-BP) is flanked by the receptor-binding and activation domains (Dedic et al. 2018; Deussing and Chen 2018).

Expression Pattern

In the human brain, gene expression can only be inferred from postmortem studies, and the availability of human brain tissue is limited. Therefore, most of the findings concerning ligand expression were gathered in rodent models. All members of the family can be found in the brain, but also in peripheral tissues. Among the family of CRH-related neuropeptides, CRH shows the most widespread expression within the brain. It is mainly expressed in the paraventricular nucleus of the hypothalamus (PVN), the central amygdala, different divisions of the bed nucleus of the stria terminalis (BNST), in the lateral part of the interstitial nucleus of the posterior limb of the anterior commissure (IPACL), in the hippocampus, in the piriform cortex, as well as throughout the neocortex. Although the identity of CRH expressing neurons has only partially been revealed so far, it is known that the majority of CRH neurons are γ-aminobutyric acid (GABA)-ergic in nature, with a few exceptions in the piriform cortex as well as the PVN, where they express the glutamatergic marker VGLUT2. The expression of UCN1 is much more restricted and can mainly be found in the Edinger-Westphal nucleus, but also in the supraoptic nucleus and lateral superior olive. UCN2 and UCN3 are also present in discrete brain regions but are more prevalent in peripheral structures like the skin, skeletal muscle, pancreas, or gastrointestinal tract (Dedic et al. 2018).

CRH-Binding Protein

The first polypeptide described to bind CRH was the CRH-binding protein (CRH-BP). Its discovery was fostered by the observation that the massive increase of circulating CRH levels during pregnancy neither increases adrenocorticotropic hormone (ACTH) nor cortisol levels. Sequestration of CRH by the CRH-BP was identified as the underlying mechanism preventing a massive activation of the HPA axis during pregnancy. The 37 kDa glycoprotein is found in the placenta, liver, circulation, pituitary and brain. It is able to bind CRH as well as UCN1, but not UCN2 nor UCN3 (Fig. 1). CRH-BP regulates the availability of CRH and UCN1 and therefore serves as endogenous buffer, which can inhibit the biological activity of CRH as well as UCN1, thereby preventing any unwanted activation, e.g., of the HPA axis. Despite this modulatory impact of CRH-BP on HPA axis regulation as well as on central CRH circuits, the physiological relevance connected to this polypeptide remains largely unclear (Deussing and Chen 2018).

CRH Receptors

The two CRH receptors – CRHR1 and CRHR2 – are heptahelical G protein-coupled receptors (GPCRs) of the class B (secretin-like) family (Fig. 1). Both receptors show 70% AA sequence homology, with lowest identity in the N-terminal extracellular domain (ECD) (40%) and highest in

the transmembrane helices as well as in the C-terminal intracellular domain (ICD) (80–90%).

Splice Variants

The human genes encoding CRHRs comprise 14 (CRHR1) and 16 (CRHR2) exons, which span approximately 50 kb on chromosome 17 and 7, respectively. In humans, eight alternative CRHR1 splice variants have been characterized to date, of which only CRHR1α is biologically active and present in the central nervous system (CNS). All other splice variants, which were primarily isolated from peripheral tissues, are truncated forms that do not signal upon CRH binding. Expression of these splice variants directly competes with expression and thus availability of CRHR1α. In addition, CRHR1α might form hetero-oligomers with these splice variants, thereby changing receptor properties such as localization, ligand affinity, and receptor signaling. Three CRHR2 splice variants have been isolated in humans so far. Variants CRHR2α, CRHR2β, and CRHR2γ are all spliced at the 5′ end, thereby maintaining identical seven-transmembrane as well as C-terminal domains. One additional splice variant, which has only been identified in the mouse so far, is a soluble form of the CRHR2α (sCRHR2α). This splice variant exclusively encodes the extracellular ligand-binding domain of the receptor and terminates before the first TMD. This soluble variant might act as a decoy receptor. However, its exact role remains unclear as it is efficiently translated but not necessarily secreted.

Expression Pattern

Both CRHRs are expressed in the CNS, albeit CRHR1 being the predominant receptor in the brain. CRHR1 is expressed throughout the brain with highest levels, e.g., in the olfactory bulb, neocortex, hippocampus, subnuclei of the amygdala, ventral tegmental area, substantia nigra, red nucleus, and cerebellum. CRHR2 expression is more limited to specific brain areas, e.g., lateral septum, ventromedial hypothalamus, medial amygdala, BNST, dorsal raphe, and choroid plexus. In humans, the CRHR2 splice variants CRHR2β and CRHR2γ are mainly expressed in the brain, whereas CRHR2α is more prominent in peripheral tissues like the skin, heart, or skeletal muscle (Dedic et al. 2018; Deussing and Chen 2018).

Receptor Interactions

Ligand-Receptor Interaction

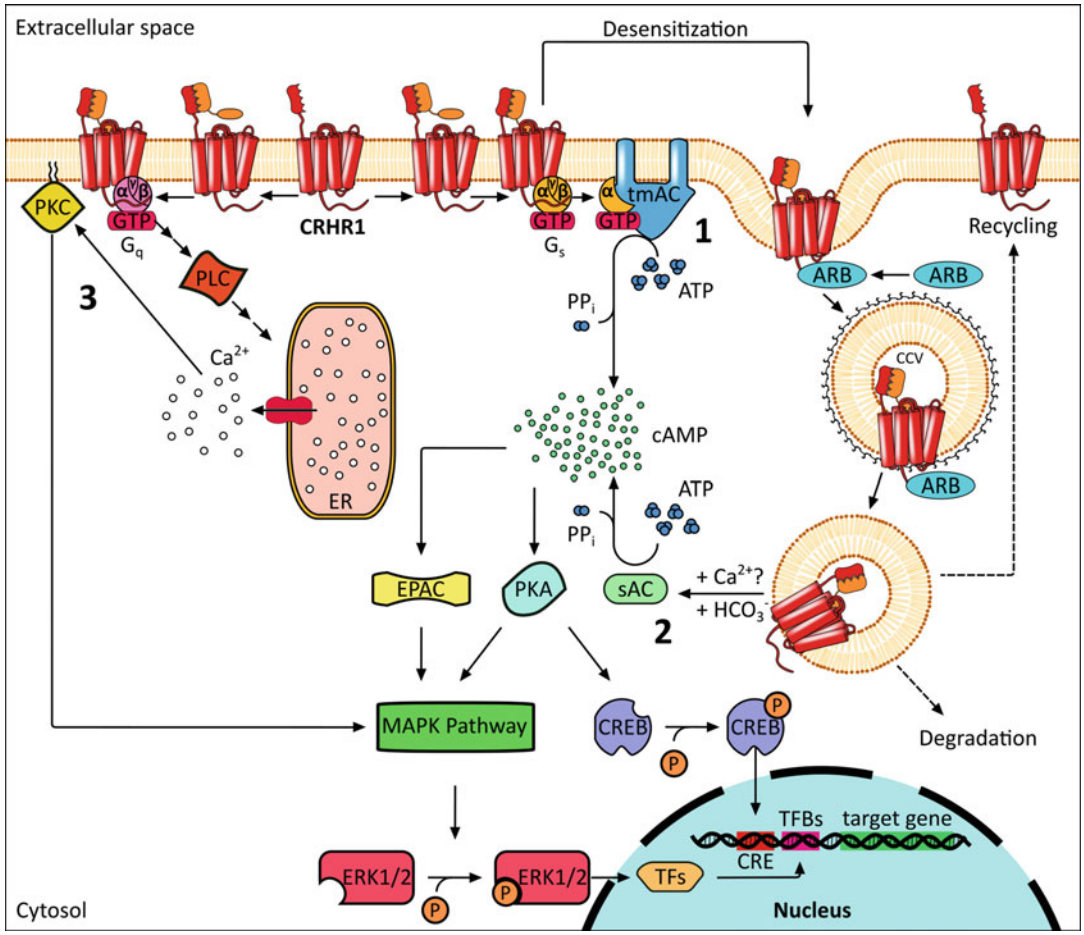
The four ligands of the CRH system (CRH, UCN1, UCN2, UCN3) bind with different affinities to the two CRHRs (Fig. 1). The main ligand for CRHR1 is CRH, which can also bind CRHR2, but with lower affinity. UCN1 shows equal affinity for both receptors, whereas UCN2 and UCN3 are exclusively bound by CRHR2. Ligand specificity is mainly determined by the N-terminal part of the receptor. The recently proposed two domain model for ligand-receptor interaction can be best described using the structure of CRH family ligands. The mature peptide consists of two receptor-interacting domains. The C-terminal domain interacts with the ECD of the receptor thereby determining the affinity and specificity of the ligand for the receptor. Once the peptide's C-terminus is bound, its N-terminal domain binds to the receptor's juxtamembrane portion and thereby activates the receptor (Deussing and Chen 2018; Inda et al. 2017).

Receptor-Receptor Interaction

CRHR1 was one of the first class B GPCRs reported to form homooligomers at the plasma membrane and in intracellular compartments. CRHR2β also forms oligomers, whereas CRHR2α only acts as a monomer. The dimer/monomer states of the receptors are proposed to play a role in G protein selectivity and thus intracellular signaling. The CRHR1 contains a C-terminal PDZ-binding domain, which is absent in the CRHR2. This domain conveys interactions with PDZ domain containing proteins such as members of the membrane-associated guanylate kinase (MAGUK) family, which serve as scaffolds for the assembly and compartmentalization of signaling complexes. Along these lines, it has been demonstrated that the PDZ-binding domain is capable of promoting the cross talk with other GPCRs such as the 5-hydroxytryptamine 2A receptor (Deussing and Chen 2018).

CRH-Dependent Signaling

CRHR-dependent signaling has been extensively studied for the CRHR1 and to lesser extent for CRHR2. Upon binding of the ligand to the CRHR, the third intracellular loop (ICL3) is bound by a heterotrimeric G protein consisting of an alpha, beta, and gamma subunit, which in turn leads to a stabilization of the receptor-ligand complex (Fig. 2). The catalytically active alpha subunit dissociates from the beta and gamma subunits and activates a membrane bound adenylyl cyclase (tmAC), which, in its activated state, generates cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP) (Fig. 2(1)).



Corticotropin-Releasing Hormone System, Fig. 2 CRHR1-induced intracellular signaling cascades: Activation of the receptor induces G protein binding (canonically Gs), which triggers the generation of cyclic adenosine monophosphate (cAMP) by membrane bound adenylyl cyclase (tmAC) (**1**). Recruitment of β-arrestin (ARB) leads to receptor internalization. Signaling continues by a yet unknown mechanism in which soluble adenylyl cyclase (sAC) generates cAMP depending on Ca^{2+} and HCO_3^- (**2**). Increased cAMP levels ultimately lead to changes in transcription by activation of Ca^{2+}/cAMP response element-binding protein (CREB) and the mitogen-activated protein kinase (MAPK) pathway. Both pathways (**1** and **2**) contribute to increased cAMP levels in the early phase of ERK1/2 activation, but only sAC (**2**) is responsible for its activation at later stages. Besides the Gs-dependent pathway transcription can also be influenced in a protein kinase C (PKC)-dependent manner induced by binding of Gq to the activated receptor, which also triggers the activation of the MAPK pathway (**3**). Receptor desensitization involves its internalization, which can result in degradation or recycling of the receptor. CCV, clathrin-coated vesicle; EPAC, exchange protein activated by cAMP 2; PLC, phospholipase C; PKA, protein kinase A; CRE, cAMP-response element; TFs, transcription factors; TFBs, TF binding sites

The second messenger cAMP can then activate different signaling pathways, ultimately modulating downstream gene expression. cAMP binds to the regulatory subunit of the protein kinase A (PKA) leading to the release of the catalytic subunit, which can phosphorylate different target proteins in the cytosol or nucleus such as the $Ca2^{+}$/cAMP response element binding-protein (CREB). Activated CREB binds to cAMP-response elements (CREs) in the promotor region of target genes, like c-*fos* to induce their expression. Besides this pathway, PKA can also activate the mitogen-activated protein kinase (MAPK) pathway by activating the MAPKKK Rap1/2. The MAPK pathway is also activated via a PKA-independent pathway. In this case, cAMP activates the exchange protein activated by cAMP 2 (EPAC2), which in turn activates Rap1/2, thereby triggering the MAPK pathway. By subsequent activation of downstream kinases (B-Raf, MEK1/2), the extracellular signal regulated kinase ERK1/2 is phosphorylated. ERK1/2 activates different transcription factors (TFs), thereby promoting the expression of genes like proopiomelanocortin (POMC).

An alternative signaling pathway leading to the activation of the MAPK pathway is triggered by the binding of a Gq protein to the ICL3 region of CRHR1 (Fig. 2(3)). The alpha subunit of the G protein activates the phospholipase C (PLC), which in turn cleaves the phosphatidylinositol-4,5-biphosphate (PIP_2) into inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 accumulates in the cytosol and activates the IP_3 receptor stimulating the release of Ca^{2+} into the cytosol. The accumulation of cytosolic Ca^{2+} from intracellular stores activates different calcium-dependent proteins including Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC), leading to the phosphorylation of c-Raf, thereby activating ERK1/2, which leads to the previously described changes in TF activation and gene expression.

A common mechanism of GPCR regulation is their desensitization, which is primarily accounted for by phosphorylation of intracellular residues by GPCR kinases (GRKs). The phosphorylation of the ICD of the CRHRs by GRK3 and GRK6 leads to the recruitment of β-arrestin (ARB), a small GPCR-binding protein. ARB leads to the termination of the receptor-G protein interaction and also serves as an adaptor protein for components of the clathrin-dependent endocytic machinery, such as clathrin or ß2-adaptin (AP-2). AP-2 is bound to the receptor and leads to its endocytosis and internalization. Upon internalization, Ca^{2+} and bicarbonate trigger the activation of the atypical soluble adenylyl cyclase (sAC), which in turn generates cAMP leading to the activation of EPAC and ERK1/2 (Fig. 2(2)).

The activation of ERK1/2 upon CRHR1 activation can be separated into two phases, the early and the late phase. The previously described G protein-dependent activation of the tmAC and the G protein-independent activation of sAC are the sources of cAMP within the early phase of ERK1/2 activation (Fig. 2(1 and 2)), but only cAMP from sAC leads to the sustained activation in the late phase (Fig. 2(1)). Although the process of sAC activation has been shown to be dependent on CRHR internalization, presence of Ca^{2+} and bicarbonate is believed to be G protein-independent; however the exact mechanisms controlling its activation are still unclear.

The intracellular signaling of CRHRs is highly dependent on the equipment of a given cell, e.g., with different G proteins, kinases, other enzymes, and transcriptional regulators. In addition, the binding of different ligands might promote a bias toward specific downstream signaling pathways. It is of note that the experimental evidence for the described signaling pathways was gathered from different cell lines, not necessarily expressing CRHRs endogenously. Hence, the components of the signaling machinery in these cells might differ from the in vivo situation, i.e., cells, expressing CRHRs endogenously. Therefore, these pathways have to be further validated in primary cells as well as in the in vivo context (Deussing and Chen 2018; Inda et al. 2017).

The CRH System and Its Connection to Stress

Hypothalamic-Pituitary-Adrenal Axis

The HPA axis is a neuroendocrine system crucially involved in regulating homeostasis and an

integral part of the organism's stress response system. Its circadian regulation and activation in response to internal or external challenges are specifically controlled by CRH. CRH is expressed in parvocellular secretory neurons with glutamatergic identity in the PVN where it is stored in large dense core vesicles. As a consequence of physical or psychological stressors, CRH is released from axon terminals in the external zone of the median eminence into the portal vasculature. The release of CRH results in a local activation of CRHR1 in anterior pituitary corticotrophs, which triggers the release of ACTH into the general circulation. Upon its arrival at the adrenal cortex, it activates the ACTH receptor, stimulating the synthesis and secretion of glucocorticoids (human, cortisol; mouse, corticosterone). The effects of glucocorticoids are manifold leading to an increase in the catabolic metabolism by promotion of gluconeogenesis in the liver, lipolysis in fat, and amino acid mobilization in muscle tissue. Glucocorticoids also create an anti-inflammatory environment in peripheral tissues. The whole stress response via the HPA axis is a self-regulated process in which glucocorticoids act in a negative feedback loop inhibiting further generation and secretion by acting on glucocorticoid receptors (GRs) in the anterior pituitary and different brain regions. The CRHR2 plays also a role in HPA axis regulation and is particularly involved in the initiation and termination of HPA axis activity. In contrast to CRH, the contribution of UCNs to HPA axis regulation is rather limited (Deussing and Chen 2018; Dedic et al. 2018).

Behavioral Stress Responses

Besides its elementary role in HPA axis, regulation the CRH system is also involved in modulating complex behavioral responses upon stressful stimuli as elucidated from a wealth of studies involving genetic mouse models or behavioral pharmacology. CRH and the CRHR1 are both widely expressed throughout the brain including nuclei not connected to the HPA axis implying an additional role of this system in brain physiology. The central application or overexpression of CRH in rodents causes reactions resembling the behavioral and physiological stress response. In contrast, the disruption of CRH/CRHR1 signaling by pharmacological or genetic means promotes anxiolytic effects. The anxiolytic consequences of CRH/CRHR1 loss-of-function approaches as well as the stress-like phenotypes originating from gain-of-function experiments support a prominent role of the extrahypothalamic CRH system with regard to the behavioral reaction to stressors. In recent years, it has become clear that the central CRH system is more complex than originally envisioned. The original concept of a dualism of a stress-initiating/anxiogenic CRHR1 and a stress recovery-related/anxiolytic CRHR2 has largely been revised. The function of CRHRs and their ligands is critically determined by their cellular localization and previous stress exposure or experiences. To fully understand the role of CRH-related neuropeptides and their receptors in brain physiology requires more detailed understanding of related neurocircuits and their dynamics (Deussing and Chen 2018).

Therapeutic Potential of the CRH System in Stress-Related Disorders

In 1999, the CRH hypothesis of depression was proposed, based on the following findings regarding the CRH system in MDD patients: (i) elevated levels of CRH in the cerebrospinal fluid, (ii) increase in CRH secreting neurons in the PVN, (iii) reduction of CRH-binding sites in the frontal cortex secondary to elevated CRH levels, and (iv) elevated urinary cortisol levels due to increased ACTH and cortisol pulses. All of these findings, together with the potential role of CRH in emotional behaviors, support the assumption that the HPA axis and CRH itself are causally involved in the etiology of MDD and other stress-related disorders. Therefore, the CRHR1 was seen as a promising target for the development of new antidepressants, which led to the generation of different small molecule antagonists that have been tested in clinical trials (Holsboer 1999; Spierling and Zorrilla 2017).

Drugs

The two domain model of ligand-receptor interaction states that there are two potential targets for

the inhibition of the CRHR1, the N-terminal ECD, and the juxtamembrane domain. For these two targets, different drug types are suitable: (i) peptides for preventing ligand-receptor interaction and (ii) non-peptide antagonists for the inhibition of receptor activation.

An example for a peptide antagonist is the N-terminally truncated CRH analogue alpha-helical CRH (αhCRH), which shows approximately the same binding affinity to CRHRs as the full-length CRH. However, since it cannot cross the blood-brain barrier, it needs to be applied at the location of interest in the brain. Accordingly, peptide antagonists for the inhibition of CRHRs are mainly used in basic research, but not for therapeutic purposes in human subjects. Therefore the development of non-peptide antagonists, so-called small molecules, was necessary for their use in clinical trials (Fig. 1 and Table 1).

All currently available small molecules with high affinity and selectivity for CRHR1 share a common structure of one or two aliphatic top units and a nitrogen in the core ring, which can directly interact with a histidine in the third transmembrane domain and in that way occupy the hydrophobic pocket of the receptor. Their structure has somewhat hampered the development of small molecule antagonists for clinical trials because of their inherent lipophilic properties, resulting in poor water solubility.

Small molecules for the inhibition of CRHR1 have been extensively tested in different mouse models of anxiety, depression, and addictive disorder, showing promising results and leading to their use in different clinical trials.

The first clinical study of a CRHR1 antagonist was a phase 2a open-label study to assess safety and efficacy of NBI-30775/R121919 (Table 1). The patients showed an improvement in the Hamilton Depression Rating Scale (HDRS) scores and no major impact on the HPA axis (tested by the CRH challenge test). The study was still discontinued because of elevated levels of liver enzymes in healthy patients. A phase 1 study with NBI-34041 showed no influence on HPA axis while demonstrating safety and tolerability, also improving resistance to psychosocial stress. In a phase 2b double-blinded, placebo-controlled study in patients with recurrent MDD using the antagonists NBI-316,311, there was no improvement in the HDRS scores and it was prematurely stopped. Similarly Pexacerfont was unable to

Corticotropin-Releasing Hormone System, Table 1 Selected clinical trials of small molecule CRHR1 antagonists (based on Spierling and Zorrilla 2017)

Indication	Drug name	Study type	Outcome	Reference (year of completion)
Major depression	R121919	Phase 2a, open label	Improvement in HDRS; discontinued: elevated liver enzyme levels	Zobel et al. (2000)
	Pexacerfont	Phase 1, double-blinded, placebo-controlled	No results reported	NCT00135421 (2007)
	PF-00572778	Phase 1, single-blinded, placebo-controlled	discontinued: elevated liver enzyme levels	NCT00580190 (2008)
	CP-316,311	Phase2b, double-blinded, placebo-controlled	No improvement in HDRS, prematurely stopped	NCT00143091 (2006)
	ONO-2333Ms	Phase 2b, quadrupole-blinded, placebo-controlled	Lacked efficacy	NCT00514865 (2008)
	SSR125543	Phase 2, triple-blinded, placebo-controlled	Lacked efficacy	NCT01034995 (2011)
Generalized anxiety disorder	Pexacerfont	Phase 2b, double-blinded, placebo-controlled	No therapeutic effect	NCT00481325 (2008)
Post-traumatic stress disorder	Verucerfont	Phase 2b, double-blinded, placebo-controlled	Lacked efficacy	NCT01018992 (2014)

National Clinical Trial Identifier (NCT)

show any therapeutic effect in a large, randomized, and placebo-controlled clinical trial of patients with generalized anxiety disorder. Other compounds like SSR125543 lacked efficacy in controlled trials and therefore were stopped. Also Verucerfont, a CRHR1 antagonist for the treatment of post-traumatic stress disorder did not show any improvement in disease-related symptoms compared to placebo.

Since none of the small molecule CRHR1 antagonists were able to enter a phase 3 clinical trial yet, the expectations of CRHR1 inhibition for treatment of stress-related psychiatric disorders were largely unmet. In this regard, CRHR1 inhibitors share the fate of many other drugs that were specifically developed for the treatment of psychiatric disorders.

The underlying reasons for the lack of translation from animal models to patient treatment are manifold. Firstly, disturbances of the CRH/CRHR system are observed in only a subset of patients. Astonishingly, this has not been taken into account in previous clinical trials but could easily be overcome, e.g., by patient stratification to select patients suitable for treatment. Moreover, the predictive sensitivity of preclinical models is good, while they are lacking sufficient specificity. Another reason for the poor performance of these antagonists may be their relatively limited binding to the receptor, as a long duration of receptor occupancy might be crucial for their efficacy. Additionally, all of the antagonists developed to date bind to the atypical allosteric binding site, with none binding at the orthosteric binding site. Studies on other GPCRs have shown that the administration of a double antagonist, against both the allosteric and orthosteric binding sites, showed the highest receptor inhibition. Besides these model- and compound-specific questions, it has to be emphasized again that different neuronal subpopulations are involved in the CRH/CRHR1 system-related behavioral stress responses. In addition, CRHR1 signaling depends highly on the components of the signaling machinery, which might differ between different neuronal populations. Thus, a general inhibition of CRHR1, without discriminating between specific CRH/CRHR1 circuits might be less effective for the treatment of psychiatric disorders. Therefore, a better understanding of the involved cellular subpopulations and related signaling pathways is crucial for the understanding of how treatment of these diseases can be improved (Deussing and Chen 2018; Spierling and Zorrilla 2017).

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COX

▶ Cyclooxygenases

CTLA8

▶ Interleukin-17

C-Type Natriuretic Peptide and Its Receptors

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Synonyms

CNP; Vascular natriuretic peptide

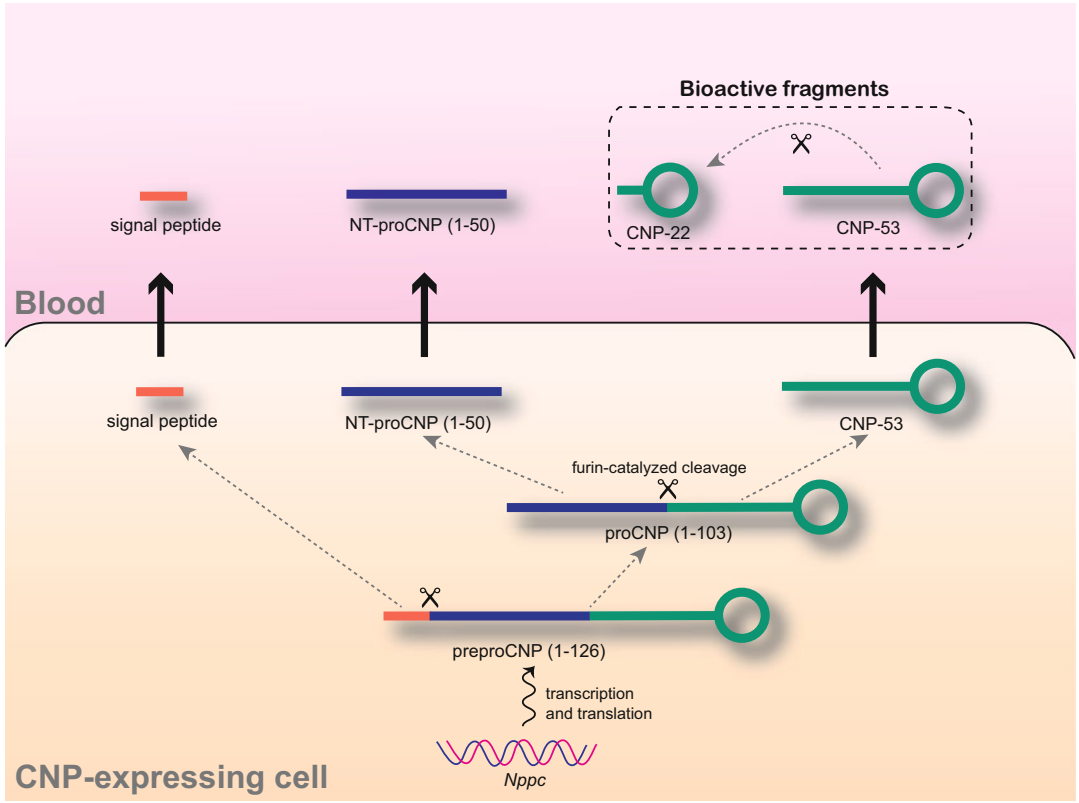
Definition

C-type natriuretic peptide (CNP) is a paracrine/autocrine peptide belonging to the family of natriuretic peptides. All natriuretic peptides share a structurally related C-terminus that constitutes the receptor-binding site in the molecules. The intracellular signaling of CNP is mediated through a specific CNP receptor, e.g., natriuretic peptide receptor B (NPR-B) as well as via the common natriuretic peptide receptor C (NPR-C), which acts both as a clearance receptor and as a trigger of intracellular signaling. CNP is synthesized in many tissues and cell types, and the CNP system accordingly plays a role in several physiological processes – including bone growth and vascular homeostasis. Different targets of the CNP system have been in recent focus for drug development, and these pharmacological interventions have shown a promising clinical potential.

Basic Characteristics

C-Type Natriuretic Peptides

In 1990, C-type natriuretic peptide was reported as the third peptide of the family of the natriuretic peptides – in addition to Atrial and B-type natriuretic peptide (ANP and BNP) (Zois et al. 2014). Subsequently, Dendroaspis Natriuretic Peptide (DNP) was isolated from the venom of the green mamba and was also included in the peptide family (Singh et al. 2006). The commonality of the members is a preserved intramolecular ring structure of 17 amino acids formed by a single disulfide bridge. Extending from cystyl residues forming the disulfide bridge, the different natriuretic peptides have variable N- and C-terminal regions. Bioactive CNP does not possess a C-terminal extension stemming from the ring structure and is found in two major forms, CNP-22 and CNP-53, with a differently sized N-terminal region (5 and 36 amino acid residues, respectively) (Prickett and Espiner 2012). Located on chromosome 2, the human CNP gene, *NPPC*, is often considered the genetic ancestor of the natriuretic peptide family (Prickett and Espiner 2012; Takei 1999). Notably, the CNP gene is extremely conserved between species, indicating a vital biological function of CNP (Lippert and Goetze 2010). From *NPPC,* the first translational product, pre-proCNP, is synthesized, and cotranslatory cleavage by the signal peptidase forms the first prostructure, proCNP, together with a hydrophobic signal peptide (103 and 23 amino acid residues, respectively) (Pemberton et al. 2014). Further intracellular processing by the endoprotease furin leads to the formation of CNP-53 and an N-terminal proCNP fragment (50 amino acid residues). After release from the cell, CNP-53 can be further processed to CNP-22 by unknown mechanisms (Prickett and Espiner 2012). Both bioactive CNP, NT-proCNP, and the signal peptide are measurable in the human circulation (see Fig. 1 and "Measurement of C-Type Natriuretic Peptides in Human Plasma"). The half-life of CNP-22 in the human circulation is estimated to be 2–3 min (Prickett and Espiner 2012).



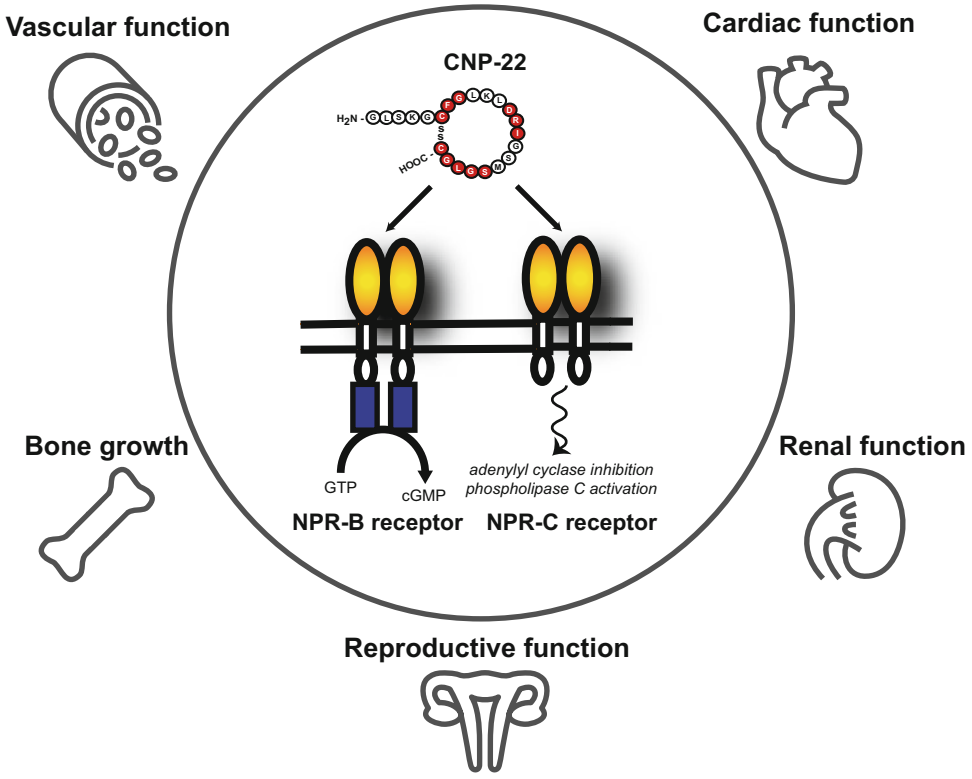
C-Type Natriuretic Peptide and Its Receptors, Fig. 1 Expression of CNP from gene transcription to circulating CNP-fragments

C-Type Natriuretic Peptide Receptors and Enzymes

From a functional point of view, CNP differs markedly from ANP and BNP. The CNP system has two types of membrane-bound receptors (see Fig. 2), where natriuretic peptide receptor B (NPR-B), also known as guanylyl cyclase B, is CNP specific. Binding of CNP leads to NPR-B receptor activation that catalyzes the intracellular conversion of guanosine triphosphate to cyclic guanosine monophosphate (cGMP) as second messenger. The other CNP receptor, natriuretic peptide receptor C (NPR-C), has affinity for all natriuretic peptides and holds both an intracellular signaling function and a clearance mechanism (NPR-C is often referred to as a clearance receptor). Intracellular signaling from the NPR-C receptor is mediated through inhibitory G-proteins and, hence, inhibition of adenylyl cyclase and phospholipase C activation (Zois et al. 2014). Furthermore, natriuretic peptides including CNP are all substrates for degradation by the broadly expressed enzyme, membrane-bound metallo-endopeptidase (EC 24.11, neprilysin) (Potter 2011), which is the target of a newly introduced treatment in heart failure (see "Inhibition of Neprilysin").

Sites of Expression and Biological Roles

In contrast to ANP and BNP – both mainly secreted from cardiomyocytes – CNP is expressed in a variety of tissues and cell types including chondrocytes, vascular endothelial cells, cardiomyocytes, reproductive tissues, and renal tissue, as well as high concentrations in the pituitary gland and brain of the central nervous system (Sellitti et al. 2011) (see Fig. 2). The blood-borne concentrations of bioactive CNP are too low for activation of the CNP receptors through the circulation. Thus, CNP is regarded as a paracrine/



C-Type Natriuretic Peptide and Its Receptors, Fig. 2 The CNP system illustrated with CNP-22, its receptors, NPR-B and NPR-C, and the organs, where the CNP system has a regulatory function (CNP-53 is not shown for illustrative purposes)

autocrine regulatory peptide (Prickett and Espiner 2012). Accordingly, CNP has been ascribed separate biological roles in different organs with functions in bone growth, vascular homeostasis/protection, and reproduction being the most extensively studied.

Reports on rare human mutations of genes specifically related to the CNP system point to a crucial role in the regulation of longitudinal bone growth, where CNP and NPR-B expressed in chondrocytes promote endochondral ossification. Loss of function of NPR-B has been shown to cause severe growth retardation, and overexpression of *NPPC* leads, correspondingly, to a phenotype with clinical resemblance to the Marfan syndrome, including tall stature. In mice, genetic manipulations of the CNP system have consolidated the pivotal role in bone growth (Prickett and Espiner 2012). It has also been shown that the intracellular signaling cascade from the activated NPR-B converges with the signaling from the Fibroblast Growth Factor 3 receptor (FGFR3), which is pharmacologically utilized in trials of treatment of achondroplasia (see "CNP Analogs").

In the cardiovascular system, implications of a complex interplay between CNP and the receptors, NPR-B and NPR-C respectively, to maintain cardiovascular homeostasis have emerged during the recent years from preclinical studies. In general, these investigations have been based on thorough experiments of tissue-specific and global knock-out of CNP and the two receptors. It has been shown that CNP produced in the vascular endothelium exerts a vasodilatory function through receptors in smooth muscle cells and pericytes of the vascular tree, where both NPR-B- and NPR-C-signaling seem instrumental. Moreover, the CNP system is mediating a cardioprotective process including reduced

endothelial dysfunction, inflammation, and atherosclerosis, as well as enhanced angiogenesis (Moyes and Hobbs 2019). Recently, it was shown that CNP-signaling mediated by NPR-C activation is important for organizing structural and functional elements in cardiac tissue, where endothelial cells, cardiomyocytes, and fibroblasts are all local sources of CNP (Moyes et al. 2019). Whether this essential cardiovascular role of the CNP system is also applicable to humans is still uncertain, but measurement in plasma indicates an independent association between CNP and heart disease.

The role CNP and its receptors in reproductive physiology represents another branch of the diverse action of CNP. Studies in female rodents have shown high concentrations of CNP mRNA in ovaries and uterus. In addition, both CNP and NPR-B follow a time-dependent expression over the estrous cycle, and estradiol rapidly upregulates CNP synthesis in the uterus (Walther and Stepan 2004). In the male reproductive tissues, the CNP system is involved in testicular regulation and penile erection (Walther and Stepan 2004). Interestingly, the CNP system has been found to play a role at another level of reproductive biology. In a recent report, pulsatile gonadotropin-releasing hormone stimulation in gonadotropic cells of the anterior pituitary gland induced enhanced expression of CNP and NPR-B and, also, CNP upregulated gonadotrope transcription factors (Mirczuk 2019). Thus, CNP and its receptors seem to be involved in multiple and distinct cellular processes of reproductive function.

Measurement of C-Type Natriuretic Peptides in Human Plasma

In spite of the low circulating concentrations, several methods exist for quantification of CNP and its molecular precursor in plasma. Radioimmunoassays for measurement of CNP-22 have been developed, and plasma concentrations for healthy adult individuals are typically in the range of ~1 pmol/L (Prickett et al. 2013). In this context, it should be recapitulated that the related peptides ANP and BNP circulate in much higher concentrations, and cross-reactivity should always be taken into account. Radioimmunoassays and enzyme-linked immunosorbent assays (ELISAs) for measurement of the more stable N-terminal proCNP fragment have revealed approximately 10–20-fold (~15 pmol/L) higher concentrations in plasma when compared with CNP-22 (Prickett et al. 2013). In addition, a modified radioimmunoassay for proCNP-derived peptides has been developed, where the assay principle and the specific molecular target ensure detection of various forms of proCNP and, therefore, measured plasma concentrations are higher than that of N-terminal proCNP concentrations, typically 30–50 pmol/L in reference individuals (Lippert et al. 2010). Lastly, an immunoassay for the signal peptide from preproCNP has shown that this "by-product" of peptide processing is also present in the human bloodstream and in remarkably high plasma concentrations of 60–90 pmol/L with no apparent correlation to N-terminal proCNP concentrations (Pemberton et al. 2014).

Although the vascular endothelium is thought to be a major source for proCNP-derived peptides in plasma, it is largely unknown in what proportion the different CNP-producing tissues contribute to the venous plasma concentrations. Nevertheless, measurement of circulating CNP and its molecular precursors as biomarkers has been the scope of different clinical studies, predominantly within cardiovascular research. In particular, N-terminal proCNP has been shown to be an informative molecular target for subgroups of patients with heart disease. High concentrations of N-terminal proCNP in plasma are associated with an unfavorable prognosis with increased risk of disease progression and death in patients with unstable angina pectoris (Prickett et al. 2017) and in patients with heart failure with preserved ejection fraction (Lok et al. 2014). Notably, this predictive potential of N-terminal proCNP measurement was beyond established cardiovascular risk factors including other natriuretic peptides (ANPs and BNPs). It remains to be clarified which risk factor(s) high concentrations of CNPs in plasma potentially reflect, and if these

biomarkers add unique information with substantial value in the clinical setting.

Drugs

CNP Analogs

The genetic disorder achondroplasia is the most common variant of dwarfism caused by a gain-of-function mutation in the FGFR3-receptor that leads to increased and unregulated intracellular signaling. Since the discovery that CNP/NPR-B-signaling inhibits the Raf-MEK½ step of the ERK½ pathway (a MAPK cascade initiated by the activated FGFR3 receptor), CNP analogs have been in focus as a drug target to attenuate the constitutively active FGFR3-signaling (Legeai-Mallet 2016). Due to the rapid removal of bioactive CNP in the human circulation, CNP treatment is challenged by the need of prolonged release and/or stabilized forms of CNP analogs. Different CNP analogs have been developed and are currently at different stages of clinical testing (Savarirayan et al. 2019; Breinholt et al. 2019). Recently, a clinical report showed promising results from a phase 2 dose-finding trial on the treatment with the CNP agent Vosoritide, in 35 children with achondroplasia aged 5–14 years. Vosoritide treatment was administrated subcutaneously once daily, the side effects were generally mild, and, most importantly, the growth velocity was increased for up to 42 months. Ongoing phase 3 studies will further clarify the effect of Vosoritide on height growth in a broader age span and evaluate the safety of the treatment (Savarirayan et al. 2019).

Other Ligands of CNP Receptors

Different pharmaceutical strategies are pursued to develop drugs to activate the CNP system as potential treatment of cardiovascular diseases. As a means to activate both the NPR-A (the ANP/BNP-specific receptor) and the NPR-B-receptor, a fused drug, Cenderitide, has been designed. The molecular structure of Cenderitide is a CNP-22 molecule, where a C-terminal extension, identical to the 15 amino acid sequence in DNP, has been attached. Thus, the drug works as a dual ligand and triggers intracellular signaling from both NPR-A and NPR-B. The clinical scope of this agent is treatment of heart failure with both reduced and preserved ejection fraction. Cenderitide has been tested by infusion in 12 patients with stable heart failure, and a safe, well-tolerated treatment was reported as well as increased cGMP plasma concentrations (Kawakami et al. 2018). Another approach is to activate the NPR-C-signaling by designing agents specific to this common natriuretic peptide receptor that seems to mediate a cardioprotective function of the CNP system. A specific NPR-C agonist, cANF4–23, has been developed, and the drug has been tested in an experimental model of secondary pulmonary hypertension due to left ventricle heart failure, where a reduction in pulmonary artery systolic pressure and enhancement of cardiac performance has been observed. To our knowledge, cANF4–23 has so far not been tested in human trials (Egom et al. 2017).

Inhibition of Neprilysin

As mentioned, neprilysin is abundantly expressed in the human organism and degrades a wide range of regulatory peptides. In particular, neprilysin has a high affinity for natriuretic peptides (CNP $\geq$ ANP $>$ BNP) (Potter 2011). Inhibition of neprilysin has been introduced in the treatment of chronic heart failure with the aim to utilize the beneficial effect of increased endogenous natriuretic peptide concentrations. Since neprilysin also degrades the potent vasoconstrictor angiotensin II (Ang II), treatment with inhibition of neprilysin by sacubitril is combined with the angiotensin-receptor blocker valsartan, in order to antagonize a potential vasoconstrictive effect of diminished Ang II degradation. Sacubitril/valsartan is currently an approved drug in the treatment of chronic heart failure with reduced ejection fraction (HFrEF) and has been shown to lower both the risk of death and the hospitalization rate compared with treatment of angiotensin-converting-enzyme-inhibition alone (McMurray et al. 2014). Moreover, a recent trial found a positive effect of sacubitril/valsartan treatment in

subgroups of patients with heart failure with preserved ejection (HFpEF) (Solomon et al. 2019). The mechanism of sacubitril is thought to be mediated by an increased bioavailability of endogenous ANP and BNP. Still, given the versatile activity of neprilysin, it remains more or less unresolved if changed activities of other peptide systems, including the CNP-system, contribute beneficially. Considering the high affinity of bioactive CNP to neprilysin and the marked vasoprotective effects of CNP in preclinical studies, there is an urgent need of examining the effect of neprilysin inhibition on the CNP system in humans.

Cross-References

► MAP Kinase Cascades

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Cyclic AMP-Dependent Protein Kinase Anchoring Proteins

▶ A-Kinase Anchoring Proteins (AKAPs)

Cyclin-Dependent Kinase Inhibitors

▶ Cyclin-Dependent Kinases as Therapeutic Targets

Cyclin-Dependent Kinases as Therapeutic Targets

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Synonyms

Cyclin-dependent kinase inhibitors

Definition

A subset of cyclin-dependent kinases (CDKs), including CDK1, CDK2, CDK3, CDK4, and CDK6, control progression through the cell division cycle by phosphorylating specific substrate proteins in an ordered, temporal sequence. Since cancer is a disease of uncontrolled cell proliferation, CDK inhibitors may be useful as anticancer therapeutics. The rationale for using small-molecule CDK inhibitors as anticancer drugs is further supported by the observation that natural CDK inhibitor proteins p15, p16, p18, p19, p21, and p27 suppress cell proliferation, and in certain contexts function as tumor suppressors (Lee and Yang 2001).

Inhibitors of Cell Cycle CDKs

Most efforts to develop anticancer CDK inhibitors have focused on CDK2, CDK4, and CDK6 due to their roles in phosphorylating the retinoblastoma tumor suppressor protein, Rb, and in driving cells through the G1 to S-phase cell cycle transition and through S-phase. The cell cycle CDKs show extensive functional redundancy as demonstrated by mouse gene knockout models (Berthet and Kaldis 2007; L'Italien et al. 2006). Only CDK1 is essential for mouse development (Diril et al. 2012). The high sequence identity (65%) between CDK1 and CDK2 has made the development of CDK1- and CDK2-selective inhibitors challenging. RO-3306 is one of the few compounds that exhibits selectivity for CDK1 over CDK2 (Vassilev et al. 2006).

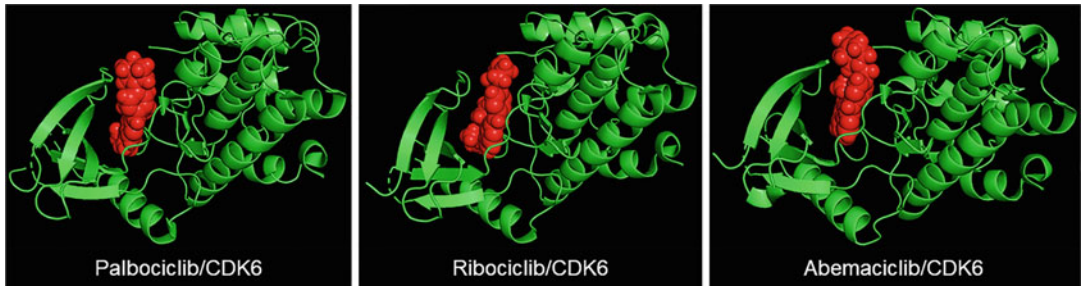
Inhibitors of Non-Cell Cycle CDKs

Non-cell cycle CDKs, including CDK5, CDK7, CDK8, CDK9, CDK11, CDK12/13, and CDK19 may also be important targets for anticancer therapeutics due to their roles in transcription, RNA processing, the DNA damage response, and the regulation of cell signaling proteins (reviewed in (Fisher 2019; Liu et al. 2017; Dannappel et al. 2018; Lui et al. 2018)).

Modes of CDK Inhibition

CDK inhibitors may function in several distinct ways as reviewed previously (Law et al. 2015). These inhibitory mechanisms are not mutually exclusive and some CDK inhibitors act via combinations of these modes.

- ATP-competitive: Most of the CDK inhibitors that have been investigated act by occupying the ATP-binding pocket of the kinases. Analysis of crystal structures of various CDKs bound to their inhibitors has shown a variety of binding modes within the ATP-binding site, including binding at the Ribose pocket, the hydrophobic region, the solvent region, and the phosphate pocket. The dual CDK4/6-specific drugs Palbociclib (Ibrance), Ribociclib (Kisqali), and Abemaciclib (Verzenio) are FDA-approved CDK inhibitors used to treat subsets of breast cancer. The structures of



Cyclin-Dependent Kinases as Therapeutic Targets, Fig. 1 X-ray crystal structures of CDK4/6 inhibitors Palbociclib, Ribociclib, and Abemaciclib (red spheres) bound to CDK6 (green ribbon diagrams)

these drugs bound in the ATP pocket of CDK6 is shown in Fig. 1 (PDB#s: 5L2I, 5L2S, and 5L2T; (Chen et al. 2016)).

- Covalent

  The potential for developing irreversible CDK inhibitors is illustrated by the identification of an inhibitor that reacts with C312 of CDK7 (Christensen et al. 2014; Kwiatkowski et al. 2014), and a compound that bonds with C218 of CDK14 (Ferguson et al. 2019).
- Allosteric

  Allosteric kinase inhibitors are an attractive option since allosteric sites may be less conserved between kinases than the ATP and substrate-binding pockets. Some precedence exists for identifying allosteric CDK inhibitors and targetable allosteric sites (Pellerano et al. 2017; Betzi et al. 2011).

Revelations from the Clinical Use of CDK4/6 Inhibitors

Expectations were that CDK4/6 inhibitors would block tumor growth by inducing cell cycle arrest. However, results from clinical studies and preclinical models have shown that these drugs act through additional, non-cell cycle mechanisms. First, CDK4/6 inhibition can induce senescence selectively in cancer cells (Rader et al. 2013; Valenzuela et al. 2017; Chen and Pan 2017; Tao et al. 2017). These "additional" mechanisms of anticancer activity result in part from CDK4/6-dependent phosphorylation of substrate proteins, including FOXM1, SMAD3, and NFAT, in addition to RB, in cancer cells (Anders et al. 2011; Deng et al. 2018; Liu et al. 2014; Liu 2006; Zelivianski et al. 2010), and the effects of inhibiting CDK4/6 in immune cells and other cell populations in the tumor microenvironment (Teh and Aplin 2019; Schaer et al. 2018; Klein et al. 2018).

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Cyclizing (E.C.4.6.1.1.)

▶ Adenylyl Cyclases

Cyclooxygenases

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Synonyms

COX; Prostaglandin G/H Synthases; Prostaglandin-endoperoxide H Synthases (PGHSs)

Definition

The cylooxygenases (COX)-1 and COX-2 are two distinct isoenzymes encoded by separate genes that catalyze the first two enzymatic steps in the biosynthesis of prostanoids from ω3 and ω6 20- and 22-carbon essential fatty acids (Smith et al. 2011). Bioactive prostanoids comprise prostaglandins (PGs) PGD, PGE, PGF, as well as prostacyclins (denoted PGIs) and thromboxanes (TXs), which display numerous physiological and pathophysiological effects mediated by specific G protein-coupled receptors (Table 1). The

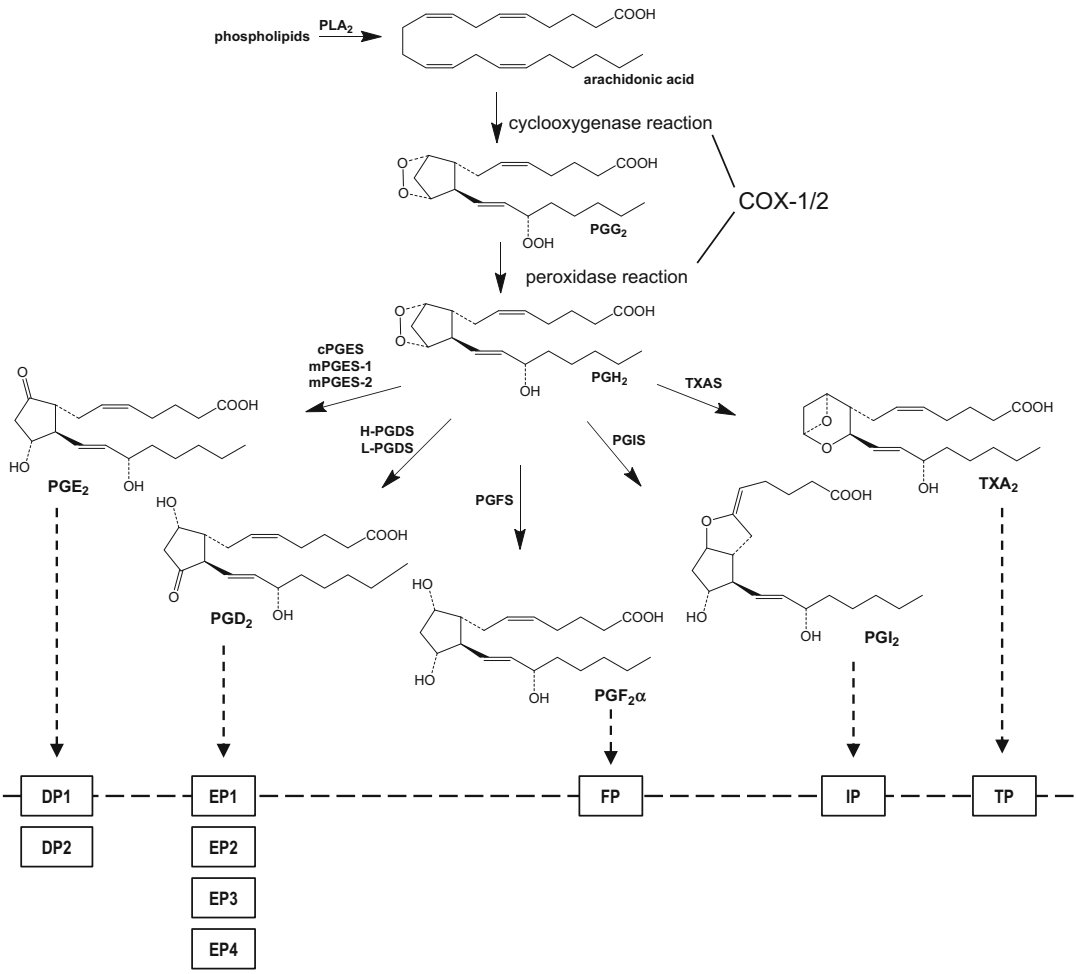
Cyclooxygenases, Table 1 Targets and biological function of prostanoids

Prostanoid	Prostanoid receptor	Target cells and tissues	Biological actions/(patho-)physiological effects
PGD_2	DP1, DP2	Mast cells, airways, brain	Vasodilatation, anti-platelet aggregation. Sleep induction, relaxation of intestinal smooth muscles.
PGE_2	EP1, EP2, EP3, EP4	Platelets, macrophages, vascular smooth muscle cells, kidney, brain	Vasodilatation, bronchodilatation, anti-platelet aggregation. Inflammation, fever, pain, protects gastric mucosa.
$PGF_{2\alpha}$	FP	Vascular smooth muscle cells, airways, eye, uterus	Vasodilatation/vasoconstriction, bronchoconstriction. Labor induction, uterus contraction, ovulation and parturition, contraction of intestinal smooth muscles.
PGI_2	IP	Platelets, endothelial cells, kidney, brain	Vasodilatation, bronchodilatation, anti-platelet aggregation. Decrease in blood pressure, mediates pain, antithrombogenic, protects gastric mucosa.
TXA_2	TP	Platelets, macrophages, vascular smooth muscle cells, kidney	Platelet aggregation, vasoconstriction, bronchoconstriction. Haemostasis, hypertension.

arachidonic acid (AA)-derived eicosanoids of the "2-series" compounds with two double bonds and 20 C-atoms are the most abundant and prominent prostanoids that are involved in inflammation, pain, fever, hemostasis and thrombosis, regulation of renal function, cytoprotection of the mucosa in the stomach, induction of labor, angiogenesis, and cancer (Smyth et al. 2009). The nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ibuprofen, diclofenac, or indometacin elicit their pharmacological effects by inhibition of COX enzymes (Patrignani and Patrono 2015). COX-1 is constitutively expressed in most tissues and mainly confers the biosynthesis of prostanoids involved in basic housekeeping functions throughout the body. In contrast, COX-2 is primarily an inducible enzyme in a variety of cells in response to endotoxins, cytokines, and mitogens, but is also constitutively expressed, for example, in the endothelium, brain, and kidney (Smyth et al. 2009). Therefore, the anti-inflammatory effect of classical NSAIDs is essentially due to inhibition of COX-2, while the ulcerogenic side effects are proposed to arise mainly from inhibition of COX-1, explaining the serious gastrointestinal toxicity of NSAIDs due to their nonselective inhibition of COX-1 and COX-2. Selective COX-2 inhibitors ("coxibs") display equivalent efficacy to traditional NSAIDs, with reduced gastrointestinal side effects but increased risk for gastrointestinal adverse events (Grosser et al. 2017; Schjerning et al. 2020).

Basic Characteristics

In eicosanoid biosynthesis, the bifunctional COXs catalyze two distinct sequential reactions (Fig. 1) that occur at physically different sites within the catalytic domain: after release of AA by a phospholipase (PL)A_2, the first reaction is a bis-oxygenation, where two O_2 molecules are inserted into the carbon backbone of AA at C11 and C15 yielding the cyclic peroxide PGG_2 (cyclooxygenase reaction); the second step (peroxidase reaction) is the reduction of the 15-hydroperoxyl group of PGG_2 leading to PGH_2 (Smith et al. 2011; Simmons et al. 2004). The highly instable PGH_2 is quickly converted by tissue-specific prostaglandin synthases: the hematopoietic PGD_2 synthase (H-PGDS) and lipocalin-type (L-)PGDS form PGD_2; the cytosolic PGE_2 synthase (cPGES) and the microsomal (m)PGES-1 and -2 generate PGE_2; the PGF synthase (PGFS)



Cyclooxygenases, Fig. 1 Biosynthetic pathway of prostanoids and their receptors. Upon release of arachidonic acid by a phospholipase (PL)A_2, COX-1 and COX-2 bis-oxygenate AA in a first step to the endoperoxide PGG_2 (cyclooxygenase reaction) and in a second step to the corresponding alcohol PGH_2 (peroxidase reaction). Specific prostanoid synthases then convert instable PGH_2 to the bioactive prostanoids PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 (prostacyclin) and TXA_2 that essentially mediate their biological effects via their cognate GPCRs

makes $PGF_2\alpha$; the PGI synthase (PGIS) produces PGI_2 (also named prostacyclin); the TXA synthase (TXAS) forms TxA_2 (Smith et al. 2011).

Structural Features

COX-1 and COX-2 from the same species possess about 60–65% amino acid identity and a high degree of structural homology. They both exist as heme-containing, monotopic membrane proteins, forming homodimers with a molecular mass of approx. 70 kDa per monomer, where human COX-1 consists of 576 and COX-2 of 581 amino acids. Each monomer consists of three distinct structural domains: (i) an N-terminal epidermal growth factor (EGF) domain, (ii) an α-helical membrane-binding motif, and (iii) a C-terminal catalytic domain, the latter accounting for about 80% of the monomer, containing the cyclooxygenase and peroxidase active sites on either side of the heme (Kurumbail et al. 2001). Both COXs carry three high mannose oligosaccharides, one of them facilitates the folding of the protein, and for COX-2 a fourth oligosaccharide regulates its degradation.

The cyclooxygenase active site lies on the opposite side of the heme from the peroxidase active site at the top of an L-shaped, long hydrophobic substrate-binding channel and a smaller amphipathic side pocket (Kurumbail et al. 2001). This channel originates in the membrane binding domain and extends toward the interior of the enzyme. The mouth of the channel consists of a large volume that narrows to a constriction, formed by Arg120, Tyr355, and Glu524, and must open for entry of substrates or inhibitors into the channel. When AA enters this channel, the C-20 methyl group is accommodated at the narrow terminus of the channel, while the carboxyl group lies at the constriction. This directs C-13 of AA for hydrogen abstraction in close proximity to the catalytically active Tyr-385. The peroxidase active site is located on the surface of the protein near the heme cofactor. The cyclooxygenase active site is composed of the L-shaped, mainly hydrophobic, substrate-binding channel and a smaller amphipathic side pocket. Since the three-dimensional structures of COX-1 and COX-2 are very similar, and both the cyclooxygenase active and the peroxidase active sites are highly conserved, it is not surprising that the two isoenzymes have comparable kinetic parameters (V_{max} and K_M) for the enzymatic conversion of AA. However, the volume of the COX-2 active site is about 20% larger than that of the COX-1, causing different properties between the isoenzymes: (i) increased promiscuity of COX-2 for fatty acid substrates, (ii) distinct susceptibilities for inhibition by aspirin that acetylates COX-1 at Ser530 and completely abrogates enzyme activity, whereas acetylation of COX-2 (also at Ser530) prompts for formation of 15*R*-hydroxyeicosatetraenoic acid instead of PGH_2, and (iii) the presence of the larger Ile523 in COX-1 versus Val523 in COX-2 limits the size of the side pocket, thus hindering the accommodation of space-demanding inhibitors, selective for COX-2.

Reaction Mechanisms

Conversion of AA to PGG_2 is initialized by the abstraction of the pro-S hydrogen atom from C-13, followed by steps that are consistent with nonenzymatic lipid peroxidation (Smith et al. 2011). Cyclooxygenase catalysis requires enzyme activation which depends on the peroxidase activity: a two-electron reduction of a peroxide substrate causes oxidation of the ferric heme to an oxo-ferryl porphyrin radical cation. The transfer of an electron from Tyr-385 to the heme generates a tyrosyl radical which is in perfect position to abstract the pro-S hydrogen from C-13 of AA. Reduction of the peroxyl radical to the hydroperoxide to form PGG_2 regenerates the tyrosyl radical. Subsequent after the cyclooxygenase reaction, the peroxidase reduces the 15-hydroperoxy group of PGG_2 to the corresponding alcohol of PGH_2.

Roles in Physiology and in Pathophysiology

COX-1 and COX-2 share the same catalytic activities and generate the same product, but they display numerous structural and functional differences with significant consequences for physiology and pathophysiology (Smyth et al. 2009). COX-1 is widely distributed and constitutively expressed in most tissues, and its gene, Ptgs-1, codes for a 2.8 kb mRNA that is relatively stable. The COX-2 gene, Ptgs-2, in contrast, is an immediate early gene that can be induced by various inflammatory and proliferative stimuli, and the 4 kb COX-2 mRNA is rather instable leading to rapid turnover. Moreover, the translation rates for the two isoenzymes differ, being superior for COX-2 over COX-1, due to differences in the signal peptides at the N-terminus, that is, 22-24 for COX-1 and 17 for COX-2. The differences in the pattern of gene expression suggest that COX-1-derived prostanoids are required for homeostatic functions, for example, gastric cytoprotection and hemostasis, whereas COX-2 predominantly may play a pathophysiological role in PG formation particular during inflammation but also during tumorigenesis (Simmons et al. 2004). In fact, COX-2 is frequently expressed in many types of cancers exerting pleiotropic and multifaceted roles in carcinogenesis and cancer cell resistance to chemo- and radiotherapy. It induces cancer stem cell-like activity and promotes apoptotic resistance, proliferation, angiogenesis, invasion, and metastasis of cancer cells (Wang and Dubois 2010). Administration of

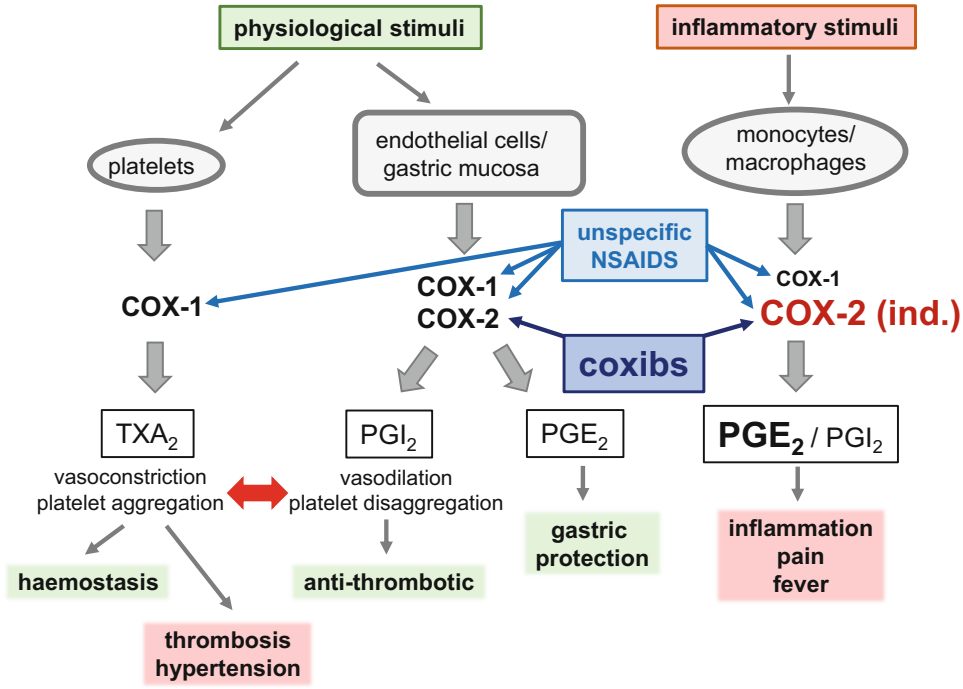
COX-2 inhibitors in a preoperative setting could reduce the risk of metastasis in cancer patients and sensitizes cancer cells to treatments like radio- and chemotherapy (Hashemi Goradel et al. 2019).

The simplified pattern of beneficial and detrimental roles of COX-1 and COX-2, respectively, is a matter of ongoing debate for several reasons. Selective pharmacological and genetic inhibition of COX-1 or COX-2 as well as clinical use of COX-2 selective inhibitors do not confirm this paradigm of COX function (Grosser et al. 2017; Schjerning et al. 2020; Patrono and Baigent 2017). In humans, COX-1 is uniformly expressed in almost all tissues, most prominently in platelets, blood vessels, smooth muscle cells, interstitial cells, and mesothelial cells. But also the COX-2 protein was found in nearly all organs, such as in brain, kidney, thymus, stomach, and the female reproductive tract, mainly localized to parenchymal cells. Genetic deletion of COX-2 indicates crucial roles of the isoenzyme in kidney development and female fertility, and prolonged therapeutic use of COX-2 selective inhibitors confirms homeostatic functions of COX-2 in the cardiovascular system. Moreover, COX-2 may play a significant role in resolution of inflammation which is particularly important in the healing of gastric ulcers, and inhibition of COX-2 can even cause exacerbated inflammation. On the other hand, COX-1 is constitutively expressed in resident inflammatory cells and can be induced during endotoxin-evoked inflammatory responses and during cellular differentiation. Moreover, pharmacological or genetic inhibition of COX-1 did not result in increased susceptibility to gastric ulceration. In fact, mRNA and protein levels of both COX-1 and COX-2 are co-expressed in circulating inflammatory cells and in the inflamed synovium of rheumatoid arthritis patients. Thus, both COX-isoenzymes may contribute to the acute inflammatory response where COX-1-derived prostanoids may be generated in the initial phase, while COX-2 upregulation is delayed for several hours being the dominant pathway in the chronic phase of the inflammatory response.

Beside the distinct tissue-specific gene expression of COX-1 and COX-2, there are additional reasons why the isoenzymes are not functionally interchangeable at the protein level (Patrignani and Patrono 2015; Smith et al. 2011). First, COX-1 and COX-2 differentially couple to downstream prostanoid synthases, for example, COX-2 with PGIS or with mPGES-1. Second, COX-2 requires lower amounts of activating hydroperoxides than COX-1 and thus COX-2 can function at lower AA concentrations than COX-1. Third, COX-2 exhibits a more promiscuous substrate specificity and can metabolize also EPA, α- and γ-linolenic acid, or linoleic acid, but also ester and amide derivatives of AA, such as the endocannabinoids 2-arachidonoyl-glycerol or arachidonoylethanolamide that are only poor substrates for COX-1. Metabolites of these endocannabinoids display a variety of physiological activities mediated by orphan GPCRs, eicosanoid receptors, or nuclear lipid receptors. Finally, acetylation of Ser530 by aspirin in the active site inhibits prostanoid formation of both isoenzymes but retains the ability of COX-2 to oxygenate AA to 15*R*-hydroperoxy-eicosatetraenoic acid instead of PGG_2. In conjunction with lipoxygenases, acetylated COX-2 can generate aspirin-triggered lipoxins, protectins, and resolvins that are inflammation-resolving lipid mediators that actively terminate inflammation and promote tissue regeneration, potentially explaining the clinical benefits of aspirin (Serhan et al. 2015).

Drugs

With the discovery that aspirin and aspirin-like drugs mediate their anti-inflammatory properties by inhibiting PG formation in 1971, COX was revealed as the major drug target of NSAIDs. Although several off-target effects of NSAIDS have been reported, most of them occurred at supratherapeutic concentrations in cell culture experiments. Today, COX inhibitors are divided into the class of traditional NSAIDs that inhibit both COX-1 and COX-2 and into the class of COX-2-specific inhibitors, referred to as coxibs (Patrignani and Patrono 2015). The serious gastrointestinal toxicity of traditional NSAIDs and the initial assumption that COX-2 is the exclusive source of prostanoids in inflammation and cancer,



Cyclooxygenases, Fig. 2 Physiological and pathophysiological roles of COX-1 and COX-2 and targeting by NSAIDs or coxibs. Physiological stimuli cause moderate formation of TXA_2, PGI_2 and PGE_2 by ubiquitously and constitutively expressed COX-1 and COX-2, mediating normal haemostasis, blood pressure regulation and gastric protection. Under inflammatory conditions, COX-2 is strikingly induced in monocytes and macrophages producing substantial PGE_2 that causes inflammation, fever and pain. Unselective NSAIDS that inhibit COX-1 and COX-2 block the formation of all prostanoids and suppress inflammatory pain and fever but may cause gastrointestinal toxicity devoid of serious cardiovascular hazards. Coxibs and COX-2-preferring NSAIDs (e.g. diclofenac) effectively block inflammation sparing some of the gastric side effect but cause cardiovascular complications due to superiority of COX-1-derived production of pro-thrombotic TXA_2 versus anti-thrombotic PGI_2

without sufficient understanding of COX-2 biology, stimulated the intensive development of coxibs with the expectation that selective COX-2 inhibition provides analgesia devoid of gastrointestinal complications. Later it was found that these actively advertised "safer NSAIDs" revealed cardiovascular adverse events, including heart attacks, which is currently the major obstacle related to the use of COX-2-selective drugs (Schjerning et al. 2020). Together, the commonly accepted rule of thumb is that COX-2 inhibition is required and sufficient for NSAIDs to exert the anti-inflammatory, analgesic, and antipyretic effects, while the variable degrees of COX-1 and COX-2 inhibition contribute to the toxicities affecting the gastrointestinal tract, the kidneys, and the cardiovascular system, eventually providing a rational for developing COX-2 selective inhibitors (Fig. 2).

Among the multiple NSAIDs, aspirin is unique as this drug is the only one that covalently and irreversibly inhibits COX-1/2 by acetylation of Ser530. All the other NSAIDs bind noncovalently as either (a) rapidly and time-dependent AA-competitive inhibitors (e.g., naproxen) or (b) time-independent tightly binding inhibitors (e.g., indomethacin) that slowly form very stable complexes with COX and are highly potent. Interestingly, no structural differences are apparent in complexes of COX-1 with a competitive inhibitor or a slow tight-binding inhibitor (Kurumbail et al. 2001).

The chemical structures of NSAIDs are diverse but exhibit the common features of fatty acid-like molecules with a hydrophobic backbone

consisting of an aromatic nucleus and an acidic head group:

- Salicyclic acids, for example, aspirin or sulphasalzine
- Phenylacetic acids, for example, diclofenac, felbinac, or bufexamac
- Hereoaryl acetic acids, for example, indomethacin or etodolac
- Arylpropionic acids, for example, ibuprofen, flurbiprofen, naproxen, dexketoprofen or thiaprofenic acid
- Anthranilic acids, for example, mefenamic acid or flufenamic acid
- Enolic acids/oxicames, for example, meloxicam, lornoxicam, or piroxicam

The crystal structure of COX-1 with various NSAIDs such as diclofenac, ibuprofen, or flurbiprofen revealed that the aromatic portion of the molecules lies in the hydrophobic substrate-binding channel of the enzyme while the acidic moiety forms hydrogen bond to the hydroxyl of Tyr338 and the guanidinium group of Arg120. The NSAID-binding site in COX-2 is identical to the one in COX-1, except the presence of the Val523 and Arg513 in COX-2 versus the more space-demanding Ile523 and His513 in COX-1. The smaller amino acids in the COX-2 active site cause a slightly larger volume of the drug-binding cavity and allow, in contrast to COX-1, the accommodation of the sulfone or sulfonamide group of coxibs (e.g., celecoxib, etoricoxib, parecoxib). This eventually enabled the development of selective COX-2 inhibitors with 10- to 100-fold superior potencies to COX-2 versus COX-1 enzymes. Notably, the ratio of COX-1/COX-2 selectivity of a given inhibitor can strikingly differ, depending on the assay system, that is, cell-free versus cell-based assays. For example, the binding affinities of celecoxib are up to 3200-fold higher for COX-2 over COX-1, while in cell-based systems (human whole blood assay) there was no marked selectivity for COX-2. Similarly, aspirin inhibits the activities of isolated COX-1 and COX-2 enzymes with similar potencies but is about 60-fold more against COX-1 in isolated human platelets as compared to COX-2 in isolated human monocytes (Patrignani and Patrono 2015).

NSAIDs block the biosynthesis of all types of prostanoids due to unselective inhibition of COX−1/2 and are efficacious therapeutics with antipyretic, anti-inflammatory and analgesic effects. However, due to inhibition of prostanoids with homeostatic functions, they are also afflicted with severe side effects. The most common on-target side effects are as follows:

- Gastrointestinal toxicity: ulceration, bleeding, perforation, erosion, and dyspepsia, mainly due to reduced PGE_2 levels
- Nephrotoxic effects: Na^+ and water retention, edema, and acute kidney failure, essentially due to lower PGI_2 and PGE_2 levels
- Cardiovascular toxicity: higher bleeding tendencies (in particular with aspirin) due to impaired TXA_2 and elevated blood pressure, heart attack, stroke, and heart failure, mainly due to increased ratio of TXA_2 to PGI_2 formation

Note that NSAIDs that even only moderately prefer COX-2 over COX-1, such as meloxicam, nimesulide, and etodolac, induced less stomach toxicity than other representatives like indomethacin or naproxen that dominate in COX-1 inhibition. In fact, the COX-1-dependent gastrointestinal toxicity of NSAIDs favored the clinical development of the COX-2-selective coxibs, in order to limit its burden. As expected, the COX-2-selective coxibs display reduced toxic effects in the stomach despite potent anti-inflammatory efficacy, but still are not entirely devoid of gastrointestinal complications. Thus, using selective inhibitors or genetic deletion of COX-2 revealed an important role of COX-2 in the healing of preexisting ulcers, suggesting that both COX-isozymes are a source of cytoprotective prostanoids. Therefore, simultaneous inhibition of COX-1 and COX-2 that profoundly suppresses prostanoid biosynthesis might be a hazard for the gastrointestinal system consistent with the finding that in mice inhibition of both COX-1 and COX-2 is required for the induction of gastric lesions.

The most problematic obstacle with coxibs is their modest but consistent increase in cardiovascular risk under chronic use (particularly under long-term use, > 1.5 years) in a small percentage of patients, which led to the withdrawal of rofecoxib (in 2004), valdecoxib (in 2005), and lumiracoxib (in 2007) from the market after slightly higher incidences of myocardial infarctions and thromoembolic events in risk patients. The increased risk of cardiovascular events under coxib treatment is seemingly due to the preferential inhibition of systemic (and renal) vasodilatory and platelet-inhibitory PGI_2 produced by constitutively expressed COX-2 in the endothelium versus platelet-derived prothrombotic TXA_2 generated by COX-1 in platelets (Fig. 2). Nevertheless, other factors may contribute including intra- and inter-subject variability in the extent of COX isozyme inhibition and COX-2 selectivity achieved at therapeutic doses of coxibs related to heterogeneity in the pharmacokinetics and pharmacodynamics.

Conclusions and Future Perspectives of COX Inhibitors

The COX-1 and COX-2 isoforms are the key enzymes in the biosynthesis of prostanoids and are the targets of NSAIDs that are effective, widely used analgesics. The ubiquitous constitutive expression of COX-1 and the inducible expression of COX-2 led to the hypothesis that COX-1 produces homeostatic PGs, while PGs produced by COX-2 are primarily pathophysiological contributing to inflammation, fever, pain, and cancer. Recent discoveries question this paradigm and reveal more complex functionalities for both isoenzymes.

After initial attempts to eliminate the gastrointestinal toxicity of unselective COX-1/2 inhibitors, considerable attention has then been focused on the cardiovascular safety of COX-2-selective drugs given the increased risks of heart failure, elevated blood pressure, and thrombotic events in a very small number of patients. Today it is accepted that gastrointestinal toxicity is evident for all NSAIDs, also for COX-2-selective drugs. The greatest risk of cardiovascular hazards concerns coxibs but also nonselective NSAIDs with strong COX-2 inhibition such as diclofenac. Thus, the use of NSAID is generally discouraged in cardiovascular risk patients but pain-relief medication is often required, but the use of NSAIDs is still likely to rise. Future studies should focus on optimizing the safe use of NSAIDs using measures of drug exposure and biochemical markers of drug activity, carefully monitored for blood pressure and renal function as functional markers of COX-2 inhibition and measurements of symptom relief. Careful risk-benefit evaluation might help at identifying those patients for which COX-2-selective inhibitors should remain the drug of choice, versus traditional NSAIDs.

Cross-References

- ▶ Analgesics
- ▶ Endocannabinoids
- ▶ Fever
- ▶ Inflammation
- ▶ Pro-resolving Mediators
- ▶ Prostanoids
- ▶ Rheumatoid Arthritis

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Cytochrome P450 (CYP)

▶ P450 Monooxygenase System

Cytochrome P450 Isozymes

▶ P450 Monooxygenase System

Cytochrome P450 Monooxygenases

▶ P450 Monooxygenase System

Cytokines

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Synonyms

Chemokines; Growth factors; Interferons; Interleukins; Lymphokines; Monokines

Definition

Cytokines are a large and heterogeneous group of small polypeptides, most of them with a Mr between 8 and 30 kDa (Table 1). They are mostly soluble extracellular mediators (some exist in membrane-associated form) and function through high-affinity cell surface receptors. Released by cells of hematopoietic origin (reflected in the terms "lymphokines" and "monokines") as well as by a wide variety of non-hematopoietic cells, cytokines are critical for the normal functioning of the immune defense, which they coordinate by communicating between participating cells.

"My private metaphor was that of a zoo of factors in a jungle of interactions surrounded by deep morasses of acronyms and bleak deserts of synonyms" (Ibelgaufts 2020).

Basic Characteristics

Historically, many cytokines were discovered by means of their immunoregulatory properties, hence, the term interleukins. However, cytokines

Cytokines, Table 1 Structural/functional groups of cytokines (examples)

Interferons	IFN-α, β, γ
Hematopoietic cytokines/colony-stimulating factors	IL-3, IL-7, erythropoietin (EPO), GM-CSF, G-CSF, M-CSF, thrombopoietin (THPO)
IL-1 family	IL-1α, IL-1β, IL-18, IL-1Ra, IL-33, IL-37
TNF family	TNFα (cachectin, TNFSF2), lymphotoxin-alfa (TNFβ,TNFSF1), CD40 ligand (TRAP, TNFSF5), CD95 ligand (FAS ligand, TNFSF6), TRAIL (Apo2 ligand, TNFSF10)
Chemokines	IL-8 (CXCL8), GROα/β/γ (CXCL1/2/3), MIP1α (CCL3), MIP3α (CCL20), MCP1 (CCL2), IP-10 (CXCL10), I-TAC (CXCL11)
Immunoregulatory cytokines	IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17, IL-23

regulate not only functions of the specific immune system but also many aspects of infection and inflammation. At the cellular level, they control proliferation, differentiation, functional activity, and apoptosis. As a whole, they are synthesized by a broad variety of different cell types. This also holds true for many cytokines individually (e.g., IL-6, IL-8, IFN-β, for further information on individual cytokines, see Ibelgaufts 2020), whereas synthesis of others is restricted to one or few cell types (e.g., IL-2, IL-4, IL-17, IFN-γ). In general, their biosynthesis is rapidly and highly inducible by changes of the cell's microenvironment. Cytokines often have a short half-life, and production is restricted to potentially pathological conditions. Uncontrolled synthesis results in the systemic inflammatory response syndrome ("cytokine storm") but more often in chronic inflammatory or other diseases. All cytokines act locally in an autocrine or paracrine manner, but some may also act systemically. Examples for the latter are the induction of fever by IL-1 and TNF and the induction of acute phase proteins in the liver by IL-6. Cytokines can therefore also be regarded as hormones. Cytokines are pleiotropic, that is, they control multiple biological responses. There is considerable functional overlap between certain cytokines (e.g., TNF and IL-1 both induce inflammation). In general, activation of a cell by a cytokine results in reprogramming of the cell's gene expression profile.

Mechanism of Action

Most cytokines – in particular the ones that are targeted in the clinics – bind to high-affinity receptors on the plasma membrane. These receptors fall into different classes, those with enzymatic tyrosine kinase activity (e.g., the receptors of certain hematopoietic growth factors (M-CSF, SCF), G-protein-coupled receptors (chemokine receptors), and several groups that bind to a variety of intracellular adaptor proteins (IL-1, TNF, interferon, and hematopoietic cytokine receptor families)). Ligand-dependent receptor clustering activates several intracellular signaling pathways. These pathways consist of consecutive interactions between protein components that in part involve enzymatic activities. The activity of many proteins in a given cytokine-activated pathway is regulated by reversible phosphorylation and by non-degradative ubiquitinylation. The NF-κB, MAP kinase cascades, and JAK-STAT signaling pathways are intensively studied examples. Certain receptors (of the TNF-R family) can activate caspases, a family of proteases that executes programmed cell death. Other protein synthesis-independent effects of cytokines include cytoskeletal changes and modulation of cell surface receptors and adhesion molecules. Most cytokine-activated intracellular pathways ultimately turn on expression of genes whose products change the biological function of the same or a neighboring cell. These include further cytokines (hence the term "cytokine network"), cell surface receptors and adhesion molecules, enzymes involved in degradative processes (e.g., collagenases), and in formation of small molecular weight mediators (e.g., cyclooxygenases, NO synthases).

Cytokines and Therapy

Knowledge on cytokines has been exploited to improve the therapy of many diseases, basically in two ways:

1. Application of cytokines as drugs. A few relevant examples will be given below. In general, treatment with cytokines turned out to be problematic due to their pleiotropic nature of function. Thus tumor necrosis factor, apart from its antitumoral effect, has severe sepsis-like systemic side effects as manifestations of its activity, which largely precludes its use as anticancer drug. Similar toxicity has been observed for several other inflammatory cytokines, in particular IL-1 family members. Selected recombinant cytokines are being used to boost immune reactions during infection and cancer (interleukin-2, interferons) or to substitute cytokine deficiencies (hematopoietic growth factors, erythropoietin).
2. Interference with cytokine action. Inhibition of cytokine synthesis has turned out to be a major

component of the activity of anti-inflammatory drugs, with glucocorticoids being the most prominent example. Because for many cytokines the molecular mechanisms of their actions have been worked out in detail, rational strategies for interfering with their biological activities have been developed. The action of cytokines can be suppressed pharmacologically by different means: (i) inhibition of their synthesis by drugs (cyclosporin → IL-2 synthesis); (ii) prevention of their interaction with cell surface receptors by soluble receptors (TNFR1 extracellular domain), by neutralizing anti-cytokine (α-TNF) or anti-cytokine receptor (α-IL-2R) antibodies, or by natural antagonists (IL-1R antagonist); and (iii) blockade of specific events in the intracellular signaling pathways by protein kinase inhibitors such as JAK inhibitors. Extracellularly acting clinically used cytokines or cytokine antagonists are proteins. Repeated application, especially for those of nonhuman origin, bears the risk of recognition by the immune system and of antibody production against them with subsequent loss of efficacy or systemic or local adverse reactions. Therefore, the proteins are nowadays genetically engineered, that is, humanized to be as homologous as possible or to be even identical to human proteins. Variants of classical antibodies include antibody (Fab) fragments, polyethylene glycol (PEG)-modified Fab fragments, single chain nanobodies, bispecific antibodies, or antibody Fc fragments fused to extracellular receptor domains. Increasingly, biosimilars are produced which mimic the protein structures and activities of approved antibodies (Shepard et al. 2017).

Clinical Use (Including Side Effects)

Examples of Recombinant Cytokines Currently in Use

Interferons

Interferon alfa-2a (Roferon A®) and interferon alfa-2b (Intron A®) are applied in the treatment of chronic hepatitis B and some malignancies, especially hairy cell leukemia. IFNα proteins induce the expression of antiviral, antiproliferative, and immunomodulatory genes. Interferon beta-1a (Avonex®, Rebif®) and interferon beta-1b (Betaferon®, Extavia®) are applied in multiple sclerosis to reduce both frequency and severity of disease incidents. In multiple sclerosis, IFNβ proteins modulate the destruction of myelin in the cause of the autoimmune reaction. Interferon gamma-1b (Imukin®) is used to reduce the incidence of infections in the treatment of chronic granulomatosis and osteopetrosis. Common side effects of interferons are flu-like symptoms, fever, myelosuppression, and skin reactions.

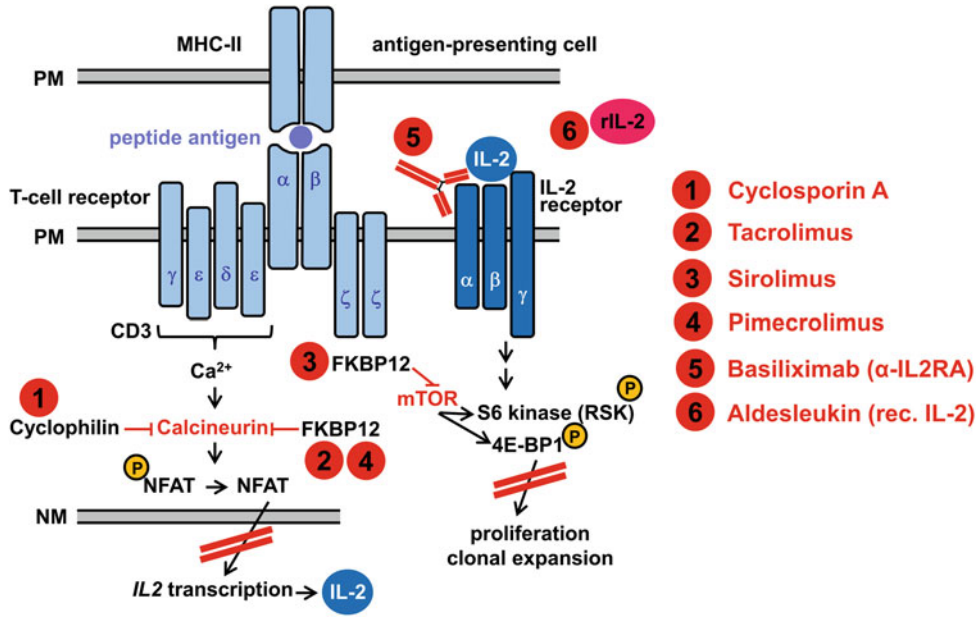
The recombinant forms of granulocyte colony-stimulating factor (G-CSF), such as filgrastim (r-metHuG-CSF, Neupogen®) and its biosimilars (Accofil®, Nivestim®, Grastofil®), or pegylated filgrastim and lenograstim (rHuG-CSF, Granocyte®), promote the differentiation of pluripotent bone marrow stem cells to leukocytes. GM-CSF (molgramostim) induces proliferation of cells of the macrophage, neutrophil, and eosinophil lineages, while G-CSF acts primarily on neutrophil precursors. They are effective in treatment of congenital and acquired neutropenias during chemotherapy of cancer or bone marrow transplantation. G-CSF is also applied to donors to increase peripheral blood progenitor cells for allogeneic bone marrow transplantation. Side effects include bone pain, fever, and myalgia. Interleukin-2 (Aldesleukin, Proleukin®) is a major growth factor and activator of cytotoxic and other T-lymphocytes. It is applied in the therapy of metastasing renal carcinoma. Side effects include hypotension, arrhythmias, edema, pruritus, erythema, central nervous symptoms, fever, and many others. Erythropoietin is physiologically produced in the kidney and regulates proliferation of committed progenitors of red blood cells. Recombinant versions (darbepoetin alfa, epoetin alfa, beta, theta, zeta) are used to substitute erythropoietin in severe anemias due to end-stage renal disease or treatment of cancer with cytostatic agents. Side effects include hypertension and increased risk of thrombosis.

Inhibitors of Cytokine Action

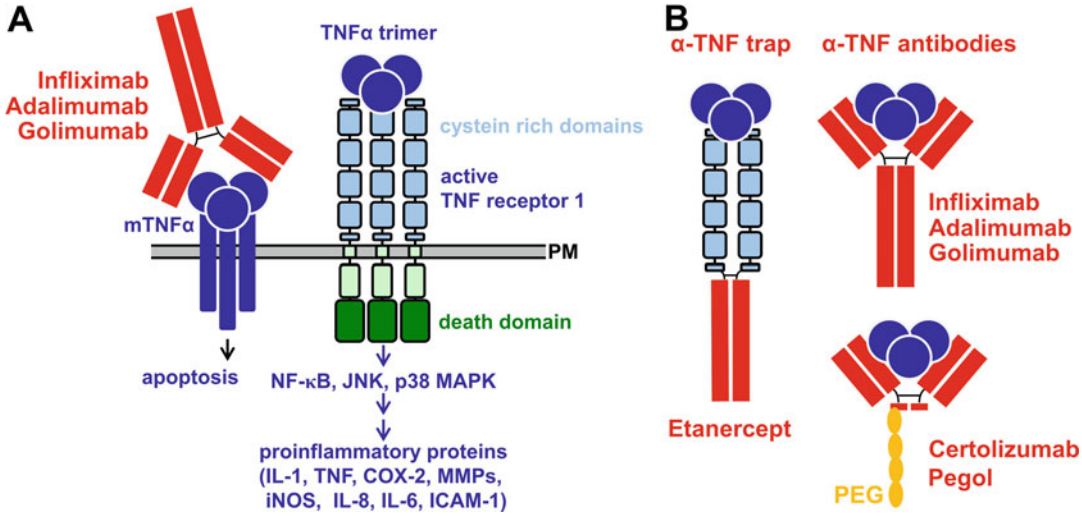
The humanized recombinant anti-IL-2 receptor antibody basiliximab (Simulect®) binds with high affinity to the IL-2 receptor alfa chain on T-lymphocytes and prevents activation and clonal expansion of anti-allograft T-lymphocytes by endogenous IL-2 during acute (kidney) allograft rejection. The main side effects are severe immunosuppression and cytokine release syndrome.

In intracellular inhibition of IL-2 production and activity, the immunosuppressants cyclosporin A (Sandimmune®), tacrolimus (Prograf®), (topical) pimecrolimus (Elidel®), and sirolimus (Rapamune®) are used for the prophylaxis of allograft rejection, for severe forms of autoimmune diseases, and for external treatment of inflammatory skin diseases (atopic dermatitis). Cyclosporin binds to the intracellular protein cyclophilin, and tacrolimus/pimecrolimus bind to the intracellular protein FK506-binding protein (FKBP) 12. The resulting complexes inhibit a serine-threonine phosphatase, calcineurin, which is required for T-lymphocyte activation and for IL-2 gene expression. Sirolimus also binds to FKBP12. However, the complex inhibits a different enzyme, the protein kinase mTOR (mammalian target of rapamycin), which is a major regulation of protein synthesis. Thereby, it inhibits IL-2 receptor-dependent progression of activated T-lymphocytes through the cell cycle. All four drugs suppress clonal expansion of antigen-activated T-lymphocytes. Cyclosporin and tacrolimus are nephrotoxic, and sirolimus causes hyperlipidemia (Fig. 1).

The monoclonal anti-TNF antibodies infliximab (Remicade®), adalimumab (Humira®), golimumab (Simponi®), and certolizumab pegol (Cimzia®) bind with high selectivity to human TNFα and neutralize its activity. Thereby, they decrease the effects of enhanced TNF levels during inflammatory disease such as production of



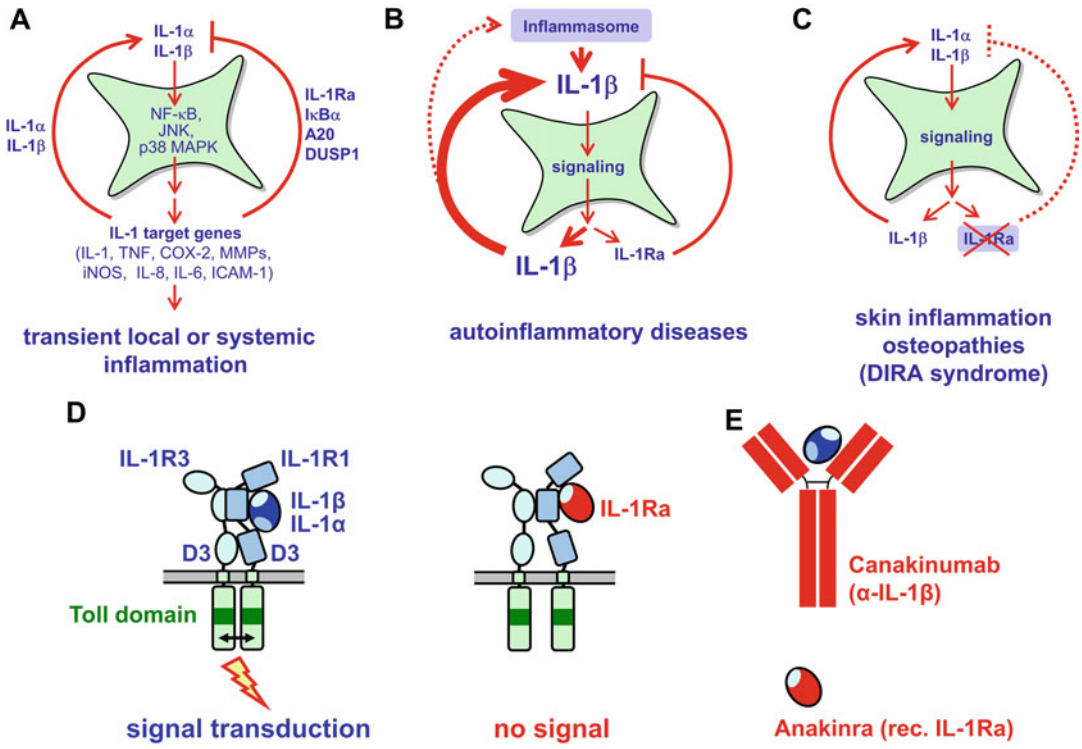
Cytokines, Fig. 1 Modulation of cytokine synthesis and function during activation of the specific immune system: antigenic activation of the multi-subunit T-cell receptor induces IL-2 synthesis which promotes clonal T-cell proliferation through the IL-2 receptor. IL-2 delivery or IL-2 receptor blockade activates or suppresses this process. The calcineurin antagonists cyclosporin A, tacrolimus, and pimecrolimus inhibit activation of cytoplasmic NFAT, a transcription factor essential for activation of the IL-2 gene (see entry ▶ "NFAT Family of Transcription Factors"). The mTOR antagonist sirolimus interferes with mTOR complex 1 (mTORC1) signaling and protein biosynthesis and thereby inhibits IL-2-dependent proliferation. *4E-BP1* eukaryotic translation initiation factor 4E-binding protein 1, *MHC* major histocompatibility complex, *NFAT* nuclear factor of activated T cells, *NM* nuclear membrane, *P* phosphorylation, *PM* plasma membrane, *rec.* recombinant, *RSK* ribosomal S6 kinase



Cytokines, Fig. 2 Modulation of TNFα: (**a**) scheme of the active trimeric TNF receptor 1 bound by a TNFα trimer upon inflammatory cell activation. Membrane-bound mTNFα can induce paracrine or reverse signaling and can be bound by α-TNFα antibodies to induce apoptosis. (**b**) Summary of TNFα antagonists. *NF-κB* nuclear factor "kappa-light-chain-enhancer" of activated B cells, *JNK* JUN N-terminal kinase, *mTNFα* membrane TNFα, *MAPK* mitogen-activated protein kinase

matrix metalloproteinases (MMPs), chemokines, adhesion molecules, cyclooxygenase-2 (COX-2) products (i.e., prostaglandins), inducible NO-synthase (iNOS), and proinflammatory molecules such as interleukin-1 and interleukin-6. The antibodies also recognize membrane-bound (m) TNFα on lymphocytes and other immune cells. These cells may subsequently become apoptotic or are eliminated via Fc-receptor-mediated phagocytosis. The anti-TNF antibodies are used to treat Crohn's disease, psoriasis (including psoriasis arthritis), spondylitis ankylosans, and rheumatoid arthritis. Side effects include immunosuppression and increased risk of infections. The recombinant soluble TNFR1-IgG1 fusion protein etanercept (Enbrel®) is a chimeric molecule consisting of the extracellular domain of the TNF receptor 1 (TNFR1) and the Fc portion of human IgG1. Two Fc domains are bound to each other via disulfide bonds, thereby yielding dimers with two binding sites for the TNF trimer. Such a construct is also called cytokine trap. Etanercept binds with high affinity to extracellular TNF and reduces TNF activity. Etanercept is not effective in Crohn's disease, possibly because it does not lead to destruction of membrane TNFα expressing cells or reverse mTNFα-mediated signaling. Indications and side effects are similar to those of infliximab and adalimumab (Fig. 2).

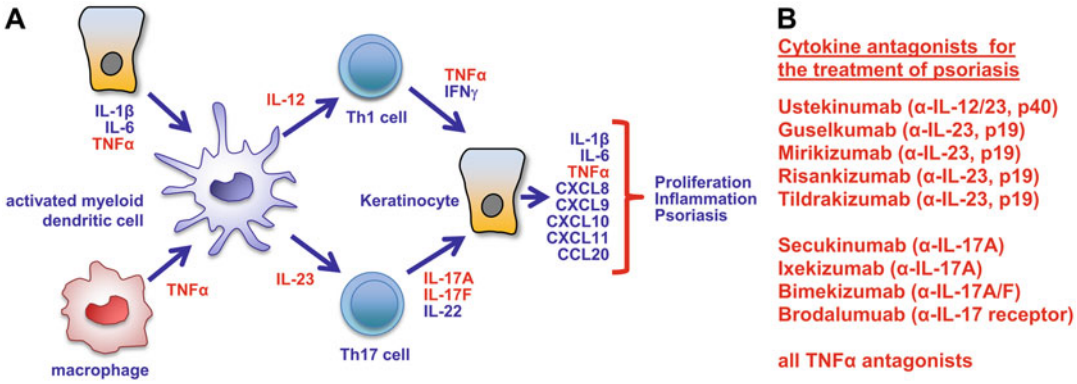
The recombinant human IL-1 receptor antagonist (anakinra, Kineret®) blocks the biological activity of both interleukin-1α and interleukin-1β by competitively inhibiting IL-1 binding to the interleukin-1 type I receptor (IL-1R1), which is expressed in most tissues and organs. The antibody canakinumab (Ilaris®) blocks secreted IL-1β. Both agents broadly reduce the proinflammatory activities of IL-1 including fever, cartilage destruction, and bone resorption and are highly effective in the treatment of gout and a number of auto-inflammatory syndromes which are mainly driven by cells of the myeloid compartment rather than by T or B cells (e.g., familial Mediterranean fever (FMF), cryopyrin-associated periodic syndrome (CAPS), and systemic juvenile idiopathic arthritis). Mild side effects include an increased risk of infections and neutropenia. The efficacy, therapeutic index, and safety of Anakinra allow using a single injection to diagnose IL-1-dependent disease (Fig. 3) (Mantovani et al. 2019).



Cytokines, Fig. 3 Modulation of the IL-1 system: (**a**) besides their own expression (as a feedforward loop), IL-1α and IL-1β induce the expression of multiple inflammatory proteins through the NF-κB, JNK, and p38 signaling pathways. The inflammatory reaction is self-limiting due to the simultaneous induction of a range of anti-inflammatory proteins, including the IL-1 receptor antagonist IL-1Ra. (**b**) Mutations in proteins of the IL-1β-releasing inflammasome complex amplify the feedforward loop, thus driving a range of the so-called auto-inflammatory disease. (**c**) Loss-of-function mutations in the IL-1Ra gene cause severe systemic inflammation including skin and bone disease by affecting negative control of endogenous IL-1 cytokines. (**d**) Scheme of the active IL-1 receptor heterotrimer composed of two subunits IL-1R1 and IL-1R3 (formerly called IL-1 receptor accessory protein, IL-RAcP) plus IL-1α or IL-1β ligands. Ligand binding causes a conformational change in the D3 domain of the IL-1R1 which promotes spatial proximity of the intracellular Toll domains to induce signaling. Binding of IL-1Ra to the IL-1R1 prevents formation of the active receptor structures. (**e**) Recombinant IL-1Ra and anti-IL-1β antibodies are highly effective in blocking IL-1-dependent diseases. *DIRA* deficiency of IL-1Ra syndrome, *A20*, also called *TNFAIP3* tumor necrosis factor alpha-induced protein 3, *DUSP* dual-specificity phosphatase, *IκBα* inhibitor of NF-κB alfa

While TNFα and IL-1α/IL-1β can be considered as master regulators of multiple proinflammatory diseases, the disruption of the IL-23/IL-17 network by antibodies has proven to be highly effective in the treatment of psoriasis and also inflammatory bowel diseases (Nestle et al. 2009). The antibodies ustekinumab (Stelara®), guselkumab (Tremfya®), risankizumab (Skyrizi®), tildrakizumab (Ilumetri®), secukinumab (Cosentyx®), ixekizumab (Taltz®), and brodalumab (Kyntheum®) interfere with this network at several levels and are in clinical use, while mirikizumab and bimekizumab are awaiting approval (Fig. 4).

Antibodies against IL-5 (mepolizumab (Nucala®), reslizumab (Cinqaero®)) or the IL-5 receptor (benralizumab (Fasenra®)) on eosinophils block differentiation and activation of these white blood cells and are used to reduce the



Cytokines, Fig. 4 Modulation of the IL-23/IL-17 cytokine network: (**a**) cell types and cytokine/chemokine mediators driving the uncontrolled keratinocyte proliferation and inflammation in psoriatic skin disease. (**b**) Approved antibodies with efficacy in blocking the actions of IL-23 and IL-17 type cytokines in psoriasis in addition to all TNFα antagonists. Note that IL-23 is composed of two subunits, p19 and p40, the latter is shared with IL-12. (**b**) Interference with the cytokine network is highly effective for treating psoriasis, a common chronic inflammatory skin disease with multiple systemic manifestations, including arthritis

severity and frequency of asthma attacks that are resistant to standard therapy.

The IL-6 receptor antibody tocilizumab (Roactemra®) is combined with methotrexate to treat severe rheumatoid arthritis, while the anti-IL-6 antibody siltuximab (Sylvant®) is used to treat Castleman disease, a lymphoproliferative disorder.

The JAK protein kinase inhibitors baricitinib (Olumiant®) and tofacitinib (Xeljanz®) suppress activation of JAK-STAT signaling and thereby the downstream effects on multiple inflammatory cytokines simultaneously. Baricitinib has a preference for JAK1 and JAK2, whereas tofacitinib preferentially targets JAK1 and JAK3. Both compounds suppress STAT1, 3, 5 phosphorylation and subsequent gene expression events. Both inhibitors affect the (patho)physiological functions of IL-2, IL-4, IL-7, IL-9, IL-10, IL-15, IL-21, IL-22, IFNα/β/γ, GM-CSF, EPO, TPO, and IL-6. Tofacitinib also suppresses IL-12 and IL-23 signaling. These drugs are alternative treatments for rheumatoid arthritis in case the abovementioned monoclonal antibodies or conventional disease-modifying antirheumatic (DMARDs) drugs fail. Tofacitinib is additionally approved for the treatment of psoriasis and colitis ulcerosa.

Outlook: Anti-cytokine therapy is now firmly established in the clinics and offers multiple new options for personalized therapy of common but also rare chronic inflammatory diseases. Improvements will involve variants of genetically engineered proteins with altered specificities and pharmacokinetic properties such as bi-specific antibodies or nanobodies (Shepard et al. 2017). Future developments will involve a better understanding of combination therapies targeting several components of cytokine networks simultaneously and the development of allosteric and irreversible (rather than ATP-competitive reversible) inhibitors of intracellular cytokine pathways (Gaestel et al. 2009).

Cross-References

- ▶ Chemokine Receptors
- ▶ Growth Factors
- ▶ JAK-STAT Pathway
- ▶ Map Kinase Cascades
- ▶ PIAS Proteins
- ▶ Rheumatoid Arthritis

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Cytoplasmic Tyrosine Kinases

► Tyrosine Kinases

D

Daily Rhythms

► Circadian Rhythms

Dalteparin

► Heparin and Related Drugs

Dementia due to Alzheimer's Disease

► Alzheimer's Disease

Depressive Illness

► Monoamine Oxidases and Their Inhibitors

Derivatives of the 7-Aminocephalosporanic Acid

► Cephalosporins

DesArg^{10}-Kallidin (Lys^{0}, desArg^{9}-Bradykinin)

► Kinins

DesArg^{9}-Bradykinin

► Kinins

Deubiquitinases

► Deubiquitylating Enzymes

Deubiquitylating Enzymes

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Synonyms

Deubiquitinases

Definition

Deubiquitylating enzymes (DUBs) are the peptidases that can remove ubiquitin from substrate

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proteins, edit ubiquitin chains, or cleave ubiquitin precursors. Human genome encodes about 100 DUBs that can be classified into six families based on similar sequence and conserved domains. These DUB families include ubiquitin-specific proteases (USPs), ubiquitin carboxyl-terminal hydrolases (UCHs), Machado-Josephin domain-containing proteases (MJDs), ovarian tumor proteases (OTUs), motif interacting with ubiquitin-containing novel DUB family (MINDYs), and JAB1/MPN/MOV34 metalloenzyme family (JAMMs). The first five DUB families are cysteine peptidases with the catalytic triad of cysteine, histidine, and asparagine/asparagine residue, whereas JAMMs are zinc metallopeptidases. DUBs exhibit strong substrate selectivity in targeting individual proteins and structural motifs. This specificity is imparted via the key domain of DUBs, i.e., a zinc finger ubiquitin-specific protease domain (ZnF-UBP domain) (Dou and Zonder 2014; Farshi et al. 2015; Harrigan et al. 2018).

Basic Characteristics

Characteristics of Ubiquitylation

Ubiquitylation exhibits vital enzymatic reactions for post-modification and turnover of substrate proteins in cellular processes. Ubiquitin, a 76-residue polypeptide, is conjugated to different residues of a variety of target proteins. In humans, approximately 600 ubiquitin E3 ligases have been identified to ensure the specificity of substrate selection. Linear ubiquitin chains exist in several formats depending on the linkage of the internal ubiquitin lysine residue (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, or Lys63). Similarly, polyubiquitin chains have different linkages that are involved in linkages between ubiquitin and ubiquitin-like proteins (UBLs) such as small ubiquitin-like modifier (SUMO) and neuronal precursor cell-expressed developmentally down-regulated protein 8 (NEDD8). Based on different types of ubiquitin and UBL modifications, the target proteins exhibit specific and diverse effects on cellular physiology. Such modifications of target protein are found in the ubiquitin proteasome system (UPS). Mostly, proteins polyubiquitylated via E3 ligases are recognized and then degraded by multimeric 26S proteasome complex; thus the ubiquitin moieties are recycled by the UPS. Currently, protein ubiquitylation process commonly exists in eukaryotic cells and is well investigated. Ubiquitylation plays a central role in regulating protein interactions, localization, and enzymatic activities and is involved in regulating DNA transcription, DNA damage and repair, cell cycle progression, endocytosis, apoptosis, and other cellular processes (Farshi et al. 2015).

Characteristics of Deubiquitylating Enzymes

Protein ubiquitylation is a reversible process conducted by DUBs. Just like phosphatases reversing protein phosphorylation, DUBs trim or remove ubiquitin chains linked to the substrate protein, act as vital regulatory components for posttranslational modifications, and add to the complexity and sensitivity of the UPS. The complex nature of UPS modulation helps the cell to accurately respond to various physiological states and stimuli through modulating the stability of key molecules in the cells.

DUBs selectively cleave ubiquitin chains to release ubiquitin. Through this critical process, DUBs modulate the degradation or activation of target proteins. Thus, diverse processing or removal of polyubiquitin moieties or chains on proteins regulated by DUBs determines the fate of targeted proteins into degradation by proteasome or another function (such as traveling to a differential subcellular localization). Importantly, DUB-mediated cleavage of polyubiquitylated chains works in a similar manner as a protein phosphatase to release phosphorylated groups in the kinase-phosphatase network. Thus, DUBs and E3 ligases determine protein fates in a similar way that kinases and phosphatases regulate protein activities. Specific cleavage along the ubiquitin chains in different locations can have differential effects on the fates of ubiquitylated proteins, which provides the cells with a complex and dynamic system to modulate protein turnover, activity, expression, and localization. In addition, DUBs regulate gene expression, apoptosis, cell cycle, DNA repair, and signaling (Harrigan et al. 2018).

DUBs in Cellular Signaling

Regulation of p53 Signaling

Deubiquitylation has emerged as a crucial process of p53 stability regulation. Several DUBs are involved in modulating the E3 ligase MDM2 (Murine double minute 2) that is a negative regulator of p53 stability. For example, OTUB1 can abrogate p53 ubiquitylation and increase p53 stability and activity via inhibiting MDM2 cognate ubiquitin-conjugating enzyme (E2) UbcH5. Through binding to E2 enzyme Ubc13, OTUB1 also modulates the K63-ubiquitylated chromatin induced by DNA damage. By cleaving ubiquitin chains on MDM2, USP7 can inhibit proteasomal degradation of MDM2 protein, leading to accumulation of MDM2 and suppression of p53 through increased ubiquitylation and degradation of p53. So, USP7 inhibition should trigger MDM2 degradation, p53 stabilization, and ultimately activation of apoptotic pathways in tumor cells. USP42 is another DUB important for p53 stability. p53 can be directly deubiquitylated by USP42 that reverses its ubiquitylation by MDM2. USP42 forms a direct complex with p53 and controls its activation in response to cellular stress; as a result it regulates p53-dependent transcription and cell cycle arrest. In addition, other DUBs such as USP2 and USP15 have been characterized to bind with p53 and deubiquitylate MDM2, thereby impeding expression of p53 (Harrigan et al. 2018).

Regulation of NF-κB Signaling

Ubiquitylation of key signaling molecules by E3 ubiquitin ligases has emerged as an important regulatory mechanism for nuclear factor-kappa B (NF-κB) signaling. DUBs counteract E3 ligases and therefore play a crucial role in the downregulation of NF-κB signaling and homeostasis. An important molecule A20, which was first identified as a zinc finger protein, can negatively regulate NF-κB signaling pathway by modifying receptor-interacting protein (RIP) with K63-ubiquitin chain and targeting RIP for degradation. Further investigating the mechanisms of NF-κB downregulation by specific DUBs such as A20 and CYLD (cylindromatosis) may provide therapeutic opportunities for the treatment of chronic inflammatory diseases and cancer (Harhaj and Dixit 2011).

Regulation of TGF-β Pathway

Transforming growth factor-β (TGF-β) pathway has a tumor suppressor role in normal and premalignant cells but promotes oncogenesis in advanced cancer cells. Several DUBs like USP4, USP11, USP15, USP18, USP9X, A20, AMSH, CYLD, and UCH37 can regulate TGF-β signaling pathway. UCH37 interacts with SMAD7 (mothers against decapentaplegic homolog 7) and deubiquitylates TβR-I (transmembrane serine/threonine kinase receptors type I) and therefore stabilizes the type I receptor and acts as the counterpart of SMAD ubiquitin regulatory factors (SMURFs) in regulating expression of TβR-I. UCH37 also increases early signaling and plays a role in TGF-β-induced migration. USP15 binds to the SMAD7-SMURF2 complex and deubiquitylates and stabilizes the type I TGF-β receptor, leading to enhanced TGF-β signaling. USP15 in glioblastoma has been identified to stabilize TβR-I and activate oncogenesis through the activation of TGF-β signaling. In addition, USP15 regulates TGF-β signaling by deubiquitylating mono-ubiquitylated receptor-regulated SMADs (R-SMADs) as well. CYLD acts as an inhibitor against JNK (c-Jun amino-terminal kinase) and NF-kB signaling and regulates TGF-β signaling in T cells. CYLD preferentially hydrolyzes or cleaves K63-linked ubiquitin chains and regulates TGF-β signaling. CYLD inhibits the TGF-β-induced development of regulatory T cells through deubiquitinating SMAD7 that inhibits the activation of transforming growth factor receptor-β-activated kinase 1 (TAK1) and p38. AMSH (associated molecule with the SH3 domain of STAM) and AMSH-LP (AMSH-like protein) bind to inhibitory SMADs (I-SMADs) and inhibit their function to enhance TGF-β and bone morphogenetic protein (BMP) signaling. Mono-ubiquitylation of SMAD4 at K519 blocks binding of SMAD4 to phospho-SMAD2; FAM/USP9x reverts this negative modification and re-empowers SMAD4 function. These findings provide a novel molecular target for the

design of inhibitors with therapeutic potential in various diseases (Iyengar 2017).

Cytokine-Inducible Deubiquitinating Enzymes

Currently, the well-characterized cytokine-inducible DUBs in murine include DUB-1, DUB-1A, DUB-2, DUB-2A, and DUB-3, which exert regulatory functions in cell proliferation and apoptosis in lymphocytes. For example, IL-3, IL-5, and GM-CSF stimulate DUB-1 expression, thereby arresting growth in the G_1 phase of the cell cycle. Essentially, the inhibitory effect of DUB-1 is specific to hematopoietic cells. Like DUB-1, DUB-2 is also stimulated by IL-2 and expressed in T lymphocytes that exhibited constitutive activation of the IL-2 signaling pathway. Interestingly, expression of DUB-2 in Ba/F3 cells can enhance the survival of these cells through strongly inhibiting apoptosis induced by cytokine withdrawal. In addition, DUB-3 induced by IL-4 and IL-6 can regulate cell growth and survival and, when constitutively expressed, results in growth suppression and apoptosis. Since cytokine-inducible DUBs play the vital role in various immune-related cells, it might be interesting to investigate cytokine-inducible human DUB homologues and define cytokine signal transduction and molecular mechanisms through the discovery of DUB inhibitors (Lim et al. 2013).

DUBs as Clinical Markers of Cancers

Several mutated DUBs serve as oncogenes (e.g., USP6 and USP28) or tumor suppressors (e.g., CYLD and A20). Moreover, activities and expression levels of DUBs frequently alter in a variety of malignant tumors. These findings exhibit the clinical relevance of DUBs in cancer development and offer valuable markers for predicting responses of cancer patients to chemotherapeutic drugs.

Somatic mutations in USP6 and USP28 have been detected in various human malignancies. For example, the overexpression of USP6 (Tre-2) is triggered by chromosomal rearrangements in the osteoblast cadherin 11 gene (CDH11) promoter region, which turns USP6 into an oncogene associated with neoplastic aneurismal bone cysts. Mutated USP28 and CYLD are widely found in cases of lobular breast cancer and familiar cylindromatosis, respectively. In several lymphoma subtypes, the chromosomal deletions and inactivating mutations of A20 have been detected as well.

A variety of DUBs are linked to cancer through altered expression patterns. For instance, the DUB dysregulation of human cancers can be screened through in situ hybridization on tissue microarrays. In non-small cell lung carcinomas, the significantly elevated expression levels of JOSD1, CSN5, UCHL1, and USP9X are observed, whereas USP10, USP11, USP22, USP48, and CSN5 are upregulated in malignant melanoma. USP10, USP11, and USP22 levels are significantly higher in metastatic melanoma compared with benign nevi and primitive tumors. Therefore, these DUBs might offer useful indicators for diagnostic/prognostic evaluation or as new therapeutic targets. Similarly, USP22 is overexpressed in several cancers including colorectal carcinomas, liver and breast cancer, as well as oral squamous carcinoma and esophageal squamous cell carcinoma; therefore this DUB serves as a new molecular marker to predict the prognosis of human metastatic cancers. Interestingly, USP2 expression is downregulated in breast carcinomas. Conversely, USP2 is overexpressed in ovarian and prostate carcinomas and is associated with lesions of poor prognosis. Moreover, overexpression of USP2 protects prostate cancer cells from apoptosis and confers them resistance to chemotherapeutic agents through decreasing p53 stability, suggesting that USP2 has dual properties (protumor or antitumor) exhibited in a tissue-specific manner. USP8 levels are upregulated in cervical squamous cell carcinoma (CSCC) tissue samples compared to healthy tissues. Importantly, elevated levels of USP8 promote cell proliferation, migration, and invasion of CSCC cell lines. Thus, high expression of USP8 correlates with tumor stage and might be used as an independent prognostic marker for CSCC. USP7 overexpression in prostate cancer is also connected with tumor aggressiveness. USP4 is overexpressed in several types of human cancer, but

downregulated in small-cell lung cancer cell lines. Moreover, elevated expression levels of USP17 have been identified in primary lung, colon, esophagus, and cervix tumor biopsies. Furthermore, USP15 is downregulated in paclitaxel-resistant ovarian cancer, and reduced expression of CYLD is found in melanoma and other malignant tumors. USP28 can stabilize the levels of Myc through an interaction with FBW7α (F-box and WD40 domain-containing protein 7α) and is highly expressed in colon and breast carcinoma. In addition, USP14 expression is high in different cancers including ovarian and colorectal cancer. The upregulated USP14 expression levels in colorectal cancer are associated with the pathologic stages as well as liver and lymph node metastases. CXCR4 (C-X-C motif chemokine receptor 4) degradation and chemotaxis are controlled due to its deubiquitylation by USP14. In gastric cancer, USP14 also serves as a novel prognostic indicator to encourage cisplatin resistance via Akt/ERK (serine-threonine kinase/extracellular signal-regulated kinase) signaling pathways (Fraile et al. 2012; Harrigan et al. 2018).

In addition, DUBs serve as the key regulatory components in various tumor metastatic events. These events in the metastatic progression include epithelial mesenchymal transition (EMT), the extracellular matrix (ECM) degradation, cell fate regulation, as well as resistance of apoptosis in the circulation. Currently, several well-characterized DUBs such as USP4, UCHL1, UCHL3, USP22, and USP47 are involved in modulating EMT process. USP6, USP22, USP2, and USP42 participate in matrix metalloproteinase (MMP) regulation as well. USP21, USP13, USP1, PSMD14, USP7, USP15, and USP21 are important regulatory DUB members found in cell fate determinants. Furthermore, DUBs like USP9X and ataxin-3 exhibit regulatory activities in cell apoptosis process (detailed review in citation (He et al. 2017)).

DUBs in Immunity and Inflammation

Mutations in specific DUBs are associated with chronic inflammation, autoimmunity, infectious disease, cancer, and neurodegeneration. Several DUBs such as A20, CYLD, and OTULIN are well characterized as vital regulators of inflammatory signaling and cell death (Harrigan et al. 2018).

A20 exhibits negative feedback of NF-κB activation in response to various proinflammatory stimuli including TNF, IL-1, LPS (lipopolysaccharide), T-cell and B-cell receptor antigens (e.g., pathogens), and nucleotide-binding oligomerization domain-containing protein 2 (NOD2) ligands. A20 specifically removes K63-linked polyubiquitylation chains from multiple NF-κB signaling factors including NF-κB essential modulator (NEMO), receptor-interacting serine/threonine protein kinase 1 (RIPK1), and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), thereby restricting the Toll-like receptor (TLR) signaling and maintaining immune homeostasis. In addition, A20 binds to polyubiquitin chains via its seventh zinc finger motif (ZnF7) and then is recruited to NEMO. This A20-NEMO interaction impairs the IκB kinase (IKK) phosphorylation by its upstream kinase TAK1, thereby reducing NF-κB activation. Furthermore, A20 exerts crucial roles in controlling inflammatory responses and might be an important determinant for multiple autoimmune diseases. In macrophages, A20 regulates IL-1β/IL-18 release by controlling NLRP3 (nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3) inflammasome activity and CXCL9/CXCL10 (C-X-C motif chemokine 9/10) production through STAT1 (signal transducer and activator of transcription 1) signaling. In essential, the autoinflammatory arthritis phenotype is restricted by these pathways. In dendritic cells, A20 regulates the expression of co-stimulatory molecules (e.g., CD80/CD60), proinflammatory cytokines (e.g., IL-6), and anti-apoptotic proteins, thereby exhibiting in vivo T-cell and B-cell homeostasis. A20 regulates necroptosis and autophagy in $CD4^+$ T cells, as well as necroptosis, IL-2, and IFNγ release in $CD8^+$ T cells. Furthermore, A20 perturbs regulatory T-cell development. A20 in B cells governs co-stimulatory molecule expression, IL-6

production, and Bcl-x survival protein expression, thereby blocking autoreactive B-cell generation leading to an autoimmune phenotype such as systemic lupus erythematosus.

CYLD is a negative regulator of RIG1 (retinoic acid-inducible gene-1-like receptor 1)-mediated antiviral response. CYLD can bind to RIG1 and thereby inhibit ubiquitylation and signaling functions of RIG1. CYLD also inhibits the ubiquitylation of TBK1 (TANK-binding kinase 1) and IKKε, which are implicated in the negative regulation of IFN responses. In dendritic cells, CYLD deficiency triggers constitutive activation of TBK1 and IKKε. Furthermore, CYLD-deficient cells and mice are more sensitive to viral infection due to downregulated interferon (IFN) receptor signaling and antiviral gene expression stimulated by IFNβ. Interestingly, CYLD-deficient mice exhibit disorder of thymocyte development and activation of T cells associated with bowel inflammation and autoimmune responses. In essential, CYLD interacted with lymphocyte-specific protein tyrosine kinase (LCK). The active LCK is recruited to its substrate, zeta-chain-associated protein kinase 70 (ZAP70), and then activates downstream T-cell receptor (TCR) signaling events. Also, CYLD exerts key regulatory effects on B-cell function. Lack of CYLD expression can induce constitutive activation of the canonical NF-kB signaling cascade in B cells. Furthermore, CYLD-deficient B cells exhibit defects in B-cell maturation and homeostasis. These defects are characterized by hyperproduction of marginal zone B cells and expansion of B cells in peripheral lymphoid organs. Similarly, abnormalities of B-cell function have been observed in $CYLD^{ex7/8}$ knock-in mice with a shorter isoform of CYLD (sCYLD).

Modification of proteins with M1-linked polyubiquitin chains exerts important roles in regulating immune and inflammatory signaling cascades. M1-linked polyubiquitin chains can form from the linear ubiquitin chain assembly complex (LUBAC). This assembly complex consists of heme-oxidized IRP2 ubiquitin ligase 1 (HOIL1, also known as RBCK1), HOIL1-interacting protein (HOIP, also known as RNF31), and SHANK-associated RH domain-interacting protein (SHARPIN). Importantly, LUBAC can be recruited to a wide range of immune receptors and ubiquitylate target proteins, especially RIPK1, RIPK2, MYD88, interleukin-1 receptor-associated kinases (IRAKs), and NEMO. Furthermore, deficiency of M1-linked ubiquitin chains perturbs immune signaling in LUBAC component-deficient mice. To date, OTULIN has been identified to specifically cleave M1 linkages. Knockdown of OTULIN results in M1-ubiquitinated protein accumulation. Conversely, overexpression of OTULIN attenuates TNF-induced NF-κB activation and proinflammatory signaling. Furthermore, phosphorylation-dependent regulation of interaction between OTULIN and HOIP governs the functions of OTULIN and offers a subtle control for M1-linked polyubiquitin signaling. The roles of A20, CYLD, and OTLIN in NF-κB signaling and cell death are so similar, but the nonredundant functions of these DUBs are determined by the cellular context. The cross talk between these DUBs needs to be further investigated.

In addition, USP4 can stabilize the nuclear receptor retinoid-related orphan receptor γt (RORγt) in TH17-activated T cells and offer a potential therapeutic target for rheumatoid arthritis. In dendritic cells, TRABID is required for TLR-mediated expression of the inflammatory cytokines IL-12 and IL-23. USP18 regulates the TAK1-TAB interaction, which is required for TH17 cell differentiation and the autoimmune response. The DUB cellular zinc finger anti-NF-κB protein (CEZANNE, also known as OTUD7B) is participant in T-cell receptor signaling. It binds to ZAP70, thereby perturbing the interaction of ZAP70 with negative regulatory phosphatases. USP10 can deubiquitylate and stabilize T-bet, leading to enhanced secretion of IFNγ. Moreover, elevated expression of USP10 is found in peripheral blood mononuclear cells from patients with asthma compared with healthy donors. Thus, the expression of DUBs in various immune cells will be a new area for further investigation.

DUBs in Infectious Diseases

DUBs can function as potential therapeutic targets for various infectious diseases including viral infections, bacterial infections, and parasitic infections (Harrigan et al. 2018). For example, both severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) are highly pathogenic human coronaviruses, which contain papain-like cysteine proteases termed SARS-CoV PLpro and MERS-CoV PLpro, respectively. These proteases have structural similarity to the USP family of DUBs based on their X-ray structures. SARS-CoV papain-like proteases (PLPs) negatively regulate antiviral defenses by disrupting the STING (stimulator of interferon genes)-mediated IFN induction. Bacterial effectors often target kinase cascades involved in inflammation or the eukaryotic Ub system to promote bacterial pathogenicity. DUBs in *Shigella* (ShiCE) and *Rickettsia* (RickCE) showed strong preference for the K63-linked ubiquitin chains. Therefore, bacterial DUBs are potential therapeutic targets. The deubiquitylating enzyme PfUCH54 identified from *Plasmodium falciparum* has deubiquitylating activity and can remove adducts of the UBL NEDD8. Similarly, PfUCHL3 identified from *Toxoplasma gondii* has the dual specificity of the enzyme that is orthologous to mammalian UCHL3 and found to be required for parasite survival. Taken together, it will be interesting to further investigate DUB inhibitors with anti-infective activities.

Drugs

The development of selective small molecule inhibitors that targeted DUBs might be encouraging for anticancer therapeutics. Bortezomib is the first 20S proteasome inhibitor (PI) approved by the FDA in 2003 for the treatment of multiple myeloma (MM) and mantle cell lymphoma. However, the extended treatments of bortezomib result in several adverse effects due to its nonselectivity. Researchers have been looking for strategies to improve PI-based therapies. A variety of DUBs such as USP4 (UNP), USP6 (Tre-2), USP8 (UBPY), USP14, USP28, and UCHL5 (UCH37) have been found to function as crucial modulators in cancer development and progression. Moreover, the crystal structures of USP and UCH subfamilies (e.g., USP2, USP7, USP8, USP14, and USP21) have been well characterized, thereby attracting molecular recognition profiling investigations of these DUBs in their active states. These types of investigations have helped the rationale to design and develop DUB inhibitors. Thus, targeting specific upstream components of the UPS such as DUBs and E3s will be able to improve the current therapeutic treatments for cancer and other diseases (Dou and Zonder 2014; Farshi et al. 2015; Fraile et al. 2012; Harrigan et al. 2018; Komander et al. 2009; Ndubaku and Tsui 2014).

A small molecule PR-619 with moderate inhibitory activity is selective for DUBs over other cysteine proteases. HBX 41,108 originally identified as a USP7 inhibitor is a nonselective DUB inhibitor. HBX 41,108 can stabilize p53 in HEK293 cells and lead to caspase-3 and poly (ADP-ribose) polymerase (PARP) cleavage in both p53þ/þ and p53/HCT-116 cells. But HBX 41,108 can also inhibit USP5, USP8, UCH-L3, and caspase-3 with a potency (70–200 nmol/L) greater than its activity against USP7 (530 nmol/L). Considering the key regulatory roles played by USP7 in the cell physiological processes, there is growing interest in investigating USP7-specific inhibitors. The novel small molecule USP7-specific inhibitor P5091 identified by Progenra can stabilize p53, can impede cancer cell proliferation, and is also an effective antitumor agent in various tumor models. For example, P5091 can trigger cell apoptosis in multiple myeloma cells resistant to conventional and bortezomib therapies. Interestingly, P5091 is well-tolerated in animals, inhibits tumor growth, prolongs survival, and enhances synergistic anti-multiple myeloma activity in combination with other chemotherapeutic agents such as lenalidomide, histone deacetylase (HDAC) inhibitor, or dexamethasone. Hit-to-lead optimization identified additional

analogs of P5091 (e.g., aqueous soluble derivative P045204) that increases the steady-state levels of p53 and its transcriptional target p21 in a time-dependent manner in HCT-116 cells.

b-AP15 (or VLX1500) is characterized as a member of a unique class of proteasome inhibitors. It has potent inhibitory effects on the 19S regulatory particle-associated UCHL5 and USP14. Importantly, b-AP15 triggers apoptosis in tumor cells regardless of their p53 or BCL2 accumulation and potently blocks tumor progression and dissemination in multiple solid tumor mouse models and an in vivo AML model.

The molecules like pimozide, GW7647, and ML323 are potent inhibitors of the USP1-UAF1 complex. Pimozide or GW7647 can non-competitively bind with the USP1-UAF1 complex and then exhibit in synergy with cisplatin to inhibit cell proliferation in cisplatin-resistant non-small cell lung cancer (NSCLC) cells. In addition, pimozide induces degradation of USP1 substrate ID1 and therefore attenuates leukemic cell growth. ML323 exerts excellent selectivity for DUBs. In non-small cell lung cancer and osteosarcomas, ML323 potentiates cisplatin-induced cytotoxicity. ML323-mediated inhibition of USP1 inhibits deubiquitylation of Fanconi anemia group D2 protein (FANCD2) and proliferating cell nuclear antigen (PCNA) and compromises translesion synthesis (TLS) and Fanconi anemia (FA) pathways. Thus, ML323 may provide a means to sensitize cancer cells to platinum-based therapies.

The small molecule inhibitor WP1130, also known as Degrasyn, is derived from a compound with JAK2 (the Janus kinase 2) kinase inhibitory activity. WP1130 rapidly induces accumulation of polyubiquitylated proteins, resulting in induction of apoptosis. WP1130 is a partially selective inhibitor that directly inhibits the deubiquitylating activity of USP9X, USP5, and USP14, all of which regulate survival protein stability and proteasome function. In particular, WP1130-mediated USP9X inhibition induces apoptosis by reducing MCL-1 levels and increasing tumor cell sensitivity to chemotherapy. Moreover, USP9X inhibition by WP1130 can inhibit the growth of ERG-positive tumors in vitro and in mouse xenograft models of prostate cancer.

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Diabetes Mellitus

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Definition

Diabetes mellitus is defined as hyperglycemia (fasting >7 mM and/or 2 h postprandial >11.1 mM) due to absolute or relative lack of insulin. The most common forms are type 1 diabetes (prevalence ~0.5%), with absolute lack of

insulin, and type 2 diabetes (prevalence ~8.5%) which is due to the combination of insulin resistance and insufficient insulin secretion. Among people diagnosed with diabetes ~5.5% have type 1 diabetes and ~ 90% type 2 diabetes.

Basic Mechanisms

Type 1 Diabetes (Previously Insulin-Dependent Diabetes Mellitus, IDDM, or Juvenile Diabetes)

Progredient destruction of insulin-secreting β cells by an autoimmune mechanism during a period of several years (Jahromi and Eisenbarth 2006). The diagnosis is confirmed by detection of antibodies against β-cell proteins (glutamic acid decarboxylase, tyrosine phosphatase IA-2, insulin). Islet cell destruction is probably mediated by activated T lymphocytes. There is a genetic susceptibility to type 1 diabetes, and several predisposing loci (e.g., the MHC locus) and alleles of genes associated with the disease risk (the insulin gene, PTPN22, CTLA-4) have been identified (Jahromi and Eisenbarth 2006). In addition, genetic and epidemiological data indicate a strong environmental factor in the pathogenesis.

Type 2 Diabetes (Previously Non-Insulin-Dependent Diabetes Mellitus, NIDDM)

Type 2 diabetes is a frequent consequence of the metabolic syndrome (obesity, insulin resistance, dyslipidemia, hypertension). Obesity causes insulin resistance which is initially compensated by hyperinsulinemia, mediated via increased insulin biosynthesis and insulin secretion and/or a β-cell proliferation. In the course of insulin resistance and under specific genetic and epigenetic predisposition, the β-cell function deteriorates and insulin secretion is reduced, leading to relative insulinopenia. In particular, the initial response (first phase insulin secretion) of islets to a glucose load is impaired during the first stages of the disease. Fasting hyperglycemia is preceded by a variable period of impaired glucose tolerance (IGT) during which secondary complications (micro- and macrovascular) start to develop. Thus, in many type 2 diabetics, secondary complication can be diagnosed at the time of the detection of hyperglycemia; these are accelerated and aggravated by other components of the metabolic syndrome (dyslipidemia, hypertension).

Obesity and insulin resistance appear to be due to a heterogeneous combination of genes involved in the control of appetite and thermogenesis. Several genes have been identified that are involved in the regulation of food intake and of body weight (e.g., leptin, the leptin receptor, melanocortin receptors; see chapter on appetite control). However, an increasing number of genes responsible for the polygenic obesity have been identified by genome wide association studies (GWAS), as well as by exome and whole genome sequencing. Currently about 300 obesity (Goodarzi 2018) and 400 diabetes genes (Mahajan et al. 2018) are known which in each case lead to the disease by a different set and combination of genes. Data obtained from rodent models indicate that islet cell failure is produced only under conditions of obesity. This interaction of two different genetic constellations (obesity and diabetes genes) explains the heterogeneity of the type 2 diabetes with regard to its association with body weight.

The molecular pathology of the β-cell destruction in the course of insulin resistance is not fully understood. It has been suggested that the constant hyperstimulation of the β cell by glucose ("glucose toxicity"), elevated fatty acids ("lipotoxicity"), or a combination of both ("glucolipotoxicity") may lead to cell damage, for example, via ER stress, reactive oxygen species, and hypoxia (Fonseca et al. 2011; Gerber and Rutter 2017).

Pharmacological Intervention

Type 1 diabetes requires a lifelong treatment with insulin. The goal of therapy is to maintain near-normoglycemia, to avoid acute complications, and to prevent long-term micro- and macrovascular complications. Insulin can be administered subcutaneously by a range of insulin types (rapid, short, intermediate, and long-acting, see Table 1) and delivery methods, for example, via multiple daily injections (MDI) and continuous

Diabetes Mellitus, Table 1 Pharmacokinetic characteristics of the most commonly used insulin preparations and analogs

	Onset of action	Maximum	Duration of action
Rapid and short-acting insulins:			
Insulin lispro	15 min	½–1 h	2–5 h
Insulin aspart	15 min	½–1 h	2–5 h
Insulin glulisine	15 min	½–1 h	2–5 h
Regular insulin	30 min	1–2 h	5–8 h
Long-acting insulins:			
NPH insulin	30–60 min	2 h	11–20 h
Insulin glargine	60 min	4 h	12–30 h
Insulin detemir	60 min	5–8 h	12–22 h

subcutaneous insulin infusion (CSII), also designated insulin pump therapy (Malik and Taplin 2014). Both therapies have to be supported by comprehensive education and depend on self-monitoring of blood glucose and/or continuous glucose monitoring (CGM) to avoid hypoglycemia and glucose variability. Recent advances in diabetes technology have resulted in the development of a commercially available, hybrid artificial pancreas (AP), also known as the closed-loop system or automated insulin delivery, which is already applied in the United States and several countries in Europe. The AP incorporates a sensor for CGM, an insulin pump to deliver insulin and an algorithm connecting the two devices, which directs the pump to deliver insulin based on the real-time glucose readings from the sensor (Ramli et al. 2019).

To achieve an optimal glycemic control, several different insulin analogs were developed which allow to simulate endogenous insulin secretion under changing circumstances (Table 1). When patients are treated by MDI, basal insulin requirements are given as an injection of long- or intermediate-acting insulin analogs (e.g., insulin glargine), while of rapid-acting insulin analogs (e.g., insulin lispro) are injected after a meal. CSII provides a 24 h preselected but adjustable basal rate of rapid-acting insulin, along with patient-activated mealtime bolus doses and thereby avoiding periodic injections.

Insulin preparations (Table 1): Recombinant human insulin is used predominantly. Retarded preparations are generated by addition of protamine (*NPH insulin*, neutral protamine Hagedorn) which may provide the "basal" supply in both MDI and CSII. Five insulin analogs with altered amino acid sequence and different pharmacokinetic characteristics are available: *insulin lispro* (swap of B28 proline and B29 lysine), *insulin aspart* (exchange of aspartate for B28 proline), and *insulin glulisine* (exchange of lysine for asparagine B3 and glutamate for lysine B29) exhibit a more rapid onset and a shorter duration of action because of a faster dissociation of the hexameric insulin–zinc–phenol complex. *Insulin glargine* is a long-acting analog which is soluble at low pH because of two additional arginines at the C terminus of the B chain. After injection, the analog precipitates from its solution and forms a long-acting depot with a steady absorption kinetic. *Insulin detemir* lacks threonine B30 and is myristoylated at lysine B29. Binding of the analog to serum albumin is responsible for its longer duration of action which is comparable to that of *insulin glargine*.

Type 2 diabetes: After clinical diagnosis, weight loss by hypocaloric diets and exercise, and to quit of smoking may normalize glucose homeostasis for a period of several years. Most successful weight reduction strategies are food substitution by formula diets for 3–5 months and sustained and intensive counseling over 6–12 months. Aerobic exercise, resistance training, and the combination of both are effective to improve glycemic control. However, because the "lifestyle changes" are difficult to maintain, and because of the progression of the islet cell failure, pharmacological intervention will usually become inevitable and has to be intensified

throughout the course of the disease. Initially, normalization of blood sugar can be accomplished with oral antidiabetics. Later on, a combination of oral antidiabetics with insulin or insulin alone (similar to treatment of type 1 diabetes) will be required.

The American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) developed guidelines for treatment of type 2 diabetes and recommend to start pharmacological treatment with metformin. A treatment with sodium-dependent glucose transporter 2 (SGLT2) inhibitors or glucagon-like peptide 1 (GLP-1) receptor agonists is recommended for patients with clinical cardiovascular disease because of a proven cardiovascular benefit. For patients with risk of chronic kidney disease (CKD) or clinical heart failure and atherosclerotic cardiovascular disease (ASCVD) an SGLT2 inhibitor is recommended. GLP-1 agonists are generally suggested as first injectable medication. If further intensification is required or one of the abovementioned drugs not tolerated, a dipeptidyl-peptidase-4 (DPP4) inhibitor is recommended followed by basal insulin, a thiazolidinedione (TZD) and a sulfonylurea (SU) (Davies et al. 2018).

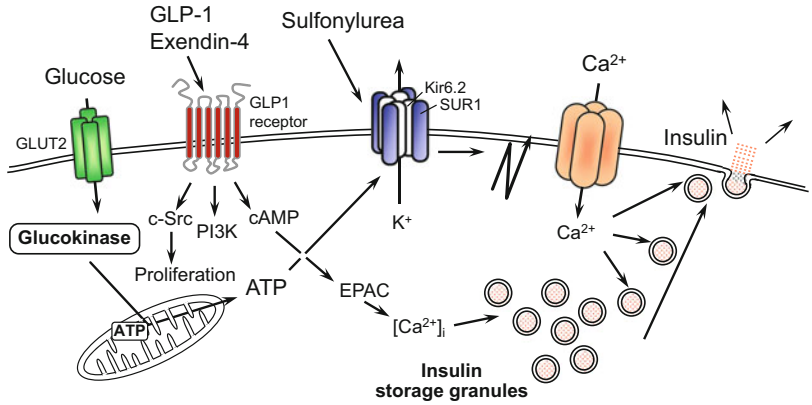
Metformin: The biguanide metformin lowers blood sugar mainly by inhibition of glucose output from the liver. It is generally accepted that this effect reflects a marked inhibition of gluconeogenesis and lipogenesis, presumably mediated by a stimulation of the AMP-dependent kinase (Zhou et al. 2001). Other effects of metformin (stimulation of glucose transport in muscle, inhibition of intestinal glucose absorption) require higher concentrations and appear to contribute little to the in vivo effect.

Under certain circumstances, and very rarely, the inhibition of gluconeogenesis by metformin may suppress lactic acid metabolism and precipitate a potentially fatal lactic acidosis. Impairment of renal function, liver disease, alcoholism, and conditions that give rise to increased lactate production (e.g., congestive heart failure, infections) are therefore contraindications for the application of metformin.

Oral formulations of metformin are rapidly and completely absorbed. The agent is poorly bound to plasma proteins; its duration of action is determined exclusively by renal elimination. Higher concentrations of metformin than in most tissues are found in the intestinal mucosa, giving rise to common side effects (irritation, diarrhea, etc.). Because of its high efficacy in lowering HbA1c, good safety profile, and low cost, metformin is the first-line medication for type 2 diabetes treatment (Davies et al. 2018).

SGLT2-inhibitors: The structure of SGLT2 inhibitors is based on phlorizin, a natural phenolic O-glucoside that competitively inhibits human SGLT2 and SGLT1 (Vallon 2015). They are oral medications that lower plasma glucose by enhancing urinary glucose secretion by about 75–120 g per day. This can be translated into a calorie loss of 308–490 kcal per day. SGLT2 inhibitors reduce HbA1c by 0.7–0.8%, lower body weight by ~5% and reduce systolic and diastolic blood pressure by an average of 4.0–4.7 mmHg and 1.9 mmHg, respectively (Seufert 2015). Furthermore, the SGLT2 inhibitors empagliflozin and canagliflozin have cardiac and renal benefits in patients diagnosed or at high risk of ASCVD and do not increase the risk for hypoglycemia. SGLT2 inhibitors are associated with a risk for mycotic genital infections and an increased risk of acute kidney injury, dehydration, and orthostatic hypotension.

GLP-1 receptor agonists: Glucagon-like peptide 1 (GLP-1) is an incretin released by enteroendocrine L-cells in response to nutrient ingestion. The activation of the GLP-1 receptor via GLP-1 receptor agonists increases glucose-dependent insulin secretion, induces β-cell proliferation and neogenesis, enhances resistance to β-cell apoptosis (Fig. 1), and reduces glucagon secretion by α cells. It also improves satiety (Baggio and Drucker, 2007). Because native GLP-1 is degraded rapidly, degradation-resistant GLP-1 receptor agonists have been developed for the treatment of type 2 diabetes. Exendin-4 is a 39 amino acid peptide originally isolated from the venom of the *Heloderma suspectum* lizard, exhibits 53% amino acid identity with native GLP-1, and is a potent agonist at the mammalian



Diabetes Mellitus, Fig. 1 Mechanism of insulin secretion and its stimulation by GLP-1 receptor agonists and β-cytotropic agents. Glucose enters the β cell via GLUT2, is phosphorylated by glucokinase, and generates ATP. Rise in the ATP-ADP ratio results in closure of ATP-dependent K^+ channels, membrane depolarization, and opening of voltage-gated Ca^{2+} channels. Ca^{2+} influx activates the exocytosis of insulin-containing secretory granules. The ATP-dependent K^+ channel consists of Kir6.2 (a K^+ inward rectifier) and SUR (sulfonylurea receptor). Sulfonylureas block the ATP-dependent K^+ channels by enhancing the effect of ATP. GLP-1 receptor-dependent intracellular signal transduction activates insulin secretion and β-cell proliferation

GLP-1 receptor. Exenatide, the synthetic peptide of exendin-4, mimics the GLP-1 effects in stimulating glucose-dependent insulin secretion from β cells and inhibiting glucagon secretion from α cells (Baggio and Drucker 2007). Exenatide is injected subcutaneously twice daily, other related peptides like liraglutide and lixisenatide are administrated once daily, and dulaglutide and semaglutide are available as a once-weekly formulation. All GLP-1 receptor agonists improve glycemic control in type 2 diabetics and cause a significant reduction of body weight. Liraglutide and Semaglutide have been shown to improve cardiovascular outcomes. The most common side effects of GLP-1 receptor agonists are nausea, vomiting, and diarrhea; however, these effects tend to diminish over the time. They also exhibit a minimal effect of hypoglycemia and may increase the hypoglycemic potential when combined with insulin or sulfonylureas (Davies et al. 2018).

DPP-4 inhibitors: Inhibition of the dipeptidyl peptidase 4 (by sitagliptin or vildagliptin) prolongs the duration of action of endogenous GLP-1, and thereby increases insulin secretion, reduces glucagon secretion, and improves glycemic control, either alone or in combination with other antidiabetic agents (Holst 2006; Henness and Keam 2006). DPP-4 inhibitors are oral medications that are well tolerated, have a neutral effect on body weight and minimal risk of hypoglycemia when applied as monotherapy. In combination with sulfonylurea DPP-4 inhibitors increase the risk for hypoglycemia by 50% in comparison to sulfonylurea alone (Davies et al. 2018).

Thiazolidinediones (PPARγ-agonists): Thiazolidinediones (TZDs; pioglitazone, rosiglitazone) are oral medications that increase insulin sensitivity and lower blood glucose levels in insulin-resistant patients. They are agonists of the peroxisome proliferator-activated receptor γ (PPARγ). Because they enhance the effect of insulin and reduce serum insulin levels in insulin-resistant patients, TZDs are usually referred to as "insulin sensitizers." PPARγ is a transcription factor which controls the expression of enzymes and proteins involved in fat and glucose metabolism, and thereby facilitates differentiation of mesenchymal stem cells into adipocytes, promotes lipogenesis in peripheral adipocytes, decreases hepatic and peripheral triglycerides, decreases activity of visceral adipocytes, and increases adiponectin. It is believed that the formation of additional, small fat cells lowers ectopic fat

storage in the liver, skeletal muscle, myocardial muscle, and endothelial cells, thereby correcting insulin resistance (Lebovitz 2019). TZDs increase HDL-cholesterol and pioglitazone was shown to reduce cardiovascular end points and hepatic steatohepatitis. The use of TZDs has been limited because some studies indicated safety concerns in respect to fluid retention, congestive heart failure, weight gain, and bladder cancer. However, recent studies indicated that cardiovascular toxicity with rosiglitazone and increase in bladder cancer with pioglitazone are no longer significant issues (Lebovitz 2019).

Sulfonylureas: Sulfonylureas are oral inexpensive medications (Aguilar-Bryan and Bryan 1999) that bind to the sulfonylurea receptor 1 (SUR1) which regulates the activity of the ATP-dependent potassium channel of the pancreatic β-cell (Kir 6.2). Binding of ATP, or of a sulfonylurea, to SUR reduces the potassium current and causes membrane depolarization, subsequently opening of voltage-gated calcium channels, and calcium-stimulated exocytosis of insulin-containing granules. Thereby, sulfonylureas stimulate insulin secretion and lower blood glucose (Fig. 1).

The commonly used sulfonylureas are the long-acting derivatives glibenclamide (US synonym: glyburide), glipidzide, gliclazide, and glimepiride. They are associated with weight gain and a risk for hypoglycemia, which is however lower than with insulin. Sulfonylureas remain a reasonable choice among glucose-lowering medications, particularly, when costs play an important role (Davies et al. 2018).

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Diabetes-Associated Peptide

▶ Amylin

Diagnostics

▶ Ca^{2+}-Binding Proteins
▶ S100 Proteins

Differentiation Factors

► Growth Factors

Digitalis

► Cardiac Glycosides

Disease-Modifying Anti-rheumatic Drugs

► Rheumatoid Arthritis

DNA Damage Response

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Synonyms

Base-excision repair; Cell cycle checkpoints; Homologous recombination deficiency; Non-homologous end joining; Synthetic lethality

Definition

The DNA damage response (DDR) involves a complex network of genes responsible for sensing and responding to specific types of DNA damage, comprising specific machineries mediating DNA repair, cell cycle regulation, replication stress responses, and apoptosis. Defects in the DDR give rise to genomic instability in cells which leads to cancer initiation and progression via mutation accumulation. This also provides targetable vulnerabilities relatively specific to cancer cells which can then be utilized for clinical benefit with the use of DDR inhibitors.

Basic Mechanisms

DNA damage occurs constantly in cells due to a number of exogenous and endogenous stressors. Consequently, cells have evolved a complex and coordinated DNA damage response (DDR) that involves several interdependent signaling pathways and machineries. The importance of the DDR in preserving cell viability and preventing neoplasia is emphasized by the vital additional roles these pathways play in regulating the cell cycle, chromatin remodeling, metabolism, immunogenicity, and apoptosis (Roos et al. 2016). For instance, the detection of DNA damage results in the activation of checkpoints that induce cell cycle arrest to provide the time necessary for DNA repair before cell division. DDR pathways are also closely linked with the apoptotic pathway in order to enable the elimination of cells with unrepaired DNA damage. The DDR pathways, therefore, facilitate cell survival in the face of genomic instability and replicative stress or direct irreparably damaged cells to undergo senescence or programmed death (Pilié et al. 2019).

Genomic instability is a key hallmark of cancer that arises owing to defects in the DDR and/or increased replication stress. These alterations promote the clonal evolution of cancer cells via the accumulation of driver aberrations (including gene copy-number changes, rearrangements, and mutations), generation of tumor heterogeneity, and evasion of apoptosis (Tubbs and Nussenzweig 2017). DNA damage most commonly manifests as single-stranded breaks (SSBs), although double-strand breaks (DSB) also occur and are more lethal to cells, requiring

prompt countermeasures in order to ensure cell survival (Table 1). Consequently, modern DDR-directed therapies target the signaling and repair mechanisms associated with DSBs, increase replication stress and thereby the frequency of DSBs, or inhibit cell cycle checkpoints that facilitate DSB repair (Fig. 1).

Defects in certain DDR mechanisms, including DSB repair processes involving homologous recombination (HR), increase genomic instability and lead to a greater reliance on compensatory, and often error-prone, DDR and survival pathways (Brown et al. 2017). These same defects, however, also create vulnerabilities that are relatively specific to cancer cells, which theoretically can then be exploited, to improve the efficacy of anti-cancer treatments and thus improve patient outcomes.

Historically, these vulnerabilities have been exploited in anticancer therapies through the use of DNA-damaging chemotherapy and radiation. More recent strategies include the rapid development of potent and selective molecularly targeted agents against key components of different DDR pathways (herein termed DDR inhibitors). Here, we focus on novel therapeutic strategies targeting promising DDR targets and discuss clinical efforts to develop combinatorial strategies to optimize therapeutic targeting of the DDR.

Pharmacological Intervention

Targeting the DNA Damage Response

Various inhibitors of DDR components are in preclinical and clinical development. In order to fully exploit the potential of DDR inhibitors, and to ensure their long-term clinical success, a thorough understanding of DDR pathway complexities must be combined with lessons learned from the successful registration of PARP inhibitors to date. Beyond PARP inhibitors, there is now a sizeable array of potent and relatively selective inhibitors in clinical trial testing against key targets involved in the DDR, including *ATR*, *ATM*, *CHK1/2*, *WEE1*, and *DNA-PK*. These agents are being developed for patients with molecularly selected tumors and in rational combinations with other molecularly targeted agents and immune checkpoint inhibitors.

Single-Agent Strategies Targeting DNA Damage Sensor Proteins

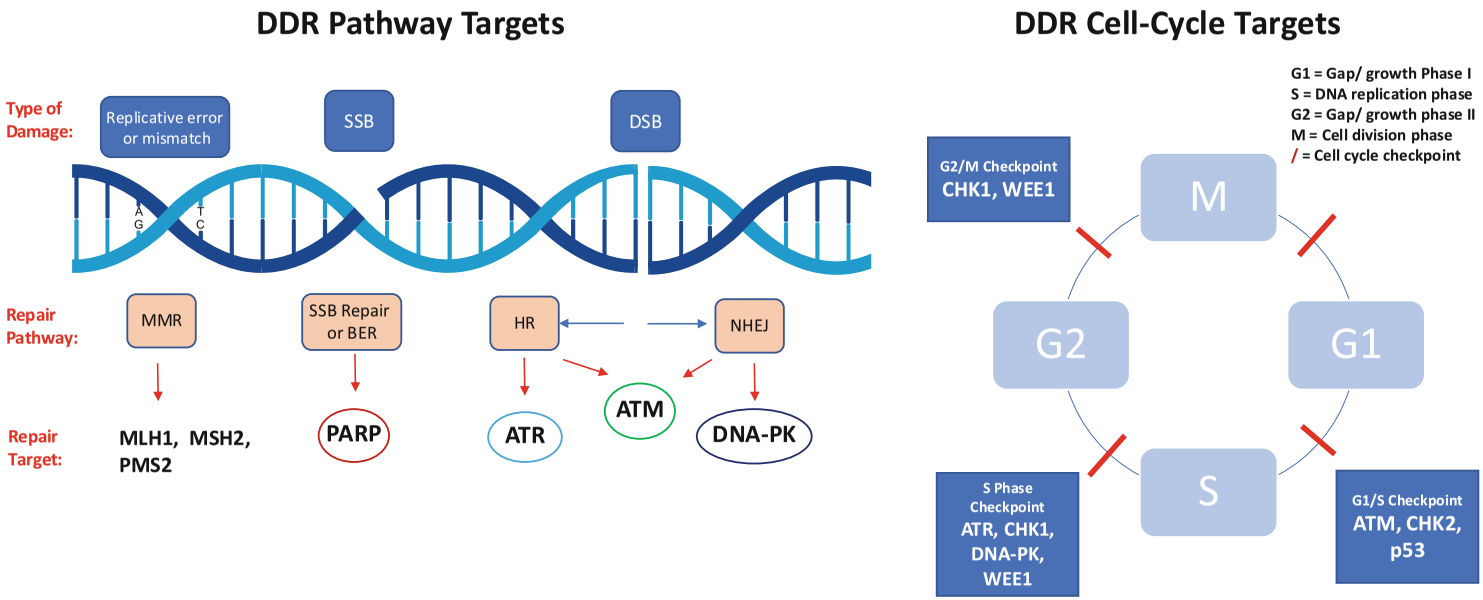
PARP Inhibitors

A well-recognized sensor of DNA damage is the protein poly(ADP-ribose) polymerase (PARP). The PARP family comprises a group of nuclear proteins that are activated upon binding to damaged DNA and have crucial roles in various aspects of the DDR (Fig. 1). Their main function is to detect DNA single-stranded breaks (SSBs) and double-stranded breaks (DSBs), recruit the DNA repair machinery, and stabilize replication forks (Helleday 2011) (Fig. 1). There are 17 PARP family members, and PARP1 has the predominant role in DNA repair. PARP2, and to a lesser extent PARP3, function in fewer, overlapping, DNA repair processes (De Vos et al. 2012).

PARP catalytic function is activated on binding to single-stranded DNA breaks, DNA nicks, or DSBs, to generate extensive poly(ADP-ribose) chains (PAR chains) on itself and proteins in the vicinity of DNA damage. These PAR chains and PARP itself then promote the recruitment of critical SSB repair proteins, such as XRCC1, to SSBs and modify chromatin structure to facilitate DNA repair. PARP auto-PARylation is also required for the dissociation of PARP from DNA damage sites (Helleday 2011). Enzymatic inhibition by PARP inhibitors therefore results in both the suppression of SSB repair and base-excision repair (BER), which molecularly converges with SSB repair in its downstream stages. Inhibition results in the trapping of PARP to SSBs, causing the stalling and subsequent collapse of DNA replication forks, resulting in replication-dependent DNA DSBs (Helleday 2011). These DSBs would typically be repaired by homologous recombination (HR); however, in HR-deficient cells, such as *BRCA1/2*-mutated tumors, less effective methods of repair are used; this leads to unsustainable levels of damage, chromosomal fusions/translocations, and ultimately cell death (Ceccaldi et al. 2016).

DNA Damage Response, Table 1 Predominant DNA repair pathways. (Adapted from Brown et al. 2017)

Double-stranded break repair pathways	
Classic (c)-NHEJ	Predominant DNA DSB repair pathway in human cells, functioning throughout the cell cycle Involves the relatively rapid ligation of broken DNA ends, mediated by the core NHEJ complex, including DNA-PK, XRCC4, LIG4, XLF, and PAXX, among others DNA end processing and DNA polymerase action may be required before ligation can occur, making NHEJ inherently error-prone NHEJ maintains genome stability, however, by rapidly repairing DSBs in circumstances where recombinogenic events would likely result in gross chromosomal rearrangements; in noncycling or G1 cells, for example
Homology-directed repair	
Homologous recombination (HR)	Relatively slow and restricted to late-S phase/G 2, as it generally relies on a homologous sister chromatid DNA strand for repair Extensive DNA end resection by helicases and exonucleases, such as DNA2, BLM, WRN, and EXO1, results in a 3′–ssDNA overhang, committing the break to repair by HR Replication protein A (RPA) coats and stabilizes the ssDNA, leading to ATR activation and subsequent signaling events BRCA2, with the help of BRCA1 and PALB2, loads RAD51 onto the RPA-coated ssDNA, leading to strand invasion, with a number of factors negatively regulating this process to prevent hyperrecombination, such as POLQ, PARI, RECQL5, FANCJ, and BLM
Alternative (alt)-NHEJ or MMEJ	Ligation pathway for DSBs when c-NHEJ is genetically compromised Occurs following limited DNA end resection Contributes to the excessive genomic deletions and chromosomal translocations seen in tumors and may also provide a backup repair pathway in HR-deficient cells
Single-stranded annealing (SSA)	Mutagenic, RAD51-independent repair pathway, involving annealing of short or longer complimentary DNA sequences on resected DNA with subsequent deletion of the intervening DNA sequence. The detailed mechanism has yet to be defined in mammalian cells
Other repair pathways	
SSB repair	SSBs usually arise following the removal of a damaged nucleotide PARP1 is the DNA damage sensor protein for DNA strand breaks. PARP1 localizes to sites of DNA damage, generating extensive PAR (poly ADP-ribose) chains Ribosylated PARP1 promotes recruitment of SSB-repair proteins to DNA damage sites
BER	DNA glycosylases recognize and remove damaged bases leading to basic sites that are processed by APE1 Results in SSB generation, repaired using SSB repair pathways
Mismatch repair (MMR)	MSH2, MSH3, and MSH6 recognize base-base mismatches and insertion/deletion loops, where they recruit MLH1 and PMS2 to damaged sites. The concerted actions of the MMR proteins engage EXO1 to remove the mismatch and then POLD and LIG1 to fill the gap and seal the nick, respectively
Interstrand cross-link (ICL) repair	ICLs cause DNA replication fork stalling and collapse, resulting in DNA DSBs ICLs are recognized by the FANCONI core complex, which engages HR, TLS, and NER pathways to repair the DNA lesion
Translesion synthesis (TLS)	DNA damage tolerance pathway that helps prevent replication fork stalling Engages low-fidelity DNA Y-family polymerases (e.g., REV1, POLH, POLI, and POLK) that accommodate the damaged lesion, replicating past it, at the expense of increased mutagenesis
Nucleotide excision repair (NER)	Removes helix-distorting lesions from DNA, in particular the UV-induced photo lesions Involves removal of a short oligonucleotide, including the damaged lesion using structure-specific endonucleases and subsequent restoration of the DNA sequence by DNA polymerases



DNA Damage Response, Fig. 1 DNA damage response pathways being targeted in the clinic. Specific types of DNA damage – mismatches due to replication, single-stranded DNA breaks (SSBs), or double-stranded DNA breaks (DSBs) – result in the activation of specific signaling and repair cascades. DNA damage response (DDR) pathways mitigate replication stress and repair DNA; thus, deficiencies in these pathways result in the accumulation of SSBs and DSBs and increased immunogenicity owing to the generation of neoantigens from mutant proteins. Poly(ADP-ribose) polymerase (PARP) enzymes are key to activating a host of downstream repair mechanisms and are primary proteins involved in SSB repair or base-excision repair (BER). The repair of DSBs occurs predominately through the rapid, error-prone nonhomologous end joining (NHEJ) repair pathway in conjunction with the much slower higher-fidelity, error-free homologous recombination (HR) repair pathway. DNA replication is a necessary component of DNA repair, and thus cell cycle regulation and replication stress responses are intertwined with DDR pathways. The kinases ATR and ATM have crucial roles in DDR signaling and in maintaining replication fork stability while also working together via their downstream targets, CHK1 and CHK2, respectively, to regulate cell cycle control checkpoints. The kinase activity of DNA-PK is essential for NHEJ and V(D)J recombination. WEE1 is a distinct nuclear kinase that regulates mitotic entry and nucleotide pools in coordination with DDR

The discovery that selected HR-deficient (HRD) tumors, initially those with germline *BRCA1* or *BRCA2* mutations, are exquisitely sensitive to PARP inhibition has pioneered a new era of research on biomarker-driven synthetic lethal treatment strategies for different cancer subtypes. This concept of "synthetic lethality" between *BRCA1/2* genetic defects and pharmacologic PARP inhibition was first presented in 2005, when the first preclinical data suggested that there may be single-agent activity with this class of agents. In "synthetic lethality," the combination of a functional genetic defect in an HR-related gene and pharmacological inhibition of a compensatory DDR pathway component, such as PARP, causes insurmountable genomic instability, mitotic catastrophe, and cell death (Lord and Ashworth 2017).

PARP inhibitors are the best-studied class of DDR inhibitors, with robust preclinical and clinical data. Indeed, several PARP inhibitors have now been FDA-approved or are undergoing testing in late phase clinical trials as single agents in various disease and treatment settings.

Current Licensed PARP Inhibitor Indications

All PARP inhibitors currently used in the clinic have a similar capacity to inhibit the catalytic activity of PARP because they share a nicotinamide moiety that competes with NAD+ for binding to this enzyme. There are differences, however, with regard to (1) the dose required to inhibit PARP activity and (2) the relative selectivity for different PARP family members. The PARP-trapping abilities of the five most-studied PARP inhibitors – niraparib, rucaparib, talazoparib, olaparib, and veliparib – also vary greatly, in contrast with their ability to inhibit protein poly ADP-ribosylation (PARylation). Talazoparib, for instance, has the greatest PARP-trapping ability observed preclinically and has cytotoxic potency in the nanomolar range. In contrast, veliparib results in less PARP trapping and is inactive at 100 mM. Furthermore, the maximum tolerated dose (MTD) of these PARP inhibitors mirrors their respective PARP-trapping ability, rather than their capacity to inhibit PARP catalytic activity (Pilié et al. 2019).

Aside from veliparib, the other PARP inhibitors – olaparib, niraparib, talazoparib, and rucaparib – all have comparable single-agent activity against the advanced-stage cancers evaluated in clinical trials to date (Gelmon et al. 2011; Kaye et al. 2012; Sandhu et al. 2013; Litton et al. 2018). However, it is important to note that there have been no head-to-head comparison studies and that the heterogeneous clinical characteristics of the patient populations in these separate trials make cross-study comparisons challenging (Fong et al. 2009; Gelmon et al. 2011; Kaye et al. 2012; Sandhu et al. 2013; Coleman et al. 2017; Litton et al. 2018).

In general, PARP inhibitors have shown a favorable side-effect profile as single agents, with a shared toxicity profile including fatigue, myelosuppression, and gastrointestinal symptoms (Fong et al. 2009; Gelmon et al. 2011; Kaye et al. 2012; Sandhu et al. 2013; Coleman et al. 2017; Litton et al. 2018). Patients treated with PARP inhibitors do have a theoretical risk of secondary malignancies due to DNA damage and genomic instability generating further mutational events, consistent with patients with DDR-deficient cancers treated using DNA-damaging chemotherapies. In practice, however, the risk of secondary malignancies, such as acute myeloid leukemia, in patients treated with PARP inhibitors has generally been low (reported as $<1\%$). Accordingly, regular complete blood counts should be undertaken during therapy, with timely referral of patients with persistent cytopenias to hematology specialists.

Ovarian Cancer

The development of PARP inhibitors for the treatment of ovarian cancer has provided clear clinical proof of concept and paved the way for the development of selective DDR inhibitors in cancer care. There are now three FDA-approved agents in different therapeutic settings – olaparib, rucaparib, and niraparib.

Maintenance Setting

Olaparib was first approved in 2014, for maintenance treatment of patients with platinum-sensitive, relapsed, germline, or somatic *BRCA1/2*-mutant ovarian cancers in complete or partial remission after platinum-based chemotherapy, based on data from the randomized phase II study 19 trial (NCT00753545), which demonstrated improved progression-free survival (PFS) in olaparib versus placebo (Ledermann et al. 2012). This finding was confirmed in this population in the follow-on, placebo-controlled, phase III SOLO2/ENGOT-Ov21 study, in which the median PFS was 19.1 months in the treatment group compared with 5.5 months in the placebo group (hazard ratio [HR], 0.3), with a tolerable adverse effect profile and no detrimental effects on quality of life. In 2017, the FDA approved olaparib for the maintenance treatment of ovarian cancer, irrespective of *BRCA*1/2-mutation status, on the basis of data from these trials.

In the ARIEL3 and NOVA trials, rucaparib and niraparib, respectively, have been explored in the maintenance setting for relapsed ovarian cancer, in patients with germline or somatic *BRCA* mutations and those with wild-type *BRCA*, with patient stratification by *BRCA* mutations (Mirza et al. 2016; Coleman et al. 2017), in an effort to define the wider homologous recombination deficiency (HRD) group of these cancers. ARIEL3 prospectively used the Foundation Medicine loss of heterozygosity test established in the ARIEL2 study to stratify patients with wild-type *BRCA1/2* into HRD-positive and HRD-negative groups (Coleman et al. 2017), while the NOVA study prospectively stratified patients using the myChoice HRD test (Myriad Genetics, Salt Lake City, UT), which was initially established in triple-negative breast cancer.

ARIEL3 demonstrated a PFS advantage in all patient groups over placebo, with the greatest benefit observed in the *BRCA*-mutant group (16.6 vs. 5.4 months; HR 0.23). A benefit was also observed in the HRD (13.6 vs. 5.4 months; HR 0.32) and intention-to-treat (10.8 vs. 5.4 months; HR 0.36) populations (Coleman et al. 2017). In the NOVA study, PFS was significantly increased in all patient groups; the median PFS was 21.1 versus 5.5 months (HR 0.27) in the germline *BRCA* cohort, 12.9 versus 3.8 months (HR 0.45) in the HRD group, and 9.3 versus 3.9 months (HR 0.45) in the overall non-germline *BRCA* group. There are subtle differences between the patient groups in the studies – the NOVA trial deployed a somewhat stricter entry criteria and required patients to have excellent disease response and no measurable disease greater than 2 cm following chemotherapy. Regardless, these data are clear: maintenance treatment with a PARP inhibitor significantly prolonged PFS in all patient groups over placebo, although overall survival (OS) data are pending. Based on these data, olaparib, rucaparib, and niraparib are now approved by the FDA in this setting in the United States. Talazoparib and veliparib are currently in late phase trials in patients with newly diagnosed ovarian cancer (NCT02470585) and in other advanced-stage cancers, mainly in combination with chemotherapies for the latter agent.

Upfront Setting

In 2018, the phase III SOLO1 trial set a new standard of care with maintenance olaparib substantially extending PFS in newly diagnosed *BRCA1/2*-mutated advanced-stage ovarian cancer (Moore et al. 2018). In this study, 391 patients with advanced, high-grade, *BRCA*-associated serous or endometrioid ovarian cancer who had a complete or partial response to frontline, platinum-based chemotherapy were randomly assigned to maintenance with olaparib or placebo (Moore et al. 2018). At a median follow-up of 41 months, olaparib resulted in a lower 3-year rate of disease progression or death compared with placebo (60 vs. 27%; HR for disease progression or death 0.30, 95% CI 0.23–0.41). Recently presented data have shown that, after 5 years of follow-up, 48.3% of patients treated with olaparib have not experienced disease progression and are still living with stable disease vs. 20.5% of those taking placebo (Banerjee et al. 2020); median PFS was 56 vs. 14 months in the olaparib group, and among patients in complete response (CR) at baseline, risk of disease

recurrence or death was reduced by 63%. These data have confirmed that as a first-line therapy, olaparib can have substantial benefit for patients earlier in the cancer pathway.

The PRIMA trial established niraparib as maintenance therapy in the first-line setting, irrespective of tumor *BRCA* status (González-Martín et al. 2019). In the PRIMA trial, patients with advanced ovarian cancer who had responded to first-line, platinum-based chemotherapy were randomly assigned to niraparib or placebo for 36 months. For the subgroup with *BRCA*-associated cancers, niraparib prolonged PFS compared with placebo (22.1 vs. 10.9 months; HR 0.40, 95% CI 0.27–0.62).

Recurrent Setting

Studies have also shown that patients with advanced ovarian cancer and a germline *BRCA* mutation may respond to olaparib if they have experienced progression on multiple previous lines of chemotherapy, in platinum-sensitive or platinum-resistant disease (Matulonis et al. 2016; Kaufman et al. 2015), which has led to FDA approval in this setting. In a meta-analysis of phase I and II trials including 273 patients with advanced, *BRCA*-associated ovarian cancer naïve to PARP inhibition, in the cohort of 205 patients who had received $\geq$3 lines of chemotherapy,[32] the tumor response rate was 31% (95% CI 25–38), and median duration of response was 7.8 months (95% CI 5.6–9.5).

Rucaparib has also been FDA-approved for use in advanced ovarian cancer patients who have received two or more chemotherapies and who have deleterious germline or somatic *BRCA* mutations, in either platinum-resistant or platinum-sensitive disease. The data to support this approval come from a pooled analysis of two phase II studies in which 106 patients with *BRCA*-mutated, high-grade ovarian cancer who had received at least two chemotherapy regimens and no prior PARP inhibition were treated with rucaparib (Oza et al. 2017). In this population, the objective response rate (ORR) was 54%, and the duration of response was 9.2 months; the ORR was 25% for those with platinum-resistant disease and 66% for platinum-sensitive disease.

Beyond Ovarian Cancer

BRCA1 and *BRCA2* are the two most studied genes in the HR repair pathway. Germline variants of these genes were initially discovered in patients with hereditary breast or ovarian cancer; however, somatic and germline *BRCA1/2* mutations, as well as aberrations affecting other HR genes including *ATM*, *ATR*, *BARD1*, *BRIP1*, *CHK1*, *CHK2*, *PALB2*, *RAD51*, and *FANC*, are increasingly being detected in patients with other tumor types through NGS of paired tumor and nonmalignant DNA samples (Pritchard et al. 2016).

Breast Cancer

The first studies exploring the possible effects of PARP inhibition on patients with germline BRCA cancer included patients with both breast and ovarian cancer, and responses were also observed in breast cancer patients. Rationally, it was initially proposed that triple-negative breast cancer, being a "BRCA-like" cancer with sensitivity to platinum-based chemotherapy, may also be sensitive to PARP inhibition, though early studies did not bear this out, and clinical trials with single-agent PARP inhibitors have focused on germline *BRCA1/2* patients.

The OlympiAD, a randomized phase III study of olaparib versus placebo for patients with metastatic breast cancer with germline BRCA mutation, confirmed activity PARP inhibitors in these patients, as a doubling of the ORR, a significant PFS benefit, and a more favorable safety profile for olaparib versus single-agent chemotherapy (not including platinum-based agents) were seen in patients with germline *BRCA1/2*-mutant, HER2-negative, metastatic breast cancer (Table 1). Olaparib is now FDA approved as a treatment following chemotherapy and appropriate hormonal therapy.

The ABRAZO phase II study with talazoparib also demonstrated promising activity for this patient group, which was confirmed in the phase III EMBRACA study, in which the primary analysis showed a PFS advantage for talazoparib over placebo (8.6 vs. 5.6 months; HR 0.54,

$P < 0.0001$) in all subgroups, including those with central nervous system disease (Litton et al. 2018). Importantly, the talazoparib-treated patients in this study also had improvements in quality of life and delayed clinically meaningful deterioration compared to patients treated with standard chemotherapy, which led to the FDA approval of talazoparib as a treatment option for patients with germline *BRCA1/2*-mutated advanced HER2-negative breast cancer. Updated data from the EMBRACA trial have shown that talazoparib did not produce a statistically significant OS benefit for these patients; however, OS results may have been confounded by subsequent treatments, as most patients included in the study went on to receive subsequent systemic therapies. Nevertheless, previous data showing talazoparib improved patient-reported quality-of-life measures over available chemotherapies with an acceptable safety profile were confirmed, and thus, talazoparib remains an option for patients with advanced *BRCA1/2*mutant, HER2negative breast cancer. A randomized trial of investigator's choice chemotherapy versus niraparib (NCT01905592) is ongoing.

Substantial efforts are also being applied to early-stage clinical testing of PARP inhibitors in the neoadjuvant setting. In contrast to other agents, encouraging data have recently been reported utilizing talazoparib in the neoadjuvant setting in patients with *BRCA1/2*-mutant, HER2-negative breast cancer, with 10 of 19 patients (53%) achieving a pathologic CR (pCR) (Litton et al. 2020). Perhaps more potent PARP inhibition of *BRCA1/2*-mutant tumors in an earlier setting, when driver mutations are perhaps more limited, might prove to be particularly effective.

Emerging Monotherapy Indications

It is clear that, for patients with a deleterious germline *BRCA*1/2 mutation, there is activity across a range of cancer types beyond breast and ovarian cancers, including prostate, pancreatic, and biliary cancers. In the TOPARP-A phase II study in metastatic CRPC, all 49 patients provided a fresh tumor sample before treatment for genomic analysis. The ORR to olaparib in this patient group was 33%, and 14 of 16 patients with disease response were in the biomarker-positive group with identified deleterious deletions or mutations in DDR genes (*BRCA1*, *BRCA2*, *ATM*, *BRIP1*, *BARD1*, *CDK12*, *CHEK1*, *CHEK2*, *FANCL*, *PALB2*, *PPP2R2A*, *RAD51B*, *RAD51C*, *RAD51D*, and *RAD54L*) (Mateo et al. 2015). These data resulted in olaparib receiving breakthrough therapy designation in prostate cancer from the FDA. The TOPARP-B study confirmed the antitumor activity of olaparib in metastatic prostate cancer with defective DNA repair secondary to either germline or somatic gene inactivation, as confirmed composite response was achieved in 25 (54.3%; 95% CI 39.0–69.1) of 46 evaluable patients in the 400 mg cohort and 18 (39.1%; 25.1–54.6) of 46 evaluable patients in the 300 mg cohort. The PROfound trial led to the recent FDA approval of olaparib for patients with mCRPC after treatment with enzalutamide and/or abiraterone. This prospective, randomized phase III trial examined the efficacy of olaparib in men with mCRPC and DDR mutations in 15 genes associated with DDR; PFS was significantly longer in patient receiving olaparib than control patients (de Bono et al. 2020). Other phase III clinical trials are evaluating niraparib and talazoparib, alone or in combination with AR signaling inhibitors.

Rucaparib was granted breakthrough therapy designation by the FDA based on initial efficacy and safety results from the TRITON2 study. Preliminary published data from this study shows promising results, with 43.9% of *BRCA* patients achieving a confirmed radiographic response, with the majority of responses lasting longer than 24 weeks. Interestingly, although a high response rate was observed for patients with *BRCA1/2*-mutant CRPC, responses were not reported in those harboring tumors with *ATM* and *CDK12* mutations. Similarly, in the PROfound trial, a hazard ratio of 0.74 for radiographic PFS with wide confidence limits was noted for patients with CDK12 alterations treated with olaparib (de Bono et al. 2020; Mateo et al. 2020). Studies of other PARP inhibitor treatments

in patients with *CDK12* mutations are similarly disappointing, and alternative agents are needed in this subset of patients. These findings do suggest that patients with *ATM*-mutant or *CDK12*-mutant metastatic CRPC may require alternative therapies, such as chemotherapy, or ATR inhibitors given as monotherapy, or in combination regimens.

Pancreatic ductal adenocarcinoma is also an emerging area of potential use for PARP inhibition. The POLO (Pancreas Cancer Olaparib Ongoing) phase III trial evaluated the efficacy of olaparib as maintenance therapy in patients who had a germline *BRCA1/2* mutation metastatic pancreatic cancer and disease that had not progressed during first-line platinum-based chemotherapy and demonstrated PFS was significantly longer in the olaparib group than in the placebo group (7.4 months vs. 3.8 months; HR 0.53; 95% CI 0.35 to 0.82; $P = 0.004$). In addition, after 2 years, 22.1% of patients had no disease progression versus 9.6% of those who received placebo, and the median duration of response was 24.9 months in the olaparib arm versus 3.7 months with placebo. This has led to FDA approval of olaparib for metastatic pancreatic adenocarcinoma with germline *BRCA1* or *BRCA2* mutation – the only approved targeted medicine in biomarker-selected patients with advanced pancreatic cancer.

Beyond Monotherapy Approaches

The heterogeneous responses to PARP inhibitors, even in *BRCA1/2*-mutant tumors, indicate the existence of complex inherent and acquired resistance mechanisms. Several novel mechanisms have been suggested, such as secondary mutations in both *BRCA1 and BRCA2* that restore, at least partially, HR capabilities and allow tumors to escape inhibition by PARP inhibitors, mitigation of replication stress, promoter demethylation of *BRCA1/2* genes, point mutations in PARP1, and drug-efflux pumps affecting drug pharmacokinetics (Pilié et al. 2019). Thus, strategies to overcome such resistance mechanisms have included the development of multiple drug combinations, which can also synergize antitumor efficacy.

Combination Strategies

Combination strategies using PARP inhibition have included multiple chemotherapeutic agents, molecularly targeted agents, and immunotherapeutics. Mechanisms resulting in synergy or additive activity of PARP inhibitor and chemotherapy combinations are multifactorial and depend on the specific combination and tumor in question. For example, chemotherapy can increase DNA damage, while PARP inhibitors can diminish the ability of PARP enzymes to repair DNA damage. Early promising efficacy has been observed with these combinations; however, tolerability of such regimens has been mixed. For example, although the concurrent addition of olaparib to carboplatin and paclitaxel for patients with advanced ovarian cancers showed an improved PFS in the combination group (12.2 months vs. 9.6 months), grade 3 neutropenia was reported for 43% of patients in the combination arm versus 35% in the chemotherapy-only arm, and a substantial proportion of patients required dose reductions or treatment delays as a result of myelosuppression (Pilié et al. 2019).

Combinations with Other DDR Pathway Inhibitors

The pairing of PARP inhibitors with other DDR pathway inhibitors, such as ATR and WEE1 inhibitors, is a rational approach currently under investigation. Preclinical studies have shown that olaparib-resistant cancer models may be resensitized to olaparib when combined with a WEE1 inhibitor or an ATR inhibitor (Pilié et al. 2019). There are also other potent inhibitors that target different key nodes along the DDR cascade in clinical development, including *CHK1/2*, *DNA-PK*, *ATM*, and *POLθ,* which may represent other potential combination partners for PARP inhibitors (Pilié et al. 2019). In view of the likely overlapping mechanism-based bone marrow toxicity with such agents, careful thought must be given to the concurrent development of such combinations, especially with regard to the doses, schedules, and sequences.

Combinations with Immune Checkpoint Blockade

There is now convincing preclinical evidence supporting the combination of DDR pathway inhibitors and immune checkpoint inhibitors as a rational antitumor strategy. PARP inhibition has been shown in preclinical models to inactivate GSK3β and upregulate PD-L1, suppressing T-cell activation and increasing cancer cell killing (Jiao et al. 2017). In addition to this, PARP inhibition generates cytosolic double-stranded DNA, which activate cGAS-STING signaling and its associated transcription programs, and induces IFN-mediated antitumor immune responses. These critical changes amplify STING signaling and promote tumor infiltrating lymphocytes and antitumor immunity, which can then be further enhanced through immune checkpoint blockade (ICB) (Shen et al. 2019). PARP inhibitor treatment has also been shown to enhance tumor susceptibility to ICB, regardless of the *BRCA1/2* status, in vitro and in vivo (Shen et al. 2019).

In the recently reported MEDIOLA phase II basket trial investigating olaparib and the PD-L1-inhibitor durvalumab in advanced solid tumors, in patients with advanced germline *BRCA1/2* mutation breast cancer, 24 out of 30 patients had disease control at 12 weeks, and 50% had disease control at 28 weeks. In the platinum-sensitive *BRCA1/2* mutation relapsed ovarian cancer cohort of the MEDIOLA study, ORR was 63%, while disease control rate (DCR) at 12 weeks was 81%. The combination of olaparib and durvalumab was safe and well-tolerated, with a low incidence of grade 3 and all-grade immune-related toxicities.

These data are in keeping with the TOPACIO study, the first study to report the safety and efficacy of combining PARP inhibitors and immuno-oncology checkpoint therapy in patients with metastatic TNBC, with or without *BRCA1/2* mutations. Here, Vinayak et al. investigated niraparib in combination with pembrolizumab in patients with advanced or metastatic TNBC and demonstrated promising antitumor activity, with numerically higher response rates in patients with *BRCA1/2* mutations: the ORR included 7 of 15 patients with *BRCA1/2* (47%; 90% CI, 24%–70%), and the DCR, 12 of 15 (80%; 90% CI, 56%–94%), with 2 confirmed complete responses, 5 confirmed partial responses, and 5 with stable disease. Among the 27 patients without *BRCA1/2* mutations, the ORR included 3 (11%; 90% CI, 3%–26%) and the DCR included 9 (33%; 90% CI, 19%–51%), with 3 complete responses and 6 with stable disease.

In the phase I/II TOPACIO/KEYNOTE-162 study, the clinical activity and safety of the niraparib and pembrolizumab combination in patients with platinum-resistant ovarian cancer were investigated. Overall efficacy data showed ORR of 18% (90% CI, 11%–29%), with a disease control rate of 65%, including 3 (5%) patients with complete responses, 8 (13%) with partial responses, 28 (47%) with stable disease, and 20 (33%) with progressive disease. The ORR was consistent across subgroups based on platinum sensitivity, prior bevacizumab, and tumor *BRCA1/2* or HRD status. Specific to advanced TNBC, the TOPACIO study showed a 28% ORR and PFS of 8.3 months with the combination of niraparib and pembrolizumab for patients with *BRCA1/2*-mutated tumors. Interestingly, responses were also seen in non-*BRCA* advanced TNBC patients, with and without HRD.

Conclusion

Over the past decade, significant advances have been made in successfully targeting the DDR in cancer medicine, with multiple selective and potent DDR inhibitors being developed in the clinic. PARP inhibitors have emerged as a well-tolerated single-agent strategy for the treatment of ovarian cancer, germline *BRCA*-mutant breast cancers, and now pancreatic and prostate cancer. Future efforts will aim at developing more inclusive and functionally based predictive biomarker assays, improving our understanding of resistance mechanisms and optimizing treatment strategies through the use of genomic-driven innovative, longitudinal trial designs to truly personalize antitumor DDR therapies, ultimately leading to improved outcomes for cancer patients.

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DNA Gyrase Inhibitors

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Synonyms

DNA topoisomerase II inhibitors

Definition

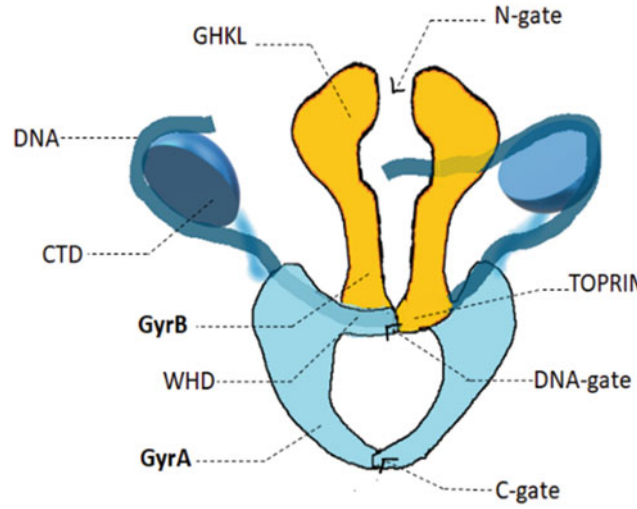
The emerging bacterial resistance makes several existing antibiotic therapies ineffective. As per the World Health Organization (WHO), approximately 7,00,000 people die annually due to drug-resistant diseases and the WHO has named bacterial resistance as one of the three major public health crises of the twenty-first century (World Health Organization 2018). To combat this, novel molecular targets are being explored for discovery of new antibacterial agents. One of the molecular targets is the prokaryotic topoisomerase that is implicated in several biological processes such as transcription, replication of DNA, and gene expression. The first topoisomerase was discovered by James Wang in 1971, followed by the discovery of *E. coli* DNA gyrase in 1976. DNA gyrase is a type II topoisomerase mainly present in prokaryotes; it overhauls the bacterial chromosome by introducing negative supercoils in the DNA via an ATP-dependent reaction. DNA gyrase introduces breaks in the two helices of the double stranded DNA. It plays an imperative role in compacting genome and resolves topological challenges related to transcription, recombination, and replication of DNA (Barančoková et al. 2018). DNA gyrase is not found in eukaryotes; hence, it is an attractive target for design and development of new chemical entities as potential antibacterial agents. This chapter presents some of the important clinically used DNA gyrase inhibitors along with an overview of new chemical entities targeting DNA gyrase.

DNA Gyrase Inhibitors

DNA gyrase inhibitors belong to three major families: quinolones, coumarins, and cyclothialidines. There is an emerging new class of DNA gyrase inhibitors in clinical trials for bacterial resistance strains. The mechanism of action of the various classes of DNA gyrase inhibitors is summarized below.

Mechanism of DNA Supercoiling by DNA Gyrase

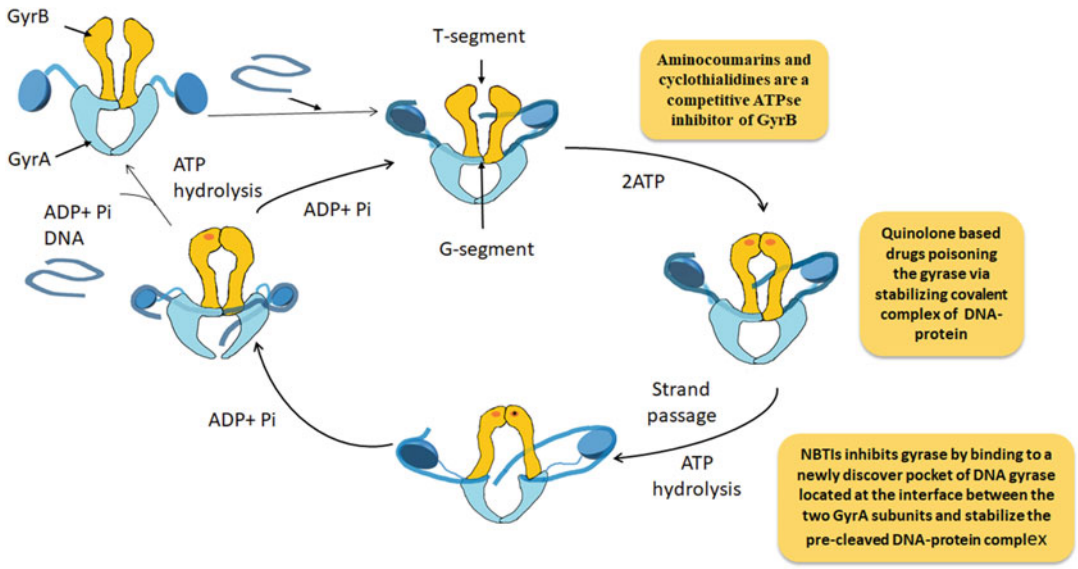
DNA gyrase is an A2B2 heterotetramer, comprised of two subunits, that is, A (encoded by *gyrA*, 97 KDa in *E-coli*) and B (encoded by *gyrB*, 90 KDa in *E-coli*). It has three domains like GyrB, GyrA, and C-terminal domain. GyrB belonging to GHKL family (GyrB, heat shock protein 90 (Hsp90)-histidine kinase/serine protein kinase, the DNA mismatch repair protein *MutL*), contains N-terminal domain for ATPase function (Fig. 1). The C-terminal domain (CTD) of GyrA forms six blades propeller kind of closed circular structure with blades 1 and 6 were connected by GyrA box. In the DNA-DNA gyrase complex, three major protein-protein interfaces forms called as gates. The gates can momentarily close and reopen for allowing passage of double-stranded DNA. The N-gate is formed by the N-terminal ATP-binding GHKL domain of GyrB that acts as an ATP-operated clamp for allowing the entry of double-stranded DNA. The central DNA gate is formed by GyrB and GyrA, specifically



DNA Gyrase Inhibitors, Fig. 1 Structure of DNA gyrase

C-terminal TOPRIM (topoisomerase primase) of GyrB along with N-terminal of WHD domain (Winged helix domain) of GyrA. This enables the catalytic tyrosine for DNA strand cleavage. Besides this, the C-terminal or exit gate is formed by GyrA (Gubaev et al. 2016).

The strand passage mechanism involves synchronized opening and closing of the N-, central DNA-, and C gate. Here, the GyrA subunit implicated in breakage and reseal of double stranded DNA while the GyrB subunit catalyzes the binding and hydrolysis of ATP. The segment of double stranded DNA intended to be cleaved known as G segment that binds at the central DNA gate encompassing the active site tyrosine that creates a transient 5′-phosphotyrosylprotein-DNA linkage in the cleaved state. A second segment of DNA is called as the T or transfer segment which pass through the upper cavity gap of GyrB, that is, N-gate. Consequently, the two catalytic gates of the DNA gyrase enzyme have been established specifically the N-terminal ATP gate and the central DNA-cleavage gate, each targeted by several antibacterial agents (Fig. 2) (Gubaev et al. 2016). Wrapping of the 140 base pairs segment of DNA around enzyme is facilitated by the C-terminal tail domain (CTD) of the GyrA protein resulted into a positive supercoil and binds to the central DNA-gate. N-terminal domains of GyrB form an ATP dependent protein clamp that closes due to nucleotide induced dimerization upon binding of ATP. Thus, the enzyme captures T segment of DNA in the upper cavity by N- or DNA gate and transfers it through the cleave in the G-segment of DNA forming DNA–phosphotyrosyl bonds 4 bp apart, thus creating a double-strand break resulting in covalent attachment of the active site tyrosine residues (Tyr122 in *E. coli* GyrA) from the two GyrA subunits to the 5′-phosphate groups on the cleaved ends via phosphodiester bonds with the help of Mg2+ ions coordinated by the TOPRIM domains of GyrB. The T segment of DNA is passed through the open central DNA gate and the broken G segment of DNA gets ultimately exit through the exit or C gate. DNA gyrase enzyme is resets for the next supercoiling cycle when the ATP gets hydrolyzed and released ADP opens the upper cavity N-gate for entry of next DNA. One DNA gyrase cycle of supercoiling facilitates two negative supercoils into the double stranded DNA molecule at the expenditure of 2 ATPs molecule. One thing is realized that DNA gyrase supercoiling cycle gives plenty targets for the inhibitors to show antibacterial activity via targeting ATP binding site of GyrA and interferes with the binding of DNA, stabilizing the DNA-protein complex in at cleaved DNA state. Thus, two major mechanisms can be liable for the antibacterial agents for targeting DNA gyrase functions. The first one is the inhibition of the ATPase enzymatic activity of GyrB and the second includes



DNA Gyrase Inhibitors, Fig. 2 Catalytic cycle of DNA gyrase and mode of action of DNA gyrase inhibitors

stabilization of the covalent binding of DNA gyrase–DNA complex, or poisoning of DNA gyrase (Gubaev and Klostermeier 2011).

Mechanism of Action of DNA Gyrase Inhibitors

Quinolones are a class of broad-spectrum bactericidal antibiotics that inhibit both gram- negative and gram-positive bacteria. The first-generation quinolones like nalidixic acid and oxalinic acid possessed weak activity, and development of fluoroquinolones such as norfloxacin and ciprofloxacin (second generation), levofloxacin (third generation), moxifloxacin, and gemifloxacin (fourth generation) expanded the armamentarium of antibiotics with potent activity and have enjoyed tremendous clinical success. They exert their action via binding to the cleavage binding site in a noncovalent manner and poison the gyrase by stabilizing the cleaved DNA-enzyme complex. The quinolones inhibit the replication process leading to cell death in bacteria (Collin et al. 2011).

The aminocoumarins class includes drugs like novobiocin, clorobiocin, and coumermycin A_1 which are naturally occurring DNA gyrase inhibitors isolated from *Streptomyces species*. Their binding site and mode of action differs from the quinolone class of DNA gyrase inhibitors. They are competitive inhibitors of the enzyme and binds at the ATP binding site of GyrB subunit inhibiting the ATPase activity of the gyrase. Besides these three classical aminocoumarins, only one natural aminocoumarin has been discovered – simocyclinones. The two binding sites identified for simocyclinones include the N-terminal domain of GyrA and C-terminal domain of GyrB. One binding pocket interacts with aminocoumarin moiety and the other binding pocket with angucyclinone moiety of the antibiotic. Therefore, occupancy of either site by an inhibitor would prevent DNA binding. These two binding sites are adjacent and not overlapping the binding site of fluoroquinolones (Khan et al. 2018).

Cyclothialidines are produced by *Streptomyces filipinensis*. The resorcinol ring of cyclothialidines is involved in a similar binding interaction as the adenine ring of ATP. They inhibit DNA gyrase activity via binding to the ATP binding site of GyrB (Khan et al. 2018).

Amycolamicin isolated from a soil *Amycolatopsis species* shows potent activity

against both gram-negative and gram-positive bacteria. Kibdelomycin is a broad spectrum, gram-positive antibacterial agent isolated from the genus *kibdelosporangium species*. It has a unique dual arm, U-shaped binding mode and is different from the binding modes of other gyrase inhibitors. Hence, they show no cross-resistance to known gyrase inhibitors such as quinolones, novobiocin, and coumermycin. They carry dichloropyrrole carboxylic acid moiety which acts as dual inhibitor of topoisomerase IV and GyrB subunit of gyrase (Barančoková et al. 2018).

CcdB and microcin B17 are plasmid-encoded toxins. These proteins stabilize the DNA gyrase-DNA covalent complexes leading to double-strand breaks in the genome (Collin et al. 2011).

Gepotidacin (GSK2140944), a traizaacenaphthylene, inhibits the gyrase by binding to the interface between the two GyrA subunits in the DNA-protein complex and targets the pre-cleaved conformation and zoliflodacin (ETX0914/AZD0914) is a spiropyrimidinetrione that stabilizes the DNA-protein complex after the DNA molecule has been cleaved. These two molecules constitute the novel bacterial type II topoisomerase inhibitors (NBTIs) class and have successfully completed phase II clinical trial for the treatment of *Neisseria gonorrhoeae* infections. These molecules possess nonquinolone binding sites of DNA gyrase to avoid cross resistance or over-lapping binding with existing known gyrase inhibitors such as quinolones or coumarins. They comprise of two heterocycles joined via an aliphatic linker, commonly containing a basic nitrogen. NBTIs form hydrophobic contacts with the gyrase via π-π interaction between two bases of the DNA. The linker part of NBTIs also shows hydrogen bonding with the side chain of Asp83 of gyrase at the binding site. For example, Gepotidacin contains a pyranopyridine and a tricyclic ring that are joined via amino-piperidine linker. It is also being studied for treatment of respiratory infections, skin infections, and urinary tract infections (Durcik et al. 2019).

The misuse and abuse of these antibiotics has led to development of quinolone-resistant bacterial strains. A vast majority of clinically observed resistance to quinolones arises due to mutations in single amino acids. These mutations are localized at the N-terminal domains of GyrA which extends from amino acids 67 to 106 in *E. coli*. Predominantly, mutations are observed at residues 67, 81, 82, 83, 84, and 106 in *E. coli*. Besides, resistance mutation is also localized at active site tyrosine positioned at 122, where DNA is bound. This domain of DNA gyrase is known as quinolone resistance determining region (QRDR). Novobiocin, marketed as Albamycin, was used clinically against *methicillin-resistant S. aureus* (MRSA). Some issues related to toxicity, poor oral absorption, low activity against gram negative bacteria, and development of resistance in coumarin sensitive organisms were observed with novobiocin. Hence, the use of novobiocin declined sharply during 1960s, although it was not officially withdrawn from the market until 2011 (Pham et al. 2019). However, in recent years many pharmaceutical industries have succeeded in overcoming the problems associated with classical aminocoumarins by designing and developing new ATPase inhibitors, for example, Quorex's indazole derivatives, AstraZeneca's aryl aminopyrimidines, vertex's pyrazole based inhibitors, and ethyl urea moiety containing DNA gyrase inhibitors have been reported by several companies such as benzimidazole ethyl urea by vertex, benzothiazole ethyl urea by Prolysis, pyridylurea by AstraZeneca, ethylurea imidazopyridines, and triazolopyridines by Pfizer (Table 1). All the known ATP-competitive inhibitors have similar interaction at the active site of DN gyrase. They contain a hydrogen bond donor and acceptor motif that interacts with Asp73, along with this essential water molecules and aromatic part forms π-π interaction with arginine residues. Except for benzimidazole and benzothiazole ethyl ureas, the progress of all the other ATP-competitive inhibitors mentioned above has been terminated. Moreover, AstraZeneca later developed azaindole and pyrrolamide classes of inhibitors with two of their pyrrolamides being the only ATPase inhibitors to reach clinical trials after novobiocin. The clinical development of pyrrolamides was later terminated, and in 2011, AstraZeneca withdrew all its efforts on development of GyrB inhibitors.

DNA Gyrase Inhibitors, Table 1 Recent patents on DNA gyrase inhibitors

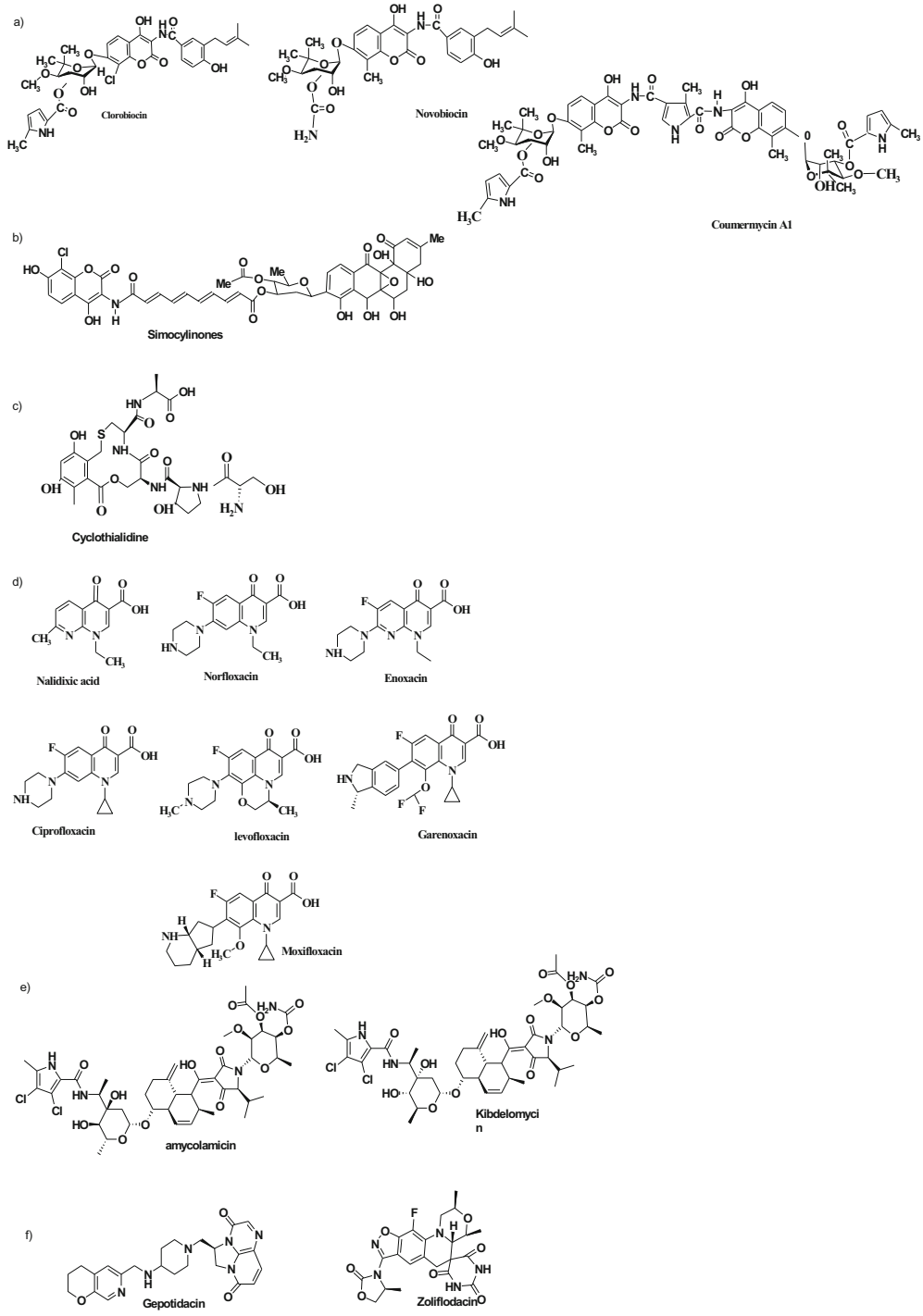
Sr. no	Patent No.	Assignee	Subject
1.	US20130030004A1	Vertex Pharmaceuticals Inc	Gyrase inhibitors and uses thereof
2.	WO 2013/138860 Al	Biota Europe limited	Antibacterial compounds
3.	WO2011032050A2	Trius Therapeutics, Inc	Gyrase inhibitors
4.	WO2017056012A1	Daiichi Sankyo Company, limited	Hydroxyalkyl thiadiazole derivatives
5.	WO201217770A1	Vertex Pharmaceuticals Inc	Phosphate esters of gyrase and topoisomerase inhibitors
6.	WO2012097269	Vertex Pharmaceuticals Inc	Pyrimidine gyrase and topoisomerase iv inhibitors
7.	US20140031318A1	Spero therapeutics Inc	Solid forms of (r)-2-(5-(2-(3-ethylureido)-6-fluoro-7-(tetrahydrofuran-2-yl)-1 h-benzo[d]imidazol-5-yl) pyrimidin-2-yl) propan-2-yl dihydrogen phosphate and salts thereof

DNA Gyrase Inhibitors, Table 2 DNA gyrase inhibitors in clinical trials

Sr. No	NCT no.	Drug	Mechanism of action	Phase	Status	Sponsor/ collaborations
1.	NCT03568942	Gepotidacin GSK2140944	Inhibits the activity of the gyrase via binding to the interface between the two GyrA subunits in the DNA-protein complex and targets the precleaved conformation	II	Completed	GlaxoSmithKline Biomedical Advanced Research and Development Authority
2.	NCT02257918 NCT03959527	Zoliflodacin ETX0914/ AZD0914	Inhibits the activity of DNA gyrase via stabilizing the DNA-protein complex after the DNA molecule has been cleaved	II III	Completed Recruiting	National Institute of Allergy and Infectious Diseases (NIAID) Global Antibiotics Research and Development Partnership

Clinical Use

DNA gyrase inhibitors are broad-spectrum antibiotics used in a variety of clinical indications. Despite the increased rate of resistance leading to re-adjustment of therapeutic strategies and applications of these inhibitors, fluoroquinolones have been used as key drugs in the treatment of urinary tract (UTIs), digestive tract, and respiratory system infections. Excessive prescription of quinolones has led to the rapid development of quinolone resistance, leading to a loss in therapeutic effectiveness of this class. Globally, high levels of resistance to *E. coli*, the predominant cause of UTIs, have led to replacement of quinolones by third-generation cephalosporins



DNA Gyrase Inhibitors, Fig. 3 DNA gyrase inhibitors. (**a**) Structure of classical aminocoumarin antibiotics. (**b**) Structure of simocyclinone. (**c**) Structure of cyclothialidines. (**d**) Structure of quinolones. (**e**) Structure of amycolamicin and kibdelomycin. (**f**) Structure of gepotidacin and zoliflodacin

for this indication. Fluoroquinolones are still used in the treatment of typhoid caused by *Salmonella* owing to resistance to earlier first line agents like chloramphenicol, trimethoprim/sulfamethoxazole, and ampicillin. Nevertheless, resistance to quinolones in *Salmonella* is rapidly being reported in South Asia, Africa, and America. Thus, *Salmonella* is now graded as a high urgency pathogen for research and development of new antibacterial agents. For respiratory tract infections, especially community-acquired pneumonia, quinolones are not recommended for first-line treatment but are reserved for serious cases. To decrease the probability of fluoroquinolone resistance development, it is recommended to limit the use of this class in the treatment of patients with less severe infections. Though it is a powerful antibiotic for treating children's infections such as diarrhea or gram-negative meningitis, the toxicity (arthralgia) of this class combined with the issues of increased resistance has reduced its use for this group. The recently reported potential of aortic rupture and dissection side effects also raises safety concerns for the use of quinolones in elderly patients, wherein they may lead to serious bleeding or even death. In addition, there are probable teratogenic and mutagenic effects, so prescription of fluoroquinolones in pregnant and breast-feeding women is limited. The use of aminocoumarins class of antibiotics has been constrained due to problems related to water solubility, activity against gram negative bacteria and toxicity. Hence, there is research impetus to explore nonquinolone binding sites of DNA gyrase to avoid cross resistance or over-lapping binding with existing known gyrase inhibitors. Until now, several molecules of the NBTIs class are under clinical trials that utilized distinct mechanism for trapping the DNA-protein complex. Two types of NBTIs have been discovered that we have discoursed in above chapter. Type 1 NBTIs inhibit the activity of the DNA gyrase via binding to the interface between the two GyrA subunits in the DNA-protein complex and target the precleaved conformation while type II NBTIs stabilize the DNA-protein complex after the DNA molecule has been cleaved (Table 2; Fig. 3).

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DNA Methylation

► Epigenetics

DNA Topoisomerase II Inhibitors

► DNA Gyrase Inhibitors

Docking Protein

► Adaptor Proteins

Dopamine

▶ Dopamine System

Dopamine System

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Synonyms

Basal ganglia; Dopamine; Dopaminergic receptors; GPCR; L-DOPA; Parkinson's disease; Substantia nigra; Ventral tegmental area (VTA)

Definition

Dopamine (dopaminergic) system plays an important role in central neural system, taking part in regulating motor control, executive functions, motivation, reinforcement, reward, sleep, feeding, attention, cognitive functions, olfaction, vision, hormonal regulation, and outside of central neural system in sympathetic regulation and penile erection. Dopamine as well influences immune, gastrointestinal, and cardiovascular systems and renal function.

Basic Characteristics

Dopaminergic Pathways

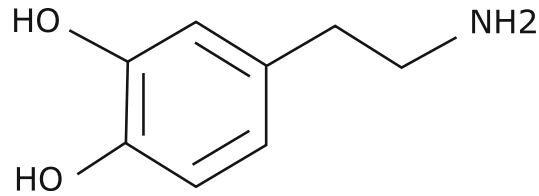
Dopaminergic neurons are a heterogenic group of cells, most of which are localized in mesencephalon and diencephalon, and a small amount is located in bulbus olfactorius. In mesencephalon cells are localized in three groups – in substantia nigra pars compacta (SN), in the ventral tegmental area (VTA), and in the retrorubral field (RRF). The axons of these neurons form four major dopaminergic pathways: nigrostriatal, mesocortical, mesolimbic (often collectively termed the mesocorticolimbic), and tuberoinfundibular pathways. The nigrostriatal pathway connects SN and dorsal striatum (caudate nucleus and putamen), and this pathway is involved in regulation of movement. Mesocortical pathway connects VTA and prefrontal cortex, and it is involved in cognitive and emotional control. The mesolimbic pathway connects VTA and ventral striatum (nucleus accumbens and olfactory tubercle), and it is involved in mediating reinforcements and reward. The tuberoinfundibular pathway is formed mostly by axons of neurons localized in arcuate nucleus in hypothalamus that end in median eminence. This pathway inhibits the secretion of prolactin from anterior pituitary gland.

Dopamine

Dopamine (DA) ($C_8H_{11}NO_2$, 3-hydroxytyramine, 4-(2-aminoethyl)benzene-1,2-diol (IUPAC)) is a catecholaminergic monoamine neurotransmitter. Chemically DA is catecholamine, in which the hydrogen at position 4 is substituted by a 2-aminoethyl group (Fig. 1).

Dopamine Synthesis

The DA synthesis consists of two reactions: (1) conversion of L-Tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and (2) conversion of L-DOPA to dopamine. Tyrosine is a nonessential amino acid that can be synthesized in the human body from the amino acid phenylalanine. This process goes primarily in the liver catalyzed by an enzyme phenylalanine hydroxylase. Then L-Tyrosine from the blood enters the brain by a low affinity amino acid transport system, and it enters the neurons by high and low affinity amino acid transporters. L-Tyrosine is converted to L-DOPA by tyrosine hydroxylase

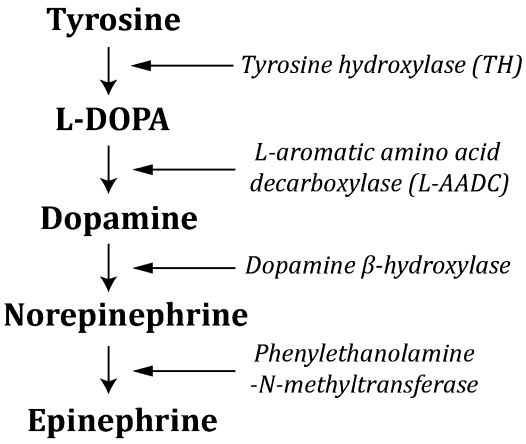


Dopamine System, Fig. 1 Dopamine structure

(TH). TH is the rate-limiting enzyme of catecholamine biosynthesis. The activity of TH is regulated by feedback inhibition of the catecholamine neurotransmitter, which binds to amino-terminal regulatory domain (R-domain) of TH, by phosphorylation by multiple kinases at four different serine residues and dephosphorylation by two phosphatases, by protein-protein interactions with enzymes in the same pathway or from the tetrahydrobiopterin pathway, by interaction with structural proteins considered to be chaperones that mediate the neuron's oxidative state, and by the protein that transfers dopamine into secretory vesicles (Daubner et al. 2011).

TH uses a cofactor tetrahydrobiopterin to convert tyrosine to L-DOPA by the addition of a hydroxyl group to the third carbon of the aromatic ring. Then the L-aromatic amino acid decarboxylase (L-AADC) removes the carboxylic acid group from the backbone of the L-DOPA and converts L-DOPA into dopamine (Fig. 2).

Dopamine is an active neurotransmitter in the brain, but at the same time, it is a precursor for norepinephrine (NE) and epinephrine in respective neurons. In the norepinephric neurons, dopamine is converted to NE by the enzyme dopamine β-hydroxylase, which can be converted further to epinephrine by the enzyme phenylethanolamine N-methyltransferase in epinephric neurons. This reaction takes part mainly within the adrenal medulla, but detectable levels of this enzyme are found in association with epinephrine neurons in the brain.



Dopamine System, Fig. 2 Catecholaminergic synthesis

After the synthesis dopamine is actively transported from the cytoplasm into the vesicles by vesicular monoamine transporter 2 (VMAT2). VMAT2 functions as a stoichiometric antiporter, transporting two H^+ ions out of a vesicle to transport one monoamine molecule into a vesicle (German et al. 2015). The activity of VMAT2 depends on posttranslational modification, protein interactions, presynaptic localization, H^+-ATPase activity, and pH in the cell (German et al. 2015). Vesicles are trafficked to the plasma membrane, neurotransmitter is released into the synapse, and then vesicles are reformed through internalization.

Dopaminergic Receptors

The physiological actions of dopamine are mediated by dopaminergic receptors (DR). DRs are G-protein-coupled receptors (GPCRs). GPCRs are a large family of receptors also called seven-transmembrane domain-containing receptors because they have seven transmembrane domains. There are five types of dopaminergic receptors, and on the basis of structural, pharmacological, and biochemical properties, they are combined in two groups: D1-like (D1, D5) and D2-like (D2, D3, D4) receptors.

There are certain differences between two groups. The genes of receptors of D1-class do not contain introns in their structure. D2-class has several introns, with six introns in D2 receptor gene, five in the gene that encodes D3 receptor, and three in D4 receptor gene. That is why all of the D2-class receptors have different splice variants. For example, D2 receptor has two splice variants, D2S (414 amino acids) and D2L (443 amino acids), and these splice variants have different features and pharmacological properties. D2S are mostly presynaptic receptors and are mostly involved in autoregulation, when D2L are presented mostly postsynaptically.

The D1-class of the receptors activates $G\alpha_{s/olf}$ family of G-proteins and stimulates cAMP production. The receptors of D2-class are coupled to $G\alpha_{i/o}$ that inactivates the cAMP.

D1 dopamine receptors are expressed at a high level of density in the nigrostriatal, mesolimbic,

and mesocortical areas, such as the caudate-putamen (striatum), nucleus accumbens, substantia nigra, olfactory bulb, amygdala, and frontal cortex, as well as at lower levels in the hippocampus, cerebellum, thalamic areas, and hypothalamic areas. D5 dopamine receptors are expressed at low levels in multiple brain regions, including pyramidal neurons of the prefrontal cortex, the premotor cortex, the cingulated cortex, the entorhinal cortex, the substantia nigra, the hypothalamus, the hippocampus, and the dentate gyrus. A very low level of expression has also been observed in the MSNs of the caudate nucleus and nucleus accumbens.

The highest levels of D2 dopamine receptors are found in the striatum, nucleus accumbens, and olfactory tubercle. D2 receptors are also expressed at significant levels in the substantia nigra, ventral tegmental area, hypothalamus, cortical areas, septum, amygdala, and hippocampus. The D3 dopamine receptor has a more limited pattern of distribution, the highest level of expression being observed in the limbic areas, such as in the shell of the nucleus accumbens, the olfactory tubercle, and the islands of Calleja. At significantly lower levels, the D3 dopamine receptor is also detectable in the striatum, the substantia nigra pars compacta, the ventral tegmental area, the hippocampus, the septal area, and in various cortical areas. The D4 dopamine receptor has the lowest level of expression in the brain, with documented expression in the frontal cortex, amygdala, hippocampus, hypothalamus, globus pallidus, substantia nigra pars reticulata, and thalamus.

D1, D2, and D4 dopamine receptors have also been observed in the retina, and prominent levels of expression of D2 dopamine receptors have been detected in the pituitary gland. In the periphery, all subtypes of dopamine receptors have been observed in the kidney, adrenal glands, sympathetic ganglia, gastrointestinal tract, blood vessels, and heart (Beaulieu and Gainetdinov 2011).

The role of dopamine and the contribution of activation of different types of DRs to various physiological processes are very wide, but among the main ones, the participation in the regulation of locomotion activity is controlled by D1, D2, and D3; significant parts of reward and reinforcement mechanisms are also controlled by dopamine with participation of D1 and D2 receptors mainly and of D3 receptors less; D1 and D2 dopamine receptors seem to be critical for learning and memory mechanisms, such as working memory, that are mediated primarily by the prefrontal cortex (Beaulieu and Gainetdinov 2011); D2 dopamine receptors play a critical role in the psychotic reactions observed in schizophrenia and bipolar disorder.

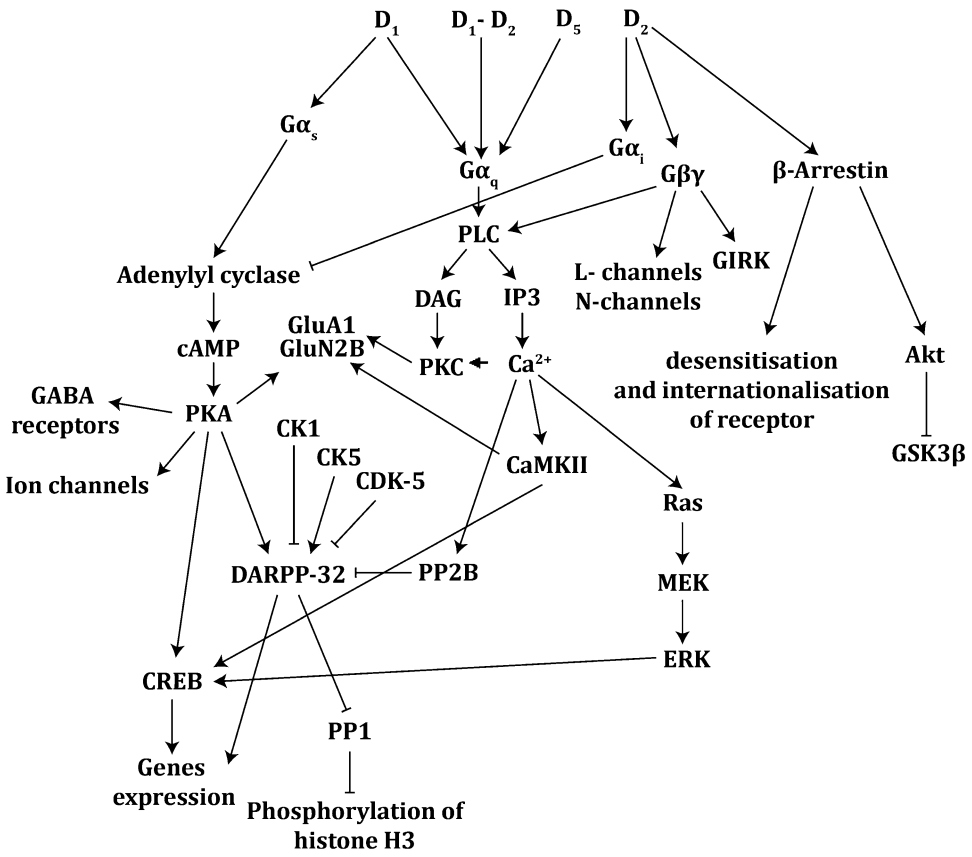
Dopaminergic receptors can form heterodimers, which lead them to the appearance of new pharmacological properties and have an effect on various signaling pathways. The following heterodimers were already described, with different types of dopaminergic receptors, D1–D2 heterodimers, D1–D3 heterodimers, and D2–D4 heterodimers, and with other types of receptors: D2-TAAR1 heterodimers, D1-NMDA heterodimers, D1-adenosine A2 heterodimers, D2-ghrelin heterodimers, D4-β1 adrenoceptor, and D4-α_{1b} adrenoceptor heterodimers (Beaulieu and Gainetdinov 2011).

Dopaminergic Signaling

Dopamine interaction with its receptors activates different signaling cascades mainly involving the G-proteins, but at the same time, it activates G-protein-independent signaling (Fig. 3).

G-proteins belong to a group of proteins which are enzymes called GTPases, because they hydrolyze guanosine triphosphate (GTP) to guanosine diphosphate (GDP). There are two classes of G-proteins, monomeric GTPase and heterotrimeric G-protein complex, consisting of three subunits: α, β, and γ. GPCR are couples with heterotrimeric G-proteins. Without a ligand agonist, the α-subunit is bound to GDP, and β and γ subunits form an inactive trimeric protein complex. Agonist binding to receptor results in GDP release and binding of GTP to the complex, which leads to dissociation of the α-subunit. This dissociation forms free α-subunit (Gα) and the β/γ-complex (Gβγ), both of which can transduce the signal and activate the effector systems.

Depending on type of α-subunit, G-proteins are classified into four families: $G\alpha_{s/olf}$, $G\alpha_{i/o}$,



Dopamine System, Fig. 3 Dopaminergic signaling

$G\alpha_q$, and $G\alpha_{12}$ (Kamato et al. 2015). Dopaminergic receptors are connected with different types of G-proteins. D1-like receptors are coupled with $G_{s/olf}$ proteins, activation of which leads to an activation of adenylyl cyclase and increase in the intracellular content of cAMP. High level of intracellular cAMP stimulates activity of protein kinase A (PKA). PKA have several targets such as cAMP response element (CREB), glutamate receptors (GluA1 and GluN2B), GABA receptors, ion channels (e.g., calcium and potassium), and dopamine- and cAMP-regulated phosphoprotein 32-kDa (DARPP-32) (Klein et al. 2019).

CREB is a cellular transcription factor involved in regulation of transcription of several genes, including BDNF, tyrosine hydroxylase, c-Fos, and some others. Phosphorylation and activation of CREB lead to an increase of gene expression. The activity of CREB also can be regulated by CaMKII and by ERK (Calabresi et al. 2014), which will be discussed later. It was shown that altered CREB signaling is involved in cognitive and neurodegenerative disorders (Saura and Valero 2011).

DARPP-32 is a multifunctional protein mainly expressed in medium spiny neurons in striatum (MSN). PKA phosphorylates DARPP-32 at Thr34. This enhances the gene expression. Also the phosphorylation of DARPP-32 at Thr34 promotes inhibitory effect of DARPP-32 on protein phosphatase-1 (PP1), which is responsible for histone dephosphorylation (mainly H3) (Klein et al. 2019). The phosphorylation of DARPP-32 at other sites can influence the phosphorylation of it at Thr34. It can be reduced by phosphorylation of DARPP-32 at Ser137 by casein kinase 1 (CK1) and enhanced by phosphorylation of DARPP-32 at Ser97/102 by casein kinase 2 (CK2). The phosphorylation of DARPP-32 at Thr75 is made by

cyclin-dependent kinase 5 (CDK-5), and it prevents the inhibition of PP1 by DARPP-32 and converts the DARPP-32 to an inhibitor of PKA (Beaulieu and Gainetdinov 2011), but this phosphorylation by CDK5 can be prevented by PKC (Sahin et al. 2008). Dephosphorylation of DARPP-32 at Thr34 can also be promoted by protein phosphatase 2B (PP2B), activated by increased level of intracellular Ca^{2+} (the causes of increasing concentration of intracellular Ca^{2+} will be discussed later). DARPP-32 can also interact with different hormones and neurotransmitters (Greengard 2001).

The activation of D1 receptors also leads to phosphorylation of ribosomal protein S6 (rpS6) at $Ser^{235/236}$ and $Ser^{240/244}$ which results in activation of CAP-dependent mRNA translation, and additional activation of PKA leads not only to phosphorylation of DARPP-32 but also to activation of mTOR complex 1 (Hutchinson et al. 2011).

In contrast to D1-like receptors, D2-like receptors are coupled to $G_{i/o}$ proteins, which have opposite effect on adenylyl cyclase, reducing its activity, which leads to a decrease in intracellular levels of cAMP, PKA activity, and phosphorylation of DARPP-32 at Thr34.

D2-like signaling also includes $G_{\beta\gamma}$ activation of phospholipase C (PLC) leading to increase cytoplasmic calcium with downstream signaling events. $G_{\beta\gamma}$ involved in regulation of activity of L- and N-type calcium channels, and G-protein coupled inwardly rectifying potassium channels (GIRKs). It was shown that these channels involved in phosphorylation of two subunits of glutamate receptors (GluA1 and GluN2B) followed by activation of signal cascades.

Another direction of D2-like dopamine signaling is signal cascade mediated through β-arrestin 2 (βArr2). Arrestins are a family of multifunctional molecular adapter proteins. Originally, it has been convincingly established that β-arrestins are involved in desensitization and internationalization of GPCRs (Ferguson 2001). However, later studies revealed that βArr2 and βArr1 can act also as molecular scaffolds for signaling molecules such as kinases and phosphatases (Beaulieu et al. 2015). In fact, βArr-dependent phosphorylation of Akt results in Thr^{308} and Ser^{473} by PDK and PDK2/rictor-mTOR, respectively (Beaulieu et al. 2015). Akt phosphorylate further several substrates including GSK3α at Ser^{21} and GSK3β at Ser^{9}, which results in their inhibition. Thus, βArr can form complexes with protein phosphatase 2A (PP2A) and Akt kinase, resulting in Akt inhibition and in subsequent GSK3β activation.

Dopamine receptors (D1, D5, or D1–D2 heterodimers) can couple also with $G\alpha_q$ protein, which leads to activation of phospholipase C_β (PLC). As it was mentioned before, PLC can also be activated by βγ subunit of G-protein. Phospholipase C_β hydrolyzes phosphatidylinositol 4,5-biphosphate into diacylglycerol (DAG) and inositol triphosphate (IP3). DAG activates number of isoforms of protein kinase C (PKC). IP3 diffuses to endoplasmic reticulum and binds to IP3 receptors on ligand-gated calcium channels, which increase calcium efflux to cell surface from the endoplasmic reticulum and from extracellular space (Kamato et al. 2015). The increased level of Ca^{2+} can activate calcium-dependent PKC variants and upregulate calcium-regulated enzymes, such as PP2B and calcium-/calmodulin-dependent kinase II (CaMKII). CaMKII can phosphorylate CREB and activate the gene expression (Calabresi et al. 2014). Also PKC and CaMKII can modulate the cross talk to cAMP signaling, by effecting glutamate receptors GluA1 and GluN2B as it is done by PKA (Beaulieu et al. 2015). At the same time, increased level of intracellular Ca^{2+} activates the MAPK signaling cascade including ERK (Cahill et al. 2014). EKR activation targets the intracellular protein CREB and also activates it (Calabresi et al. 2014).

Dopaminergic receptors can interact with other receptors like NMDA, sodium channels, GABA receptors, and Na^+/K^+ ATPase (Klein et al. 2019) and with receptor of tyrosine kinases (RTK). It was shown that RTK can be transactivated by different dopaminergic receptors: platelet-derived growth factor β receptor (PDGFβR) by D4; insulin-like growth factor receptor (IGFR) by D2; BDNF receptor by D1, D2, and D1–D2 heterodimers; and TrkB by D1. RTK transactivation by GPCR results in rapid activation of several

signaling cascades, including PI3K/Akt, Ras/MAPK, and PLC.

Disorders Associated with Dopaminergic System

Changes in the dopaminergic system play a role in a number of diseases, among which are Parkinson's disease (PD), schizophrenia (SZ) (Abi-Dargham et al. 2009), bipolar disorder (Cousins et al. 2009; Ashok et al. 2017), Huntington's disease (Chen et al. 2013), attention deficit hyperactivity disorder (ADHD) (Blum et al. 2008; Montarolo et al. 2019), Tourette's syndrome, addiction, depression, restless leg syndrome, pituitary tumors, hyperprolactinaemia, hypertension, gastroparesis, nausea, and erectile dysfunction (Beaulieu and Gainetdinov 2011).

Dopaminergic Drugs

Dopamine Precursors

L-DOPA

Dopamine Receptor Agonists

Apomorphine, bromocriptine, cabergoline, dihydrexidine, pramipexole, ropinirole, fenoldopam, piribedil, lisuride, pergolide, rotigotine

Dopamine Receptor Antagonists

Typical antipsychotics: haloperidol, chlorpromazine, fluphenazine, loxapine, molindone, perphenazine, pimozide, thioridazine, thiothixene, trifluoperazine

Atypical antipsychotics: clozapine, olanzapine, amisulpride, quetiapine, risperidone, sulpiride, ziprasidone

Antiemetics: domperidone, alizapride, metoclopramide, prochlorperazine

Reuptake Inhibitors

Methylphenidate, bupropion, amineptine, nomifensine, cocaine, methylenedioxypyrovalerone, phencyclidine

Transporter Blockers

Inhibitors of VMAT2: reserpine, tetrabenazine, deserpidine

Releasing Agents

Phenethylamine, amphetamine, lisdexamfetamine, methamphetamine, methylenedioxymethamphetamine, phenmetrazine, pemoline, 4-methylaminorex (4-MAR), benzylpiperazine

Inhibitors of Degradation (MAO Inhibitors)

Nonselective: phenelzine, tranylcypromine, isocarboxazid

MAO-A selective: moclobemide

MAO-B selective: selegiline, rasagiline, pargyline

Inhibitors of Different Parts of Dopamine Signaling

Catechol O-methyl transferase (COMT) inhibitors: entacapone, tolcapone

Dopamine β-hydroxylase inhibitors: disulfiram

Phenylalanine hydroxylase inhibitors: 3,4-dihydroxystyrene

Tyrosine hydroxylase inhibitors: α-methyl-para-tyrosine (AMPT)

Aromatic L-amino acid decarboxylase (L-AADC) or DOPA decarboxylase inhibitors: benserazide, carbidopa

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Dopaminergic Receptors

▶ Dopamine System

Drug Discovery

▶ Peptides and Peptidomimetics as Foundations for Drug Discovery

Drug Interactions

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Synonyms

Drug-drug interactions; Pharmacokinetic and/or pharmacodynamic consequence of multiple drug therapy; Pharmacokinetic and/or pharmacodynamic interactions

Background

Clinical Significance of Drug-Drug Interactions

Evidently there is an increasing risk of multimorbidity with age. As a consequence, the number of prescriptions and the number of possible related side effects rise in elderly patients. In addition, there is an increased risk of adverse events or of drug failure due to drug-drug interactions leading to changed drug concentrations or synergistic or antagonistic pharmacodynamic effects. Early studies on inappropriate drugs in the elderly stated that in average five or more drugs are taken simultaneously by patients being 65 years or older (Fialova et al. (2005); (Gallagher et al. 2008)). Drug-drug interactions were

identified as cause of adverse events leading to admission to medical wards or for prolonged hospital stays (Lazarou et al. 1998; Smith et al. 2008; Thomsen et al. 2007). These observations have been confirmed by a number of further studies. In an Austrian study, adverse drug effects were regarded as the reason in 10% for inpatient admission among 543 elderly patients (median age: 82 years), who were taking 7.5 ± 3.8 drugs at the time of their admission (Schuler et al. 2008). Moreover, the authors regarded 36% of the prescribed drugs as unnecessary and 30% as even inappropriate for elderly people according to the PRISCUS list (Holt et al. 2010). Adverse drug effects may occur also during inpatient treatment. A British study in 3695 patients showed that almost 15% of the patients suffered from adverse drug effects during their stay in hospital, causing a prolonged hospital stay in 25% out of these patients, one definition of severe adverse events. Data analysis revealed that the number of simultaneously prescribed drugs was the only significant predictor. In most frequent cases, the medication dose was incorrectly or not adjusted or renal impairment was not considered (Davies et al. 2009). Today it is estimated that drug interactions could account for 1% of hospitalizations in the general population and 2–5% of hospital admissions in the elderly (Letinier et al. 2019). However, the impact of DDI on overall health burden is difficult to assess as the clinical consequence may vary widely.

Basic Mechanisms

Pharmacodynamic Interactions

Pharmacodynamic interactions occur when two or more drugs interact directly at their site of action affecting the efficacy in the same direction (additively, synergistically) or leading to amelioration through antagonistic effects. In clinical medicine, additive or synergistic effects are often desired if a monotherapy is not indicated or efficient. For example, according to the recent guidelines of hypertension, at least two antihypertensives with different mode of action should be described to improve the speed, efficiency, and predictability of blood pressure control (Williams et al. 2018).

Similarly, potentiating strengthening (synergistic) effects are desired to treat infections or also severe pain. Although synergistic effects may be beneficial, the risk of adverse events may increase as well. This is exemplified in the treatment of heart failure with mineral corticoid antagonists such as spironolactone and ACE inhibitors, bearing the potential risk of hyperkalemia (Juurlink et al. 2004); careful monitoring however allows the application of this combination even in patients with mild to moderate kidney disease (Edwards et al. 2012). Similarly, bleeding risk is potentially elevated applying dual antiplatelet therapy with clopidogrel and aspirin while treating patients with coronary artery disease who underwent stent implantation (Sorensen et al. 2012; Lopes et al. 2019).

Severe adverse events such as QT interval prolongation are known to be (albeit rare) the result of combinations of drugs, intended to strengthen the treatment of infections such as the combination of fluoroquinolones and macrolides like erythromycin (Saxena et al. 2013). Now in the SARS-CoV2 pandemic, the use of hydroxychloroquine together with azithromycin was suggested to treat patients with acute Covid-19 symptoms. However, despite unproven efficacy of this regimen, both drugs bear the risk to augment QT interval prolongation with the risk of torsade de pointes (Chorin et al. 2020).

Pharmacodynamic Interactions as Cause of Bleeding Events

Bleeding events were identified as the most frequent cause of adverse events leading to a fatal outcome in a Swedish registry (Wester et al. 2008).

In particular gastrointestinal bleeding events could occur while patients with atrial fibrillation and coronary artery disease are in antithrombotic treatment, in particular with acute coronary syndrome or percutaneous coronary intervention (PCI). A recent meta-analysis study demonstrated that a regimen of NOACs plus an adenosine P2Y12 inhibitor was associated with less bleeding compared with a regimen of vitamin K antagonists plus P2Y12 inhibitor and aspirin (Lopes et al. 2019). However, also specific serotonin reuptake inhibitors (SSRIs) such as citalopram bear a twofold risk

of gastrointestinal bleeding events that is further elevated by concomitant administration of aspirin or another NSAIDs (Andrade et al. 2010). The serotonin uptake transporter SLC6A4 is expressed on the surface of platelet as well. Inhibition by SSRI leads to a reduction of intra-platelet serotonin concentrations with consecutive deterioration of platelet function resulting in a 3.0 (2.1–4.4) bleeding risk when SSRIs are administered together with aspirin compared to aspirin alone. Other antidepressants are not known to cause such an interaction. In addition, the SSRI-mediated loss of platelet function is augmented by anticoagulants (Schalekamp et al. 2008). Administration of proton pump inhibitors may reduce bleeding risk in those patients.

In contrast, some NSAIDs may deteriorate platelet inhibition of aspirin potentially increasing the cardiac risk in CAD patients through competition at the serine residue at position 529 of COX-1 hindering the irreversible acetylation by aspirin (Catella-Lawson et al. 2001). Clinical long-term investigations confirmed this in vitro observation (Hudson et al. 2005). Later investigations showed that more NSAIDs like naproxen, metamizole, and others may negatively interact with aspirin antiplatelet effect (Saxena et al. 2013).

Many more pharmacodynamic interactions may occur, exceeding the length of this review. In essence, there is no simple systematic to predict pharmacodynamic interactions like it is the case for pharmacokinetic interaction (see below). Hence it is still a challenge to estimate the consequences of combinations. Approaches for prediction of the magnitude of interactions using mechanism-based PK-PD modeling may help in this matter (Stepensky and Rimon 2015). This is now demonstrated in the current hasty search for best therapy regimens to treat Covid-19 (Venisse et al. 2020).

Some examples of typical pharmacodynamic drug-drug interactions are listed in Table 1.

Pharmacokinetic Interactions

Pharmacokinetic DDIs are a major cause of adverse events. As most drugs are administered orally, the different levels of ADME name absorption, distribution, metabolism, and elimination a crucial for putative drug-drug interaction leading to changes of pharmacokinetic profiles. In essence, there are three types of interactions that need to be distinguished:

(a) The PK of the investigational drug is affected by another drug (victim drug).
(b) The investigational drug inhibits drug-metabolizing enzymes or transporters leading to changes of the PK of other drugs (perpetrator drug).
(c) The investigational drug induces drug-metabolizing enzymes or transporters leading to changes of the PK of other drugs (perpetrator drug).

Knowledge about the properties of a drug being a victim or a perpetrator is of major importance in clinical routine when drug therapy regiments are changed by adding further drugs.

Regulatory Requirements

Due to the potential significance of DDI, possible interactions have to be appropriately assessed already during drug development. Particular emphasis by regulatory authorities has been given to pharmacokinetic drug-drug interactions on the level of drug metabolism and transporters. Guidelines of the US Food and Drug Administration (FDA), European Medicines Agency (EMA), and Ministry of Health, Labor and Welfare (MHLW) of Japan have been widely harmonized in the recent years proposing systematic methods and procedures for an objective and scientific evaluation of DDI potential as excellently reviewed and summarized by Iwatsubo (2020).

According to the FDA "Clinical Drug Interaction Studies – Cytochrome P450 Enzyme- and Transporter-Mediated Drug Interactions Guidance for Industry" (Sudsakorn et al. 2020), an investigational drug causing P450 inhibition can be classified as a strong, moderate, or weak inhibitor based on its effect on an index CYP substrate:

- Strong: Increment of the AUC of a sensitive index P450 substrate $\geq$5-fold
- Moderate: Increment of the AUC of a sensitive index P450 substrate by $\geq$2- to <5-fold
- Weak: Increment of the AUC of a sensitive index P450 substrate by $\geq$1.25- to <2-fold

Drug Interactions, Table 1 Examples of additive and antagonistic pharmacodynamic drug-drug interactions

Drug A	Drug B	Putative effect
Additive interactions		
NSAID	SSRI, VKI	Elevated bleeding risk
NSAID	Glucocorticoids	Elevated gastric bleeding risk
ACE inhibitor	Spironolactone, amiloride	Hyperkalemia
SSRI	MAO inhibitors	Serotonin syndrome
Tricyclic antidepressants	Low potent neuroleptics	Augmented anticholinergic effects
Fluoroquinolones	Macrolides, citalopram	QT_c interval prolongation, torsade de pointes
Hydroxychloroquine	Azithromycin	QT_c interval prolongation, torsade de pointes
Antagonistic interactions		
ASS	Ibuprofen	Attenuation of platelet inhibition
ACE inhibitors	NSAID	Attenuation of blood pressure lowering
Levodopa	Typical neuroleptics	Attenuation of dopaminergic effects
Warfarin	Vitamin K	Attenuation of anticoagulation

SSRI, serotonin reuptake inhibitor; VKI, vitamin K antagonist; MAO, monoamine oxidase; NSAID, non-steroidal anti-inflammatory drug

These categories typically describe the effect of the investigational drug when given at the highest clinical dose and the shortest dosing interval within its therapeutic dose range/dosing regimen.

DDI on the Level of Adsorption

Early interactions within the dietary tract may occur through formation of complexes that can considerably reduce the bioavailability of drugs, having the potential to interact with multivalent cations such as Ca^{2+}, Mg^{2+}, Al^{3+}, Zn^{2+}, Fe^{2+}, or Fe^{3+}. In particular bisphosphonates applied in therapy of severe osteoporosis such as alendronate are affected due to their considerably low bioavailability of only 0.5% to 2% that is further reduced in presence of calcium ions in mineral water or milk (Itoh et al. 2016). Anti-infectives such as tetracyclines or quinolones are also known to form complexes with multivalent cations that can also reduce the bioavailability of orally applied levothyroxine (Liwanpo and Hershman 2009). Further the bioavailability of drugs having a catecholamine structure as applied in Parkinson disease (levodopa, carbidopa) may be decreased iron ions (Campbell and Hasinoff 1989). As a consequence, the simultaneous intake of calcium-containing food, neutralizing antacids containing aluminum or magnesium ions, or supplements containing multivalent ion salts must be avoided.

Basic anion exchangers such as cholestyramine are not resorbed in the intestine due to their molecular structure. These amphiphilic drugs are able to build complexes with a number of cationic drugs and must be administered at least 2 h before.

In addition, the change of the gastric pH, e.g., through proton pump inhibitors like pantoprazole, may affect the bioavailability of certain drugs, such as the antiviral drug atazanavir or the bisphosphonate alendronate (Ogawa and Echizen 2011). In a clinical study investigating the protective benefit of alendronate, patients receiving proton pump inhibitors at the same time showed a higher frequency of hip fractures (Abrahamsen et al. 2011).

Interactions on the Level of Drug Transporters

Active or facilitated transport across tissue barriers is a major determinant of drug disposition in the human body. There are more than 400 transporters in the human genome, and about 30 are involved in the pharmacokinetics of drugs. Major

drug-drug interactions take place on the level of efflux transporters in the intestine, on hepatic uptake transporters, and uptake and efflux transporters in the kidney (Zhang et al. 2011). In contrast to drug-metabolizing enzymes, however, there is almost no drug which is exclusively a substrate of a specific transporters; there is partly a large overlap of specificity to between uptake and efflux transporters (Gessner et al. 2019). In addition, many drugs are substrates of drug-metabolizing enzymes as well making it difficult to distinguish clear drug-drug interactions solely based on the level of drug transporters. As a consequence, the prediction of drug transporter interactions in vivo is sometimes difficult to be made from in vitro experiments alone. Nevertheless, today firm data on the properties of drugs being a substrate, inhibitor, or inducer is mandatory during drug development process and is outlined in the drug label upon approval.

Several efflux and uptake transporters may interact with drugs in clinical use. Particular emphasis by regulatory authorities is given to P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), organic anion-transporting polypeptide 1B1/1B3 (OATP1B1, OATP1B3), organic anion transporter 1/3 (OAT1/OAT3), multidrug and toxin extrusion (MATE) proteins (MATE1/MATE2-K), as well as the organic cation transporter 2 (OCT2). A list of substrates for five classes of transporters is shown in Table 2. Substrates in bold have been recommended by the FDA as transporter-specific victim drugs to be used for DDI during clinical development of drugs (FDA 2020).

The ABC transporter P-gp (ABCB1) restricts the distribution of its substrates in the intestine and also the brain, placenta, and testes. Moreover, it acts as an excretory pump in the liver and the kidneys. It has a wide spectrum of substrates that overlap much with CYP3A4 and also to some extent with BCRP (ABCG2) (Cascorbi 2011).

Inhibition of P-gp is mostly caused through competitive inhibition by perpetrator drugs, having a low Ki. Inhibition of intestinal expressed P-gp leads usually to an elevation of C_{max} and AUC, but the clearance and related half-life is less affected (Fig. 1). Elevation of systemic exposure of more than 25% at concentrations ten times the Ki-value is considered as clinically relevant. However, as mentioned above, many compounds are also substrate or inhibitors of CYP3A4/5, affecting also the elimination. Established inhibitors are listed in Table 3; some are listed for more than one transporter demonstrating the low specificity also of many inhibitors. Some inhibitors are frequently prescribed anti-infectives such as the macrolide clarithromycin or the systemic azole antimycotic itraconazole and the antiarrhythmic drugs amiodarone and dronedarone.

Since the phenomenon of efflux transporter-mediated multidrug resistance was shown in particular in various malignancies, the idea came up to inhibit the upregulated efflux of anticancer drugs out of the cancer cell by inhibition of P-gp or BCRP. Strong inhibitors have been developed (Table 3); however since these ABC transporters are expressed at many interfaces of the body, systemic toxicity could increase as well, making this concept so far not suitable in clinical practice (Choi and Yu 2014).

The clinical relevance of P-gp inhibition has been shown for many drugs in the past but came into focus also in more recent time when novel non-vitamin K-dependent anticoagulants (NOACs) have been developed aiming to overcome the broad inter- and intra-individual variability of the vitamin K antagonists warfarin and phenprocoumon that require routine INR monitoring. Dabigatran etexilate, the oral prodrug of the thrombin inhibitor dabigatran, is a substrate of P-gp in the intestine, making it a victim drug to P-gp inhibitors, whereas the active drug dabigatran is not. Therefore, although dabigatran is not metabolized and entirely renally excreted, DDIs may take place on the level of P-gp and have to be considered (Akamine et al. 2019). According to the product information, the AUC of dabigatran (150 mg dabigatran etexilate) increases after a single dose of 40 mg ketoconazole by a clinically significant factor of 2.35, whereas 2 × 500 mg/d clarithromycin causes only an increase of the AUC by 1.19, which is considered not to be clinically relevant. The PK of the factor X inhibitors apixaban, edoxaban, and rivaroxaban are less affected by P-gp inhibition but are additionally

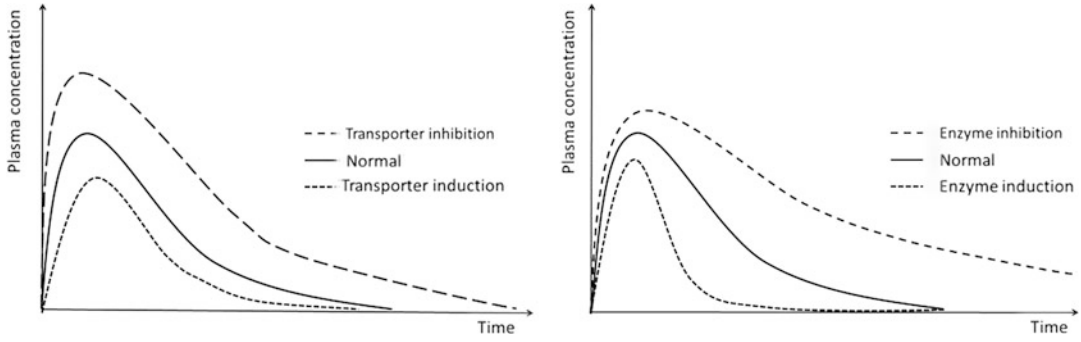
Drug Interactions, Table 2 Substrates of uptake and efflux transporters (SOLVO Biotechnology knowledge center)

OATP1B1/3	OAT1/3	OCT2, MATE-1, MATE-2 k	ABCB1 (P-gp)		ABCG2 (BCRP)
Statins	Antibiotics	Various	Opioids	Statins	Various
Atorvastatin	**Cefaclor**	Amantadine	Loperamide	Atorvastatin	**Rosuvastatin**
Pitavastatin	**Ceftizoxime**	Amiloride	Morphine	Lovastatin	Doxorubicin
Pravastatin	Cephaloridine	Berberine		Simvastatin	Imatinib
Simvastatin	Ciprofloxacin	Cimetidine	Anticoagulants	Rosuvastatin	**Sulfasalazine**
Rosuvastatin	**Penicillin**	Cisplatin	Apixaban		
		Debrisoquine	Edoxaban	Antineoplastic agents	Fluorescent dyes
Cardiac drugs	Antiviral drugs	Famotidine	**Dabigatran etexilate**	Docetaxel	Hoechst 3342
Bosentan	**Adefovir**	Guanidine	Rivaroxaban	Doxorubicin	BBR3390
Enalapril	Ganciclovir	Ifosfamide		Etoposide	
Olmesartan	**Oseltamivir**	Lamivudine	Cardiac drugs	Imatinib	
Telmisartan	Zidovudine	Memantine	Aliskiren	Irinotecan	
Temocaprilat		**Metformin**	**Digoxin**	Paclitaxel	
Valsartan	Various	Tubocurarine	Carvedilol	Seliciclib	
	Famotidine	Oxaliplatin	Celiprolol	Teniposide	
Various	**Furosemide**	Pancuronium	Talinolol	Topotecan	
Asunaprevir	**Methotrexate**	Picoplatin		Vinblastine	
Docetaxel	Pravastatin	Pindolol	Immunosuppressants	Vincristine	
Danoprevir		Procainamide	Ciclosporin		
Erythromycin		Propranolol	Tacrolimus	Various	
Fexofenadine		Ranitidine	Sirolimus	Berberine	
Glyburide		Zalcitabine		**Fexofenadine**	
Mycophenolic acid			HIV protease inhibitors	Quinidine	
Nateglinide			Indinavir		
Paclitaxel			Saquinavir	Fluorescent dyes	
Repaglinide				Rhodamine 123	
Tacrolimus					

Substrates in **bold** are recommend by the US Food and Drug Administration for clinical investigation of drug interactions during drug development (FDA 2020)

substrates of CYP3A4 (Voukalis et al. 2016). Typical examples of victim and perpetrator drug pairs on the level of intestinal distribution are exemplified for non-vitamin K-dependent anticoagulants in Table 4.

Hepatic uptake transporters facilitate the transport of anionic or cationic compounds from the portal veins into hepatocytes. Hence inhibition may cause a higher systemic exposure since hepatic metabolism or elimination into the biliary tract clearance may be decreased (Konig et al. 2013). As a consequence, C_{max}, AUC, but also the half-life may increase. A typical clinically relevant example of DDIs on the level of hepatic uptake transporters is the inhibition of OATP1B1 (SLCO1B1) by the anti-infective macrolide clarithromycin causing drastic elevation of the systemic exposure of the cholesterol-lowering drug simvastatin (Hougaard Christensen et al. 2020). In particular at higher doses, this interaction may cause side effects such as myopathy. Again, overlap with cytochrome P450 enzymes



Drug Interactions, Fig. 1 Consequences of drug-drug interactions for the pharmacokinetics on the level of drug transporters (left) and drug-metabolizing enzymes (right) as exemplified for an intestinal P-gp substrate (e.g., dabigatran-etexilate) and a cytochrome P450 3A4 substrate (e.g., cyclosporine)

Drug Interactions, Table 3 Inhibitors for drug uptake and efflux transporters

OATP1B1/3	OAT1/3	OCT2	MATE1, MATE-2 K	ABCB1 (P-gp)	ABCG2 (BCRP)
Clarithromycin	*Cyclosporine*	*Cimetidine*	Cimetidine	Amiodarone	Curcumin
Cyclosporine	Probenecid	*Cetirizine*	Dolutegravir	Carvedilol	Cyclosporin
Erythromycin	*Rifamycin*	*Naringin*	Isavuconazole	Clarithromycin	Eltrombopag
Fusidic acid	*Rifampicin*	*Pilsicainide*	Ranolazine	*Cyclosporine*	*Febuxostat*
Gemfibrozil	*Ritonavir*	*Quinidine*	Trimethoprim	Dronedarone	*Gefitinib*
Indinavir	Teriflunomide	*Rifampicin*	Vandetanib	*Erythromycin*	*Imatinib*
Rifampicin		*Ritonavir*		Itraconazole	*Omeprazole*
Rifamycin				Ketoconazole	*Ritonavir*
Ritonavir				*Lapatinib*	
Roxithromycin				Lopinavir	*FD 12–9*
Saquinavir				*Propafenone*	*Ko132*
Simeprevir				*Quinidine*	*Ko134*
Telithromycin				*Ranolazine*	*Ko143*
				Ritonavir	
				Tacrolimus	
				Telaprevir	
				Verapamil	
				Elacridar (GF120918)	
				FD 12–9	
				LY335979	
				Valspodar (PSC833)	

Drugs in roman characters: inhibitors listed by the FDA (FDA 2020). Criteria for P-gp: (1) AUC fold-increase of digoxin $\geq$2 with co-administration and (2) in vitro inhibitor. BCRP: (1) AUC fold-increase of sulfasalazine $\geq$1.5 with co-administration and (2) in vitro inhibitor. OATP1B1/OATP1B3: (1) AUC fold-increase $\geq$2 for at least one of clinical substrates in Tables 2 and 3 with co-administration and (2) in vitro inhibitor. OAT1/OAT3: (1) AUC fold-increase $\geq$1.5 for at least one of the clinical substrates in Tables 2 and 3 with co-administration and (2) in vitro inhibitor. OCT2/MATE: (1) AUC fold-increase of metformin $\geq$1.5 with co-administration and (2) in vitro inhibitor
Drugs in *italic* characters: additional inhibitors listed by (SOLVO Biotechnology knowledge center)

Drug Interactions, Table 4 Interactions of non-vitamin K-dependent oral anticoagulants (NOACs) mediated by the efflux transporter P-gp (ABCB1). (Adapted from Gessner et al. 2019)

Oral anticoagulant class	Oral anticoagulant	Perpetrator drug	PK change of victim drug	Possible clinical consequence
FII-NOAC	Dabigatran etexilate	Dronedarone	AUCa ↑ (2.1-fold)	Elevated bleeding risk
FII-NOAC	Dabigatran etexilate	Ketoconazole	AUCa ↑ (2.4-fold	Elevated bleeding risk
FII-NOAC	Dabigatran etexilate	Rifampicin	AUCa ↓ (−67%)	Elevated thrombosis risk
FX-NOAC	Apixaban	Ketoconazole	AUC ↑ (twofold)	Elevated bleeding risk
FX-NOAC	Apixaban	Rifampicin	AUC ↓ (−54%)	Elevated thrombosis risk
FX-NOAC	Edoxaban	Erythromycin	AUC ↑ (+85%)	Elevated bleeding risk
FX-NOAC	Edoxaban	Cyclosporin	AUC ↑(+73%)	Elevated bleeding risk
FX-NOAC	Rivaroxaban	Ritonavir	AUC ↑ (2.5-fold)	Elevated bleeding risk

FII-NOAC: coagulation factor II (thromboxane) inhibitor, FX-NOAC: coagulation factor X inhibitor

has to be considered since simvastatin is a CYP3A4 substrate as well and clarithromycin a CYP3A4 inhibitor. Further examples of typical victim and perpetrator drugs and their clinical consequences on the level of hepatic drug transporters are shown in Table 5.

As excellently reviewed by Gessner et al. (2019) and Ivanyuk et al. (2017), renal transporters are also involved in a significant number of drug-drug interactions. In the kidney, the competitive inhibition of transporters contributing to the secretion of drugs may lead to accumulation of the victim as exemplified for penicillin and probenecid that is a known OCT inhibitor. Whereas the penicillin/probenecid interaction was considered as beneficial in earlier years, the simultaneous administration of methotrexate and an OCT inhibitor may cause life-threatening toxicity (Titier et al. 2002). The antidiabetic drug metformin is subject to renal excretion. Hence monitoring of the kidney function is of importance to avoid side severe effects such as lactacidosis, but also drug interactions on the level of renal transporters may lead to accumulation. For example, co-administration of cimetidine leads to a 1.5-fold increase and of dolutegravir (an HIV integrase inhibitor) to a 2.5-fold increase of metformin AUC (Gessner et al. 2019). These interactions are due to inhibition of OCT2, MATE1, and MATE-2K (for more examples, see Table 6).

Pharmacokinetic Interactions on the Level of Metabolism

Drug-drug interactions caused by competitive inhibition or induction of drug-metabolizing enzymes are best investigated and categorized. Broad knowledge on the metabolic pathways of frequently prescribed drug classes as well as of specific inhibitors and inducers of the various cytochrome P450 enzymes, further phase 1 as well as phase 2 enzymes, has contributed already a lot for drug safety and lowering of unintended overdosing or lack of efficacy (Cascorbi 2012). However, there is still need of education and further development and implementation of easy to use clinical decision support system when prescribing drugs in particular to multi-morbid patients (Tolley et al. 2018).

Likewise, for drug transporters each product description of drugs contains comprehensive data on elimination pathways and putative interactions with other drugs. Hence knowledge has to be gained already during preclinical drug development and to be confirmed in clinical phases. Hereby, physiologically based pharmacokinetic modeling (PBPK) plays a major role to allow prediction of possible interactions (Min and Bae 2017). Despite renal or biliary excretion, metabolism in the liver is the predominant elimination pathway. The phase I metabolism is primarily

Drug Interactions, Table 5 Interactions of statins mediated by the hepatic organic anion uptake transporters OATP1B1, 1B3, and 2B1. (Adapted from Gessner et al. 2019)

Statin	Perpetrator drug	PK change of victim drug	Possible clinical consequence
Pitavastatin	Clarithromycin	AUC ↑ (4.6-fold)	Elevated risk of myopathy
Pravastatin	Cyclosporin	AUC ↑ (twofold)	Elevated risk of myopathy
Rosuvastatin	Cyclosporin	AUC ↑ (7.1-fold)	Elevated risk of myopathy
Rosuvastatin	Gemfibrozil	AUC ↑ (1.9-fold)	Elevated risk of myopathy
Rosuvastatin, pitavastatin	Rifampicin	AUC ↑ (5.7-fold, 4.4-fold)	Elevated risk of myopathy

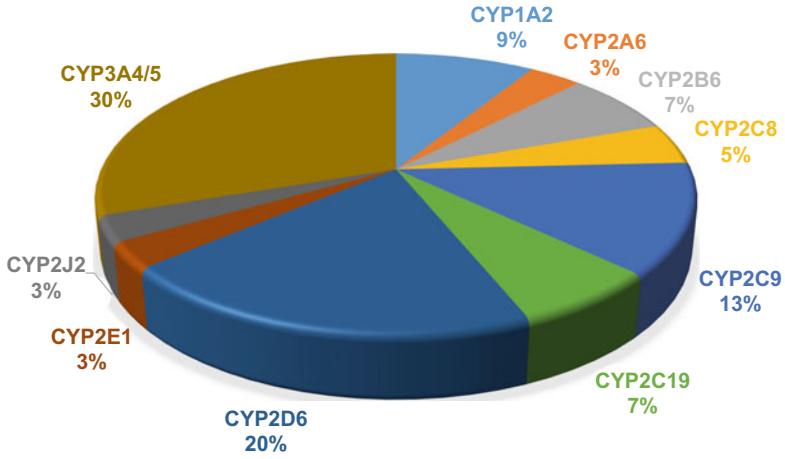
Drug Interactions, Table 6 Interactions of selected drugs mediated preferentially by the renal drug transporters adapted from Gessner et al. (2019)

Victim drug	Perpetrator drug	Transporter	PK change of victim drug	Possible clinical consequence
Metformin	Cimetidine	OCT2, MATE1, MATE2-K	AUC ↑ (1.5-fold) Cl_R ↓ (27%)	Not known
Metformin	Pyrimethamine	MATE1, MATE2-K	C_{max} ↑ (1.4-fold) AUC ↑ (1.4-fold) Cl_R ↓ (−33% to −35%)	Not known
Metformin	Trimethoprim	OCT2, MATE1, MATE2-K	C_{max} ↑ (1.2-fold to 1.4-fold) AUC ↑ (1.2-fold to 1.3-fold) Cl_R ↓ (−26%)	Not known
Metformin	Dolutegravir	OCT2, MATE1, MATE2-K	C_{max} ↑ (2.1-fold) AUC ↑ (2.5-fold)	Not known
Lamivudine	Trimethoprim/ sulfamethoxazole	OCT2, MATE1, MATE2-K	AUC ↑ (1.4-fold) Cl_R ↓ (−35%)	Not considered as clinically relevant
Chloroquine	Cimetidine	MATE1	Cl_0 ↓ (−53%) Ae ↓ (−43%)	Not known
Furosemide	Probenecid	OAT1, OAT3	Cl_R ↓ (−78%) Cl_0 ↓ (−63%)	Decreased efficacy

mediated by cytochrome P450 enzymes. As can be seen from Fig. 2, CYP3A4 and CYP3A5 are the most important ones (Zanger and Schwab 2013). About 30% of all metabolized drugs are subject to this class of oxidative enzymes. Accordingly, the majority of drugs is metabolized at least partly by these enzymes, and many have a low Ki acting as competitive inhibitors. Table 7 lists substrates of the major cytochrome P450.

It should be noted that drugs metabolized by one of these enzymes having a narrow therapeutic index are most prone to DDI. Typical examples are immunosuppressants like cyclosporine that is metabolized predominantly by CYP3A4 as well as tacrolimus and sirolimus that are metabolized predominantly by CYP3A5. The metabolism of all three drugs can by strongly affected by concomitantly administration of CYP3A4/5 inhibitors such as macrolides of azole antimycotics. Therefore, such drugs should be avoided, or dose reduction according to the actual plasma concentrations should be performed.

As noted before, simvastatin is strongly metabolized by CYP3A4. Strong inhibition bears the risk of up to 13-fold increase of the AUC leading to severe side effects such as rhabdomyolysis (Damiani et al. 2020). Such a deleterious interaction was observed when simvastatin was administered together with CYP3A4 inhibitors (Hodel 2002). Due to such severe interactions occurring



Drug Interactions, Fig. 2 Proportion (%) of hepatic cytochrome P450 enzymes to the number of drugs, undergoing phase I metabolism. (Adapted from Zanger and Schwab 2013)

in the course of concomitant treatment with simvastatin, mibefradil, a calcium T-channel blocker used in cardiovascular diseases, was withdrawn from the market in 1998.

Cerivastatin, a statin frequently earlier prescribed globally, was considered as safe due its known metabolism pathway. However, parallel use with the fibrate gemfibrozil caused a surprisingly high rate of rhabdomyolyses later known to be based on competitions on a metabolic pathway (CYP2C8) and on an uptake transporter (OATP1B1), leading to high systemic exposure. The comparably higher rate of severe side effects led to the withdrawal of cerivastatin by the manufacturer in 2001 (Jamal et al. 2004).

Despite approved drugs, some ingredients of natural products may also cause severe interaction. Best known and frequently reported also in lay press is grapefruit that contains considerable contents of the flavonoid naringin, a potent inhibitor of CYP3A4. As demonstrated in a clinical study in healthy volunteers, just one glass of grapefruit juice increased significantly the oral bioavailability of midazolam, a drug used as a probe drug for CYP3A4 (Greenblatt et al. 2003). The effect could be observed until 3 days of intake indicating an irreversible non-competitive effect of naringin. It should be noted however that other citrus fruits like lemons or oranges contain much lower amounts of naringin, causing no or very mild interactions.

Inhibition of metabolic pathways however can also lead to lack of efficacy in cases when the activation of a prodrug to the active moiety is affected. A typical example is the activation of the P2Y12 ADP receptor clopidogrel by CYP2C19. Inhibition by proton pump inhibitor omeprazole may lead to decreased platelet aggregation with a higher risk thrombosis risk in cardiovascular patients undergoing PCI or suffering from peripheral occlusive disease (Ho et al. 2009). Further, the activation of codeine to morphine through CYP2D6 can be inhibited by the antidepressant fluoxetine (Yee et al. 2014) leading to lack of analgesic efficacy.

Further groups of substrates and inhibitors are listed in Tables 7 and 8, respectively.

Induction of Transport and Metabolism

The above entries on drug transport and metabolism focused on inhibition of pathways leading in most cases to a higher risk of adverse drug events. However, induction of transporters and metabolic enzymes by selected drugs and natural products is a considerable threat to effective medication. Originally, induction is an evolutionary defense mechanism leading to accelerated extrusion or metabolic detoxification of xenobiotics. In humans, two important members of the NR1I nuclear family, the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR), function as sensors

Drug Interactions, Table 7 Interactions on the level of cytochrome P450 enzymes: selection of relevant substrates for which combinations with inhibitors or inducers of the same enzyme lead to increased occurrence of adverse or weakened effects. (Adapted to Flockhart 2007; Cascorbi 2012; FDA 2020). [a]Prodrug

CYP1A2	CYP2B6	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP2E1	CYP3A4/5	
Antidepressants	Various	Various	Antidepressants	Antidepressants	Antidepressants	Volatile anesthetics	Anticoagulants	Anticancer agents
Amitriptyline	Artemisinin	Amodiaquine	Amitriptyline	Amitriptyline	Amitriptyline	Enflurane	Apixaban	Abemaciclib
Clomipramine	Bupropion	Cerivastatin	Doxepin	Citalopram	Clomipramine	Ethanol	Edoxaban	Acalabrutinib
Doxepin	Cyclophosphamide	Dabrafenib	Fluoxetine	Clomipramine	Desipramine	Halothane	Rivaroxaban	Alectinib
Duloxetine	Efavirenz	Enzalutamide	Venlafaxine	Doxepin	Doxepin	Isoflurane	Phenprocoumon	Brigatinib
Fluvoxamine	Ifosfamide	Olodaterol		Escitalopram	Duloxetine	Methoxyflurane		Cobimetinib
Imipramine	Ketamine	Paclitaxel	**Antidiabetics**	Imipramine	Escitalopram	Sevoflurane	**Benzodiazepines**	Copanlisib
	Meperidine	Ponatinib	Glibenclamide	Moclobemide	Fluoxetine		Alprazolam	Docetaxel
Various	Methadone	Repaglinide	Glimepiride		Fluvoxamine	**Various**	Diazepam	Ibrutinib
Acetaminophen	Nevirapine	Selexipag	Glipizide	**PPIs**	Imipramine	Acetaminophen	Midazolam	Idelalisib
Apremilast	Propofol	Sorafenib	Glyburide	Esomeprazole	Minaprine	Chlorzoxazone	Triazolam	Imatinib
Caffeine	Selegiline	Torsemide	Nateglinide	Lansoprazole	Nortriptyline	Theophylline		Irinotecan
Clozapine	Sorafenib		Rosiglitazone	Omeprazole	Paroxetine		**Antidepressants**	Lenvatinib
Mexiletine	Tramadol		Tolbutamide	Pantoprazole	Venlafaxine		Amitriptyline	Neratinib
Naproxen	Velpatasvir						Citalopram	Olaparib
Olanzapine			**NSAIDs**	**Various**	**Antipsychotics**		Doxepin	Osimertinib
Ondansetron			Celecoxib	Atomoxetine	Aripiprazole		Escitalopram	Palbociclib
Tacrine			Diclofenac	Brivaracetam	Brexpiprazole		Trazodone	Panobinostat
Theophylline			Ibuprofen	Carisoprodol	Cariprazine		Venlafaxine	Regorafenib
Tizanidine			Lornoxicam	Chloramphenicol	Chlorpheniramine			Ribociclib
Triamterene			Meloxicam	Clopidogrel	Chlorpromazine		**Antipsychotics**	Rolapitant
Verapamil			Piroxicam	Cyclophosphamide	Dexfenfluramine		Aripiprazole	Romidepsin
Warfarin			Naproxen	Diazepam	Haloperidol		Chlorpheniramine	Sonidegib
Zileutin				Flibanserin	Perphenazine		Domperidone	Sorafenib
Zolmitriptan			**Various**	Hexobarbital	Pimavanserin		Haloperidol	Sunitinib
			Capecitabine	Indomethacin	Promethazine		Pimavanserin	Tamoxifen
			Clopidogrel	Labetalol	Risperidone		Pimozide	Taxol
			Fluvastatin	Nelfinavir	Thioridazine		Quetiapine	Torisel
			Irbesartan	Nilutamide	Zuclopenthixol		Risperidone	Vemurafenib
			Lesinurad	Ospemifene			Ziprasidone	Venetoclax

			Losartan	Phenobarbitone	**Antiarrhythmics**			Vincristine
			Olodaterol	Phenytoin	Encainide		**Antiviral drugs**	
			Ospemifene	Pomalidomide	Flecainide		Indinavir	**Steroids**
			Phenytoin	Primidone	Lidocaine		Daclatasvir	Estradiol
			Warfarin	Progesterone	Mexiletine		Efavirenz	Ethinylestradiol
			Tamoxifen	Proguanil	Propafenone		Nelfinavir	Dexamethasone
			Torsemide	Propranolol	Sparteine		Nevirapine	Hydrocortisone
			Valproic acid	r-Mephobarbital			Ritonavir	Progesterone
			Voriconazole	s-Mephenytoin	**Beta blockers**		Saquinavir	Testosterone
			Zafirlukast	Suvorexant	Alprenolol		Telaprevir	
				Teniposide	Bufuralol		Boceprevir	**Various**
				Tofacitinib	Carvedilol		Elbasvir/ grazoprevir	Buspirone
				Voriconazole	Metoprolol		Velpatasvir	Aprepitant
					Nebivolol			Astemizole
					Propranolol		**Calcium channel blockers**	Brexpiprazole
					Timolol		Amlodipine	Cafergot
							Diltiazem	Carbamazepine
					Opioids		Felodipine	Cariprazine
					Codeine[a]		Lercanidipine	Cilostazol
					Dextromethorphan		Nifedipine	Clopidogrel
					Tramadol[a]		Nisoldipine	Dapsone
							Nitrendipine	Deflazacort
					Various		Verapamil	Eliglustat
					Amphetamine			Eplerenone
					Atomoxetine		**Immuno-suppressants**	Esomeprazole
					Clonidine		Ciclosporin	Finasteride
					Debrisoquine		Sirolimus	Flibanserin
					Deutetrabenazine		Tacrolimus	Isavuconazonium sulfate
					Donepezil			Ivabradine
					Eliglustat			Lansoprazole

(continued)

Drug Interactions, Table 7 (continued)

CYP1A2	CYP2B6	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP2E1	CYP3A4/5	
Antidepressants	Various	Various	Antidepressants	Antidepressants	Antidepressants	Volatile anesthetics	Anticoagulants	Anticancer agents
							Macrolide antibiotics	
					Ibrutinib		Clarithromycin	Lidocaine
					Letermovir		Erythromycin	Nateglinide
					Methoxyamphetamine		Telithromycin	Netupitant/ Palonosetron
					Metoclopramide			Omeprazole
					Minaprine		**Opioids**	Ondansetron
					Netupitant/ palonosetron		Alfentanil	Pantoprazole
					Ondansetron		Codeine	Propranolol
					Phenacetin		Fentanyl	Quinidine
					Phenformin		Methadone	Quinine
					Pomalidomide		Naldemedine	Salmeterol
					Ponatinib		Naloxegol	Selexipag
					Rucaparib		Tramadol	Sildenafil
					Tamoxifen			Suvorexant
					Umeclidinium		**Statins**	Valbenazine
					Valbenazine		Atorvastatin	Voriconazole
					Zuclopenthixol		Lovastatin	Zaleplon
							Simvastatin	Zolpidem

Drug Interactions, Table 8 Inhibitors of major cytochrome P450 enzymes: strength of inhibition is indicated in red (strong: increment of the AUC of a sensitive index P450 substrate ≥5-fold), orange (moderate: increment of the AUC of a sensitive index P450 substrate by ≥2- to <5-fold), and yellow (weak: increment of the AUC of a sensitive index P450 substrate by ≥1.25- to <2-fold) according to (FDA 2020). Additional drugs in *italic* are derived from Flockhart (2007)

CYP1A2	CYP2B6	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP2E1	CYP3A4/5
Aciclovir	Clopidogrel	Clopidogrel	Amiodaron	*Chloramphenicol*	Abiraterone	*Disulfiram*	*Amiodarone*
Allopurinol	*Rucaparib*	Deferasirox	*Belinostat*	*Cimetidine*	Amiodaron	*Ribociclib*	Aprepitant
Amiodarone	Tenofovir	Gemfibrozil	*Capecitabine*	*Citalopram*	Buproprion		*Atomoxetine*
Cimetidin	*Thiotepa*	*Glitazones*	*Clopidogrel*	*Esomeprazole*	Celecoxib		Boceprevir
Ciprofloxacin	Ticlopidine	*Letermovir*	Disosmin	Felbamate	*Chlorpromazine*		*Chloramphenicol*
Citalopram	Voriconazole	*Montelukast*	Disulfiram	Fluconazole	*Chlorpheniramine*		Chlorzoxazone
Efavirenz		*Perampanel*	*Efavirenz*	Fluoxetine	Cimetidine		Cilostazol
Enoxacine		*Quercetin*	*Fenofibrate*	Fluvoxamine	Cinacalcet		Cimetidine
Fluvoxamine		Teriflunomide	Fluconazole	*Indomethacin*	*Citalopram*		Ciprofloxacin
Furafylline		Trimethoprim	Fluvastatin	*Isoniazid*	*Clemastine*		Clarithromycin
Interferon beta			Fluvoxamine	*Ketoconazole*	Clobazam		Clotrimazol
Methosalen			*Isoniazid*	*Lansoprazole*	Cobicistat		Cobicistat
Melixetin			*Lovastatin*	*Luliconazole*	*Clomipramine*		Conivaptan
Mibefradil			*Metronidazole*	*Modafinil*	*Diphenhydramine*		Crizotinib
Ofloxacin			Miconazole	Omeprazole	*Doxepine*		Cyclosporine
Levofloxacin			*Orivatancin*	*Oritavancin*	*Dororubicin*		*Delaviridine*
peginterferon alpha-2a			*Paroxetine*	*Oxcarbazepine*	Duloxetine		Diltiazem
Piperidine			*Phenylbutazone*	*Pantoprazole*	Escitalopram		Dronedarone
Ribociclib			Piperidine	*Probenicid*	Fluoxetine		Erythromycin
Rucaparib			*Probenizid*	*Rucaparib*	Fluvoxamine		*Esomeprazole*
Simeprevir			Voriconazole	Ticlopidine	*Halofantrine*		Fluconazol
Ticlopidine				*Topiramate*	*Haloperidol*		Fluvoxamine
Zileutin				Voriconazole	*Hydroxyzine*		Fosaprepitant
					Labetalol		*Gestodene*
					Levomepromazine		Grapefruit juice (Naringin)
					Lorcaserin		Idelalisib
					Methadone		Imatinib
					Metoclopramide		*Indinavir*
					Mibefradil		Istradefylline
					Midodrine		Itraconazol
					Mirabegron		Ivacaftor
					Moclobemide		Ketoconazol
					Panobinostat		*Lesinurad*
					Paroxetine		Lopitamide
					Perphenazine		*Mibefradil*
					Promethazine		*Mifepristone*
					Quinidine		Nefazodone
					Ranitidine		Nelfinavir
					Ritonavir		*Netopitant/Palonosetron*
					Rolapitant		*Norfloxacin*
					Rucaparib		*Norfluoxetine*
					Sertraline		*Omeprazol*
					Terbinafine		*Pantoprazol*
					Ticlopidine		Posaconazole
					Verumafenib		Ranitidine
							Ranolazine
							Regorafenib
							Rigociclib
							Ritonavir
							Saquinavir
							Telaprivir
							Telithromycin
							Ticagrelor
							Tofisopam
							Troleandromycin
							Verapamil
							Voriconazole

of toxic byproducts of endogenous metabolism and of exogenous chemicals and enhance their elimination (Wang et al. 2012). There is current knowledge that PXR is involved in the regulation of more than 500 genes; it acts in upregulation but also in the downregulation of the expression in primary human hepatocytes (Kandel et al. 2016).

After activation by a ligand such as rifampicin, PXR forms a heterodimer with 9-cis retinoic acid receptor RXR and binds to a hormone-responsive

Drug Interactions, Table 9 Inducers of major cytochrome P450 enzymes. Colored roman drugs: red, strong inducers (≥80%); orange, moderate inducers (≥50% to <80%); yellow, weak inducers (≥20% to <50%) that decreases the AUC of sensitive index substrates of a given metabolic pathway according to FDA (FDA 2020). Additional drugs in *italic* are derived from Flockhart (2007)

CYP1A2	CYP2B6	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP2E1	CYP3A4/5
Carbamazepine	*Artemisinin*	Rifampicin	Apalutamide	Apalutamide		*Ethanol*	Armodafinil
Insulin	Carbamazepine		Aprepitant	*Carbamazepine*		*Isoniazid*	Apalutamide
Modafinil	*Dabrafenib*		Carbamazepine	Efavirenz			Bosentan
Nafcillin	Efavirenz		Dabrafenib	Enzalutamide			*Brigatinib**
Omeprazole	*Letermovir*		Enzalutamide	*Letermovir*			Carbamazepine
Phenytoin	Nerivapine		*Letermovir*	*Norethindrone*			Efavirenz
Rifampicin	*Perampanel*		*Nevirapine*	*Prednisone*			Enzalutamide
Ritonavir	*Phenobarbital*		*Phenobarbital*	Phenytoin			Etravirine
Rucaparib	*Phenytoin*		Rifampicin	Rifampicin			*Glucocorticoids*
Teriflunomide	Rifampicin		Ritonavir	Ritonavir			Mitotane
	Ritonavir		*Secobarbitatl*	*St. John's wort (Hypericin)*			Modafinil
Aromatic hydrocarbons			*St. John's wort (Hypericin)*				*Nevirapine*
							Oxacarbazepine
Broccoli							Phenobarbital
Brussel sprouts							Phenytoin
							Primidone
							Rifabutin
							Rifampicin
							Rufinamide
							St. John's wort (Hypericin)
							Troglitazone

(also phenobarbital-responsive) element of the respective DNA (details are reviewed by (Pavek 2016). For drug metabolism and transport, most important is the induction of CYP3A4/5 and P-gp, but CYP2B6, CYP2C8, CYP2C9, and CYP2C19 as well as BCRP and some uptake transporters such as OATP2 are upregulated as well. CYP2D6 expression is not affected by PXR.

Induction of the elimination pathways may lead to ineffective drug concentrations of a large number of victim drugs of P450 enzymes and transporters. Hence knowledge about PXR ligands is crucial. Typical inducers are the old anticonvulsants phenobarbital, phenytoin, and carbamazepine, the tuberculostatic drug rifampicin, and hypericin, a constituent of the herbal drug St. John's wort. Later on, additional drugs were identified such as the anti-HI-viral drug efavirenz and the androgen receptor antagonists used in prostate cancer enzalutamide and apalutamide (see Table 9).

The induction of gene expression takes place after 1–2 days, and stable induction can be observed after repeated intake of, e.g., 600 mg rifampicin over 1 week. To classify an investigational drug as inducer in vitro, several methods have been established, e.g., determination of the fold-change of mRNA levels (≥2-fold and concentration-dependent changes or <2-fold and ≥20% of positive control) and correlation methods by calculating relative induction scores (Iwatsubo 2020). Distinction on a clinical level is made into strong, moderate, and weak inducers that decrease the AUC of sensitive index substrates of a given metabolic pathway by ≥80%, ≥50% to <80%, and ≥20% to <50%, respectively (FDA 2020).

Interactions between inducers and victim drugs may lead to life-threatening events. For example, the biodistribution of the immunosuppressants cyclosporine, tacrolimus, and sirolimus can be considerably reduced by co-administration of CYP3A4 inducers leading to acute transplant rejection. This can be even the case with self-administered herbal drugs as shown by St. John's wort in patients who had undergone heart transplantation (Ruschitzka et al. 2000). Later on it was demonstrated in clinical studies in healthy volunteers that the PK of drugs like digoxin (Johne et al. 1999) or amitriptyline (Johne et al. 2002) is significantly affected by the intake of St. John's wort. Attention should be also taken to other CYP3A4 and P-gp substrates such as the oral anticoagulants phenprocoumon or warfarin (Barnes et al. 2001), possibly also to NOACs.

Less strong are the effects of extracts of the herbal plant *Echinacea purpurea* (Awortwe et al. 2019); further clinical evidence of this frequent over-the-counter drug is required.

In addition, the aromatic hydrocarbon receptor (AhR) factor acts in a similar way by inducing enzymes involved in oxidative pathway of

aromatic xenobiotics and some drugs. More recently, also a number of endogenous ligands were identified (Kawajiri and Fujii-Kuriyama 2017). Some drugs like omeprazole, but also cigarette smoke or dietary plants like broccoli, may induce CYP1A2 leading to lower levels of theophylline or fluvoxamine (Tables 7 and 9).

Conclusions

Drug-drug interactions contribute to adverse events and consequently to global health burden; hence knowledge on the interaction potential of drugs currently on the market is required to avoid unintended adverse events by the choice of the right drug and the right dose for the right patient. However, with increasing polypharmacy in an increasing proportion of elderly people, the ideal combination of drugs is a challenge that often requires clinical decision support. In addition, pharmacogenetic traits contribute further to inter-individual variation of drug response. Although information on potential interaction is given comprehensively in the drug label, its relevance is often unclear and requires further explanation. Hence certain software approaches have been implemented in electronic prescription systems assisting physicians while prescribing medications. However, even the best programs can't work efficiently if not all information on patient's medications is available; moreover multiple pairs of drugs are still difficult to assess. Furthermore, multiple (and sometime false) alarms may lead to an attenuation of alertness. Hence both education on major drug-drug interactions and help from clinical decision support systems based on reliable data sources are required to further improve drug safety.

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Drug Receptor Theory

▶ Drug–Receptor Interaction

Drug Screening

▶ Small-Molecule Screens

Drug Targets

▶ Ca^{2+}-Binding Proteins
▶ S100 Proteins

Drug-Drug Interactions

▶ Drug Interactions

Drug–Receptor Interaction

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Synonyms

Drug receptor theory; Quantification of drug effect

Definition

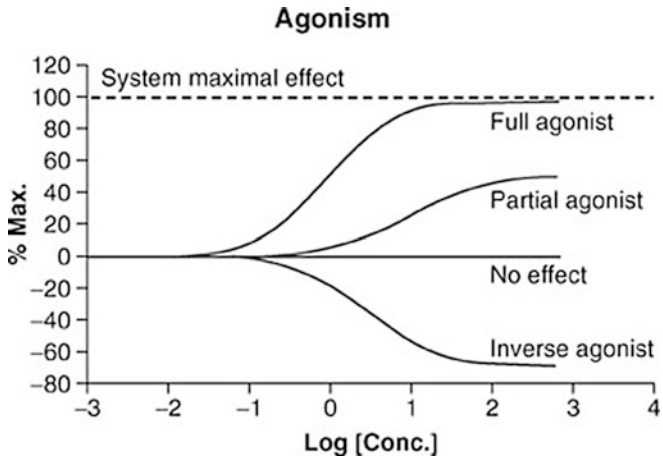
Mathematical models of the interaction between drugs and receptors, based on Michaelis–Menten kinetics, are utilized to create the quantitative tools currently used in receptor pharmacology to quantify drug effect in biological systems. Such tools are necessary since drugs almost always are tested in surrogate systems until they are known to be sufficiently active and safe for therapeutic use. The aim of receptor pharmacology is to define the molecular properties of affinity and intrinsic efficacy of drugs; these can be used to predict drug effect across different biological systems. It will be seen that the properties of affinity and efficacy are sufficient to describe the case of the interaction of a single molecule, target receptor, and cell. For an extended system of two interacting molecules (e.g., agonist and antagonist), target receptor and cell, it is useful to know additional information, namely knowledge as to whether the interaction is orthosteric or allosteric, and (optionally) the kinetics of interaction of the molecules with the target (target residence time) in order to fully characterize drug activity.

Basic Mechanisms

A basic premise in receptor pharmacology is that all drugs have affinity for receptors (the chemical property that unites the drug with the receptor), and some drugs have efficacy, the chemical property that causes the receptor to change its behavior toward its host cell. Drugs that have efficacy can produce concentration-dependent responses in physiological systems, characterized by a concentration–response curve (also often referred to as a dose–response curve).

Drugs that produce pharmacological activation of a system are called *agonists*, those that inhibit activation of a receptor system are called *antagonists*, and those that reverse spontaneously active receptor systems (Constitutive receptor activity) are called *inverse agonists* (Fig. 1). This latter class of drugs reduce elevated basal responses. Agonism is the observed effect of a ligand-producing stimulus to a receptor. The host cellular system processes that stimulus and yields an observable response.

The common currency of drug receptor pharmacology is the dose–response curve, as it defines the relationship between concentrations of drug and the resulting effect. Dose–response curves have three basic properties with which they can be described: threshold abscissal value, slope, and maximum asymptote. The location parameter of the dose–response characterizes drug potency; most often this is quantified as the EC_{50} or the molar concentration of drug producing half the maximal effect to that drug. Given tissue systems have maximal capabilities to return drug response. When an agonist produces a maximal response that is equal to the system maximal response it is referred to as a *full agonist* (Fig. 1). If an agonist produces a submaximal system response it is called a *partial agonist* (Fig. 1). While the potency of an agonist is quantified by the location



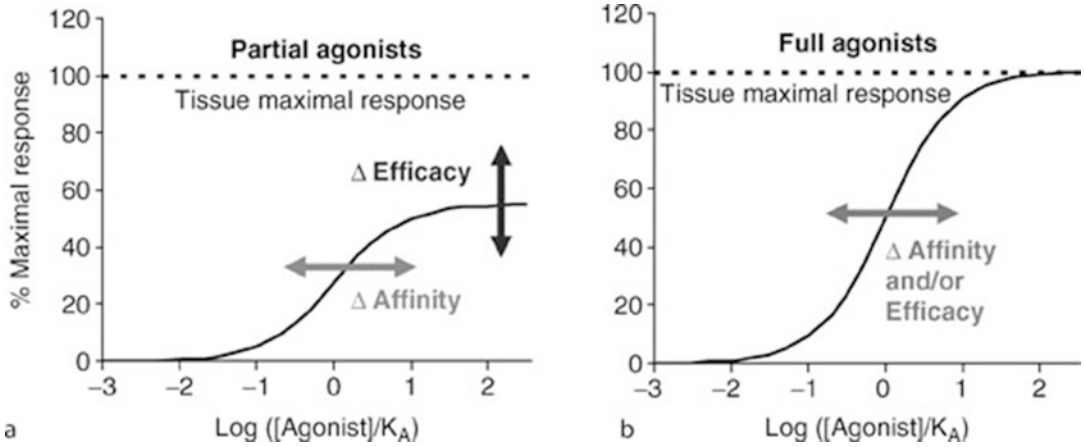
Drug–Receptor Interaction, Fig. 1 Dose–response curves to drugs that have direct effects on physiological systems. A drug that produces the maximal effect capable from the system is a *full agonist*. A submaximal effect is produced by a *partial agonist*. Drugs may not produce directly observable effects but may be present on the receptor to produce antagonism (see Fig. 3). If a system is constitutively active and shows an actively elevated basal response, then a drug that reduces this is termed an *inverse agonist*

parameter of the dose–response curve (EC_{50}), a reflection of (but not a direct measure of) the intrinsic efficacy of an agonist is given by its maximal response.

The term efficacy for this discussion is defined as the change in behavior of the target (receptor) towards its host (the cell) produced by the binding of a ligand. In this sense there are many efficacies possible for receptors including production of second messengers in cells, internalization from the cell surface, internalization with subsequent coupling to response elements, etc. It is important to note that both the quality and quantity of biological response obtained with a given drug depends very much on the assay system used to make the measurement. If the assay does not have the means to detect a given efficacy, then none will be observed; this should not be taken to mean that the drug does not have that particular efficacy. A common case in point is the failure of nonconsitutive receptor systems to detect negative efficacy (inverse agonism). For this particular efficacy to be detected, an especially engineered assay must be used. Similar effects are observed in the receptor-mediated signaling effects. While most agonist-bound receptors activate G-proteins, some initiate the binding of intracellular proteins such as beta-arrestin to induce kinase signaling. There are cases, such as for the β-adrenoceptor antagonist ICI 118,551, where the same drug will have a negative efficacy for one response system (i.e., G-proteins) and positive efficacy for another (beta-arrestin kinase stimulation).

In general, if a drug generates a direct response in a system, and if that response is lower in magnitude than the system maximal response (i.e., it is a partial agonist, see Fig. 1), then the location of the concentration–response curve along the concentration axis is determined by the affinity of the agonist and the maximal response is determined by the magnitude of the efficacy (Fig. 2a). If the agonist produces the full system maximal response (it is a full agonist), then it is not possible to assess efficacy (since the maximal response is constant) and the location of the concentration–response curve is determined by a complex function of both affinity and efficacy (Fig. 2b).

The inhibition of agonist response is termed antagonism. The effect that a given antagonist has on the dose–response curve to an agonist can be a clue to the mechanism of action of that antagonists' interaction with the receptor. In the presence of an antagonist, more agonist must be present in the receptor compartment to produce a response that would be necessary in the absence of the antagonist. A singular characteristic of an



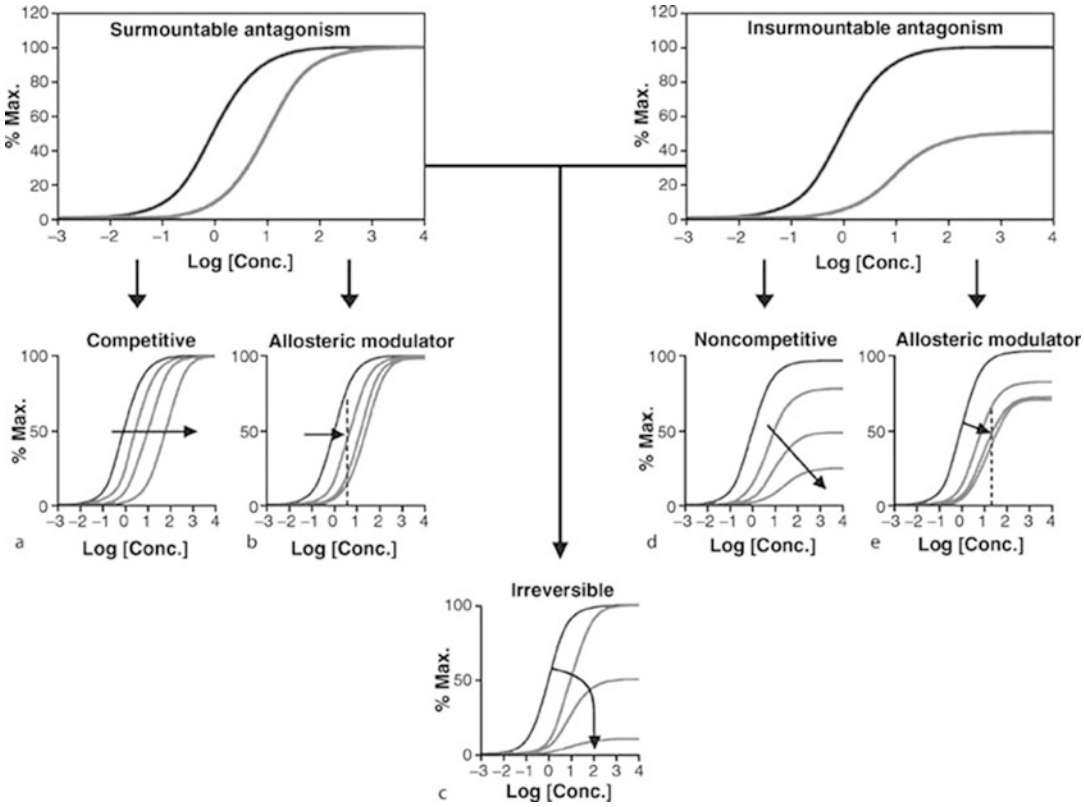
Drug–Receptor Interaction, Fig. 2 Relationships between affinity and efficacy with different agonist response patterns. (**a**) For partial agonists, differences in maximal responses between agonists relate to differences in efficacy. Differences in the location parameter of the concentration–response curve (potency) indicate differences in affinity. (**b**) For full agonists, differences in potency indicate differences in either affinity, efficacy, or both

antagonist is its effect on the maximal capability of an agonist to overcome the presence of the antagonist and produce the system maximal response. If enough agonist can be added to produce the agonist maximal response in the presence of the antagonist, the antagonism is referred to as surmountable antagonism (see Fig. 3). If no amount of agonist will produce the maximal response, the antagonism is referred to as insurmountable (see Fig. 3).

A basic concept in receptor pharmacology is the idea of orthosteric and allosteric interaction. Orthosteric interaction occurs when two molecules compete for a single binding domain on the receptor. With allosteric interactions two molecules each have their own binding domain on the receptor and the two interact through effects on the protein (conformational change). Thus, with orthosteric interactions only one molecule may occupy the receptor at any one instant whereas with allosteric interactions both molecules can bind to the receptor at the same time. There are implications for pharmacological activity, especially for antagonists, that arise from these two molecular mechanisms (vide infra).

There are certain molecular mechanisms of antagonism associated with these observed patterns on dose–response curves. Thus, competitive antagonists produce parallel shifts to the right of agonist dose–response curves with no diminution of maximal response through an orthosteric interaction of antagonist and agonist (Fig. 3a). Theoretically, there is no limit to the degree of dextral displacement a given competitive antagonist can produce on a dose–response curve. Schild analysis is used to measure the affinity of competitive antagonists. This same pattern of response also can occur with allosteric modulators (Fig. 3b) but in this case the shift of the agonist dose–response curve is limited to a maximal value defined by the molecular cooperativity factor of the antagonist. Thus, a hallmark of allosteric inhibition is that it is saturable and reaches a maximal asymptotic value. In some cases, irreversible antagonists can produce parallel shifts to the right of dose–response curves if there is a receptor reserve for the agonist (Fig. 3c). This latter mechanism can be detected with increasing concentrations of irreversible antagonist since these eventually cause depression of the maximal response.

In cases where insurmountable antagonism is observed, this can be evidence of noncompetitive antagonism. This can result from an inhibiton of



Drug–Receptor Interaction, Fig. 3 Various patterns of antagonism of drug effect. Antagonism is classified according to effects on the dose–response curve to the agonist (blue lines). Two general classifications are surmountable antagonism (maximal response to the agonist retained – top left panel) or insurmountable antagonism (depressed maximal response to the agonist – top right panel). These general patterns can be the result of different molecular mechanisms. (**a**) Orthosteric competition between the agonist and antagonist for the same binding site on the receptor. (**b**) Binding of an allosteric modulator to its own binding site to modify the affinity or efficacy of the agonist in a saturable manner. (**c**) Irreversible blockade of receptors in a system with *receptor reserve* (parallel shift to the right followed by depression of the maximal response at higher concentrations of antagonist). (**d**) Blockade of receptor function or access of agonist to the receptor either orthosterically or allosterically in a system with little receptor reserve for the agonist. (**e**) Saturable allosteric modulation of receptor function by an antagonist in a system with little receptor reserve for the agonist

receptor function (either orthosteric or allosteric) or be due to antagonist-mediated modification of receptor reactivity to the agonist (Fig. 3e). The *method of* Gaddum is used to measure the affinity of noncompetitive antagonists for receptors. Parenthetically, similar effects on dose–response curves are produced by irreversible antagonists when there is no receptor reserve for the agonist.

While antagonist potency (quantified as the equilibrium dissociation constant of the antagonist–receptor complex, specifically the rate of offset of the antagonist away from the receptor divided by the rate of onset toward the receptor) usually is used as a measure of the therapeutic utility of an antagonist (i.e., high potency leads to the use of low dose with the concomitant avoidance of high concentrations that may produce side effects), the kinetics of receptor antagonism also are important. Thus, if antagonist negation of receptor function is the desired effect, then for two antagonists of equal potency, the one with the most persistent binding (slowest rate of offset from the receptor) is preferred. This is referred to as target residence time.

Pharmacological Relevance

An important tenet of receptor pharmacology states that the molecular properties of a drug, namely affinity and intrinsic efficacy, are interpreted and reflected by physiological systems and that this process *controls* what is observed as drug effect. For example, a physiological system requires a certain sensitivity to return response from stimulation by a weak agonist, i.e., the receptor coupling of that system must be of sufficient efficiency to amplify the stimulus into an observable response. When the same weak agonist is tested in a physiological system of lower sensitivity, it might be an antagonist. Thus, it can be seen that the monikers of agonist and antagonist can be system dependent and unreliable as molecular labels for drugs. In the same manner that a system must be of sufficient sensitivity to detect efficacy and return response, it also can be overloaded by a strong stimulus. When this occurs, the system returns the system maximal response and the agonist demonstrates full agonism. A series of agonists of differing intrinsic efficacy may all overload a given system and all return the same (system) maximal response (all be full agonists); this does not imply that these agonists are of equal efficacy but only that the system was unable to discern different efficacies beyond a certain level. For this reason, the labels of full and partial agonist are also system dependent and not useful for molecular characterization of drugs.

Since drugs are tested in many surrogate systems, it is necessary to develop methods to quantify drug effect in a system-independent manner. Absolute scales are not practical, or in some cases, even possible, in this process. As discussed previously, while an absolute potency for an agonist can be determined by the EC_{50}, the magnitude of this value depends on the sensitivity of the particular measuring system and thus it cannot be extrapolated to other systems. Rather, the relative potency of agonists (ratios of EC_{50}values) is used to quantify agonist power to induce response. This process utilizes the null method and isolates only the intrinsic ability of the agonists to produce response at the receptor level. This allows for the resulting potency ratios to be a measure of relative agonist activity that is comparatively independent of the system in which the measurement is made. This ratio transcends the particular system in which it is measured and is applicable to all systems in which the agonists produce maximal response. Under optimal conditions, the therapeutic profile of the standard agonist will be known in humans, therefore the agonist potency ratio can be used to gauge the expected activity of the experimental agonist in the therapeutic arena. It should be noted that there are exceptions to this rule for agonists that produce biased signaling whereby, through the formation of a unique receptor active state, the agonist activates some cellular pathways at the expense of others to produce a skewed signal.

In the case of antagonists, absolute measures of potency are theoretically possible since these are chemical terms describing the affinity of the drugs for receptor protein. However, physiological systems can also control the observed antagonism. For example, a noncompetitive antagonist will produce a diminution of the maximal response to an agonist in a system where the response is linearly related to the receptor occupancy (Fig. 3d). However, if the receptors in a system are coupled with high efficiency and the agonist has high efficacy, then maximal responses may be achieved with less than maximal agonist receptor occupancy, i.e., there may be a receptor reserve for the agonist. Under these circumstances the agonist may still produce the maximal response even when the antagonist completely inactivates a portion of the receptors. When this occurs the antagonism resembles competitive antagonism at low concentrations and noncompetitive antagonism at higher concentrations (resembling the profile in Fig. 3c). This would be system dependent and not necessarily indicative of the molecular mechanism of the antagonist.

Another example of where the set point of the physiological system can change the observed behavior of drugs is the absence of direct effects of inverse agonists in nonconstitutively active

receptor systems. A receptor system must be constitutively active (elevated basal response) to detect inverse agonism (Fig. 1). In non-constitutively active receptor systems, inverse agonists behave as simple competitive antagonists.

In general, receptor theory uses indirect mathematical models to estimate descriptors of drug effect. These descriptors still must be used with the proviso that biological systems may still modify drug effect in a system-dependent manner and thus predictions of therapeutic effect must be made with caution across different systems.

Cross-References

- ► Drug Interactions
- ► G-protein-Coupled Receptors

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Drugs for the Treatment/Management of Gout and/or Hyperuricemia

- ► Antigout Drugs

E

Early Drug Discovery

► High-Throughput Screening (HTS) Technology

EC 2.1.1.6

► Catechol-*O*-Methyltransferase

EF-Hand

► Ca^{2+}-Binding Proteins
► S100 Proteins

EFNA1, EphrinA1, Ephrin-A1

► Eph Receptors and Ephrins

EFNA2, EphrinA2, Ephrin-A2

► Eph Receptors and Ephrins

EFNA3, EphrinA3, Ephrin-A3

► Eph Receptors and Ephrins

EFNA4, EphrinA4, Ephrin-A4

► Eph Receptors and Ephrins

EFNA5, EphrinA5, Ephrin-A5

► Eph Receptors and Ephrins

EFNB1, EphrinB1, Ephrin-B1

► Eph Receptors and Ephrins

EFNB2, EphrinB2, Ephrin-B2

► Eph Receptors and Ephrins

EFNB3, EphrinB3, Ephrin-B3

► Eph Receptors and Ephrins

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Eicosanoids

► Leukotrienes

Emesis

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Definitions

Emesis usually refers to vomiting, the forceful oral expulsion of gastric contents; an "emetic" stimulus (often a chemical) is one capable of inducing vomiting. Paradoxically, treatments described as "antiemetics" are considered to affect both vomiting and the sensation of nausea which usually precedes. Nausea is a self-reported unpleasant sensation associated with an awareness of an urge to vomit that is commonly accompanied by cold sweating, facial pallor, increased salivation, and introversion. The sensation is often perceived in the pharyngeal area but the substernum, epigastrium, and head are also sites of perception; it is not clear how these sites relate to the underlying cause (Stern et al. 2011; Koch and Hasler 2017). When protracted, nausea waxes and wanes, described as coming in "waves" and hypothesized to reflect relative sympathetic and parasympathetic nervous system activity (see below), changes in the level of visceral perception ("interoception" or degree of disruption of gastric motility, but these proposals await confirmation. Distinguishing between vomiting and nausea has implications for diagnosis of the cause and the choice of treatment, since while the initiating stimuli can be identical, the ultimate mechanisms are different; the sensation of nausea is generally harder to treat than the motor event of vomiting.

Biologically, vomiting and diarrhea provide routes by which food contaminated by toxins not detected by vision, smell, or taste can be ejected in bulk prior to substantive absorption. Nausea provides a warning (analogous to pain) resulting in cessation of feeding and of equal significance, which leads to learned aversion to the food and future avoidance. However, the systems which evolved to defend against ingested toxins can also be activated by a diverse range of therapeutic drugs (e.g., anticancer cytotoxins, the mu-opioid receptor agonist morphine, glucagon-like peptide-1 receptor agonists such as exenatide and the dopamine$_2$ receptor agonist ropinirole) and diseases (e.g., delayed gastric emptying, gastroparesis, migraine, vestibulitis, and myocardial infarct), when nausea and vomiting become side-effects and symptoms, respectively, requiring treatment with antiemetics (see below) (Sanger and Andrews 2018).

The ability to vomit is common among mammals (e.g., dogs, ferrets, and sperm whales) but rodents (e.g., rats and mice) and lagomorphs (e.g., rabbits) appear to have lost this capability during their evolution; this has implications for selection of animal models for research in this area (Stern et al. 2011). Mammals capable of vomiting show behavioral changes prior to vomiting and in rodents these may be the only external indication that a stimulus capable of inducing vomiting in other species has been administered. However, as nausea is a self-reported human sensation, the nature of the experience of animals in response to a stimulus inducing nausea in humans is impossible to know (Stern et al. 2011). Nevertheless, in rodents, learned taste aversion and avoidance occur following administration of a range of emetics, suggesting a negative hedonic perception. The significance of the behavioral changes to emetics in nonhuman animals is a matter of debate but is of great importance when attempting to identify novel therapeutic approaches to treat nausea as opposed to vomiting.

Basic Mechanisms

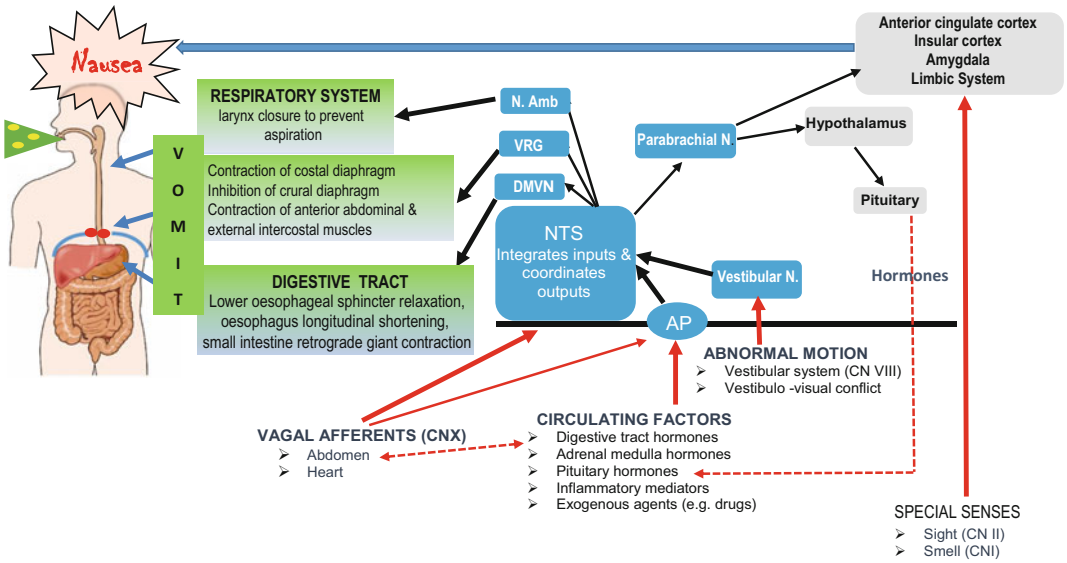
Mechanistically, vomiting is better understood than nausea. The final motor act of vomiting and

the experience of nausea involve different "integration/command" (vomiting) and "conscious sensation" (nausea) brain nuclei, with different outputs (Stern et al. 2011; Koch and Hasler 2017). However, both are induced by many different stimuli acting via the same major primary pathways.

Vomiting

Vomiting is induced by diverse stimuli activating different pathways (the sensory inputs) which converge on the *nucleus tractus solitarius* (NTS) in the dorsal brainstem, which coordinates the motor response (Fig. 1). In brief, in humans, the NTS receives major inputs from:

- The area postrema (AP), a heavily vascularized circumventricular organ not protected by the blood-brain barrier and located in the fourth ventricle. The AP is also called the "chemoreceptor trigger zone" (CTZ) for emesis, as when activated by systemic agents (exogenous or endogenous) it can induce vomiting (and nausea) via its connections with the NTS.
- Vagal afferents from the abdominal viscera (particularly stomach and small intestine) and the heart.
- The brainstem vestibular nucleus which itself receives inputs from the vestibular system (balance and orientation, acceleration) via the vestibulocochlear nerve (cranial nerve VIII).



Emesis, Fig. 1 Major pathways for the induction of nausea and vomiting. Stimuli which can induce vomiting inform the brain via activation of several pathways (red arrows) converging on the brainstem *nucleus tractus solitarius* (NTS) which integrates the inputs and coordinates the outputs for nausea and vomiting. Input pathways include vagal afferents, circulating factors acting via the area postrema (AP) and abnormal motion via the vestibular system. The NTS communicates with other brainstem motor nuclei forming a network (conceptually this network can be regarded as the "vomiting center" (VC) but it should be recognized that there is no single anatomical substrate for the VC as was previously thought) to initiate the different motor components that are required for vomiting. The main outputs for vomiting are to the digestive tract (DMVN) and respiratory system particularly the costal (◠) and crural (●) regions of the diaphragm. Note that the AP can be accessed by circulating factors which include digestive tract, adrenal and pituitary hormones, as well as exogenous drugs. The hormones may also modulate vagal afferent activity either directly or indirectly via gastric motility changes. Nuclei in blue are all in the brainstem/pons but induction of nausea requires projection of information from the NTS to "higher" brain areas particularly those involved in interoception (e.g., anterior cingulate cortex). Vomiting induced by horrific sights, unpleasant smells, and psychogenic stimuli is probably mediated by their inputs to higher brain regions which then activate the brainstem motor pathways (not shown). AP: area postrema; CN: cranial nerve; DMVN: dorsal motor vagal nucleus; N. Amb: Nucleus ambiguus; NTS: nucleus tractus solitarius; Parabrachial N: Parabrachial nucleus; Vestibular N: Vestibular Nucleus; VRG: ventral respiratory group of neurones

- The brainstem trigeminal and facial nuclei which receive inputs from cranial nerves V and VII, respectively.
- A number of "higher" brain regions including the limbic system and cerebral cortex.

The NTS is therefore provided with information on emetic stimuli that circulate in the blood (via the AP and which may be endogenous [hormones] or exogenous [drugs-see below]), originate from the gastrointestinal tract (e.g., food poisoning) and other viscera (e.g., heart; nausea preceding an infarct), arise because of abnormal motion (e.g., vestibular system and vestibulovisual conflict inducing motion sickness), result from facial pain and migraine (e.g., trigeminal nucleus), and psychogenic stimuli (e.g., horrific sights via the cerebral cortex).

The NTS integrates the signals from each input and if it reaches a threshold, sends outputs to the main motor pathways for vomiting, namely the dorsal motor nucleus of the vagus (lower oesophagus, stomach, intestine), the nucleus ambiguus (pharynx, upper esophageal sphincter), and the ventral respiratory group (connecting to cervical spinal phrenic nerve nucleus). Vomiting requires coordination between the digestive tract and the respiratory system and between both visceral and somatic divisions of the peripheral nervous system to produce the unique motor pattern. The motor events of vomiting only require the integrity of the brainstem and spinal cord with the NTS playing the pivotal role. As the NTS is also involved in nonvomiting, routine homeostatic functions (breathing, digestion), vomiting provides an interesting example of "motor program switching" by this area of the brain.

Vomiting is a dramatic, externally visible event, but as the stomach is the only site from which ingested material can be forcibly ejected, the events in the digestive tract which precede ejection are important and are listed below in temporal sequence.

- Relaxation of the proximal (corpus, fundus) stomach and inhibition of distal stomach (antrum) motility, delaying gastric emptying and reducing the movement of toxin-contaminated food to the intestine. These motor changes are mediated by vagal efferent activation of enteric inhibitory neurones (using nitric oxide in humans) and reduced drive to the enteric cholinergic neurones.
- Initiation of a single retrograde giant contraction (RGC) in the distal small intestine progressing to the stomach. This large amplitude contraction is mediated by the vagal efferents involving acetylcholine (blocked by the muscarinic receptor antagonist atropine) and pushes intestinal contents (which may contain bile) into the already-relaxed stomach.
- Once the RGC reaches the stomach, retching (derived from the Anglo-Saxon *hraecan* "to cough up phlegm") begins and while externally this appears similar to vomiting there is no ejection of gastric contents. The function of retching is unknown but unproven hypotheses include preparing the muscles for the more intense activity during vomiting, "wind up" of the central neural circuitry and testing the fluidity of the gastric contents. While some retching normally precedes vomiting it is not essential, as exemplified by projectile vomiting caused by hypertrophic pyloric stenosis and acutely raised intracranial pressure. Retching is caused by rhythmic contraction of the anterior abdominal muscles (spinal motor neurones) and contraction (descent) of the entire diaphragm (phrenic nerve) and although the stomach is compressed, material is not ejected because the antireflux mechanisms at the gastro-esophageal junction are functional. Vomiting follows retching when the crural muscle fibers of the diaphragm which encircle the lower esophagus and are differentially controlled from the remaining diaphragm, become inactive and the smooth muscle lower esophageal sphincter at the gastro-esophageal junction relaxes (via vagal efferents). These two actions, together with an overall longitudinal shortening of the esophagus, remove the resistance to expulsion of gastric contents, which are finally ejected by enhanced and protracted

abdominal muscle contraction compressing the stomach. The resulting intraluminal pressure in the stomach is up to 300 mmHg, ejecting liquid contents over several feet.

Nausea

Nausea is an unpleasant sensation and difficult to study experimentally in healthy volunteers. Increasing evidence obtained by neuroimaging supports the idea that the recognition of nausea occurs during increasingly intense activation of brain nuclei involved in interoception – the self-awareness of body sensations and internal physiological events. These involve, in particular, the anterior cingulate cortex ("visceromoror cortex"), inferior frontal gyrus, insular cortex, and amygdala (Stern et al. 2011; Koch and Hasler 2017) (Fig. 2). Their basic role is to process stress and the awareness of fear, generating fundamental "defensive" activities including modulation of autonomic outflows. The latter is responsible for physiological changes such as increased salivation, sweating (forehead), and reduced cutaneous blood flow (face and fingers) which accompany nausea. In humans, nauseogenic stimuli usually cause secretion of a high concentration of arginine vasopressin from the hypothalamus and although it may be causally involved in nausea (action on the AP, disruption of gastric motility), its function may also be water retention in anticipation of vomiting and cardiovascular adjustments (Koch and Hasler 2017). However, when these central pathways are activated in a sustained manner, subjects report severe nausea and strong stomach awareness. This suggests that nausea is the result of an increasingly intense and sustained input into these areas caused perhaps, by simultaneous activation of multiple mechanisms. The latter may explain why, in general, nausea is harder to treat by drugs than is vomiting.

Pharmacological Interventions

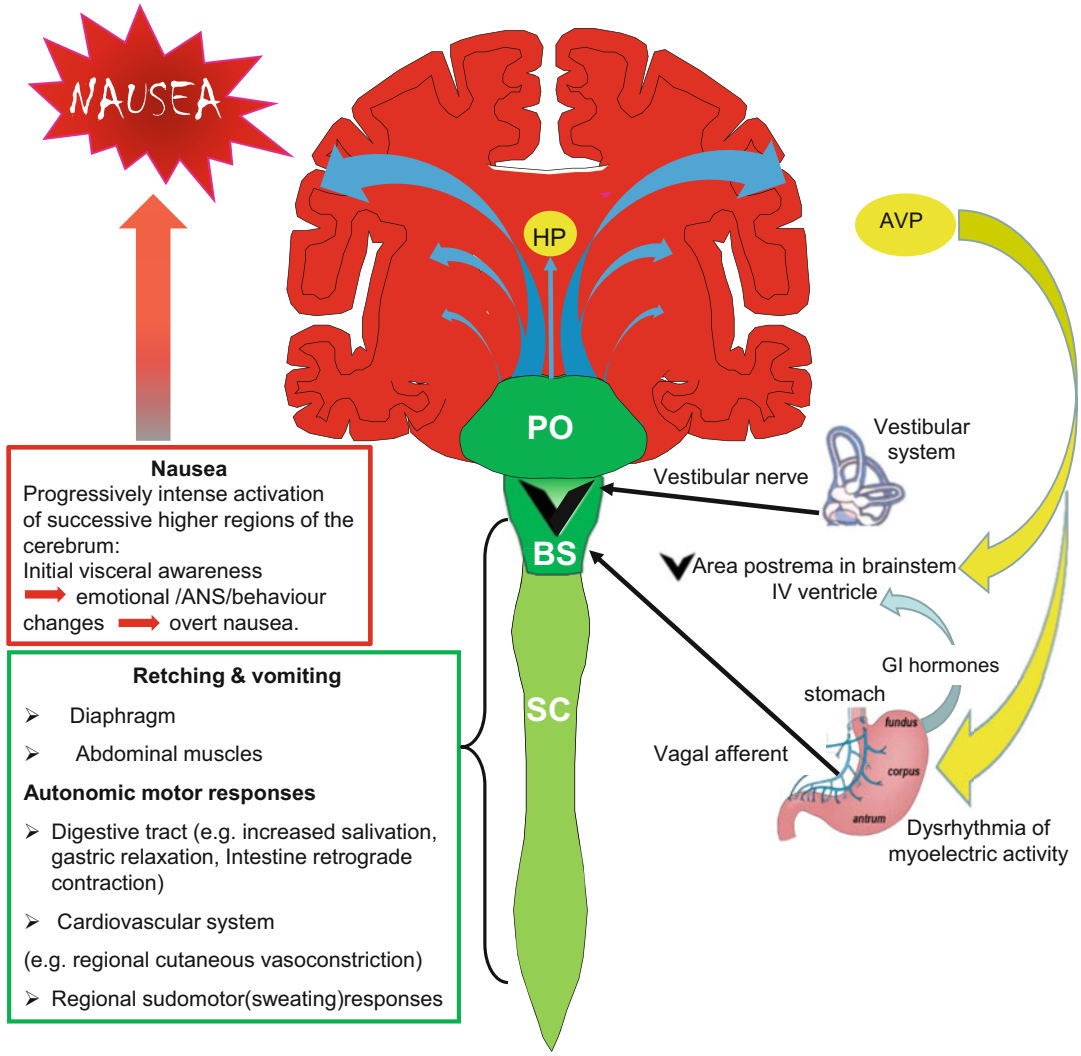
Drugs which inhibit or prevent nausea and vomiting are usually called "antiemetic" drugs. It is important to emphasize that currently there is no "universal antiemetic" drug preventing both nausea and vomiting induced by all the above pathways. Instead, particular drugs control "motion sickness" (by acting mostly within the vestibular nucleus), nausea, and vomiting originating from the viscera (by inhibiting vagal afferent functions) or induced by systemic (endogenous or exogenous) stimuli activating the AP. Table 1 lists some of the major antiemetic drugs and describes their main pharmacological actions. With few recent exceptions, most drugs were discovered by serendipity (Sanger and Andrews 2018) and tend to have multiple actions which confer both benefits and side-effects, reflecting their receptor binding profiles (Table 1).

Muscarinic Receptor Antagonists (Sanger and Andrews 2018)

Motion sickness is controlled by muscarinic receptor antagonists (e.g., hysoscine) acting primarily at the M_5 receptors in the vestibular nucleus. Although the properties of extracts of deadly nightshade (*Atropa belladonna*) and henbane (*Hyosyamus niger*) were known from antiquity, the efficacy of muscarinic antagonists was not established by formal clinical trials until World War II, when trials were conducted to improve the well-being of soldiers during transport by sea and before beach landings.

Histamine H_1 Receptor Antagonists (Sanger and Andrews 2018)

Drugs which antagonize the histamine H_1 receptor were being evaluated for the control of hay fever and urticaria in 1947 when it was observed that motion sickness could also be controlled. Today, these drugs are also readily available to aid sleep (H_1 receptor antagonism within the brain causes somnolence) but when combined with the stimulant 8-chlorotheophylline to counteract the drowsiness, drugs such as diphenhydramine became available for treatment of motion sickness. Some, such as diphenhydramine, were later found to have affinity for the muscarinic receptors (Table 1) so their precise antiemetic mechanism (s) of action is unclear. Cyclizine is another example of an H_1 receptor antagonist widely prescribed



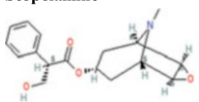
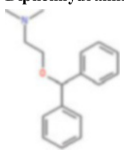
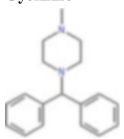
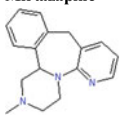
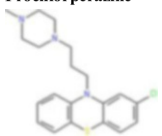
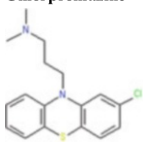
Emesis, Fig. 2 Flow of information that leads to vomiting and nausea. The motor components of retching and vomiting only require the brainstem (BS) and spinal cord (SC), triggered by the main inputs of abdominal vagal afferents, the area postrema (including by gastrointestinal hormones), and vestibular system (see text for details). Nausea requires projection of information from the brainstem to progressively "higher" regions of the brain (amygdala, insular cortex, cingulate cortex) resulting in progressively more visceral awareness and responses controlled by the autonomic nervous system (ANS; endocrine and cardiovascular). The hypothalamic pituitary axis (HP) is also activated and of particular relevance to nausea is arginine vasopressin (AVP) which is hypothesized to act on the stomach to induce gastric dysrhythmia (associated with nausea) and possibly the area postrema; the consequences of either would be to reinforce the original emetic stimuli

as a treatment of motion sickness but also as a "first line" antiemetic if the cause of emesis is uncertain (e.g., during palliative care). Although it was taken on the Apollo space missions, cyclizine is such an old drug that very little is known about it!

Dopamine D_2 Receptor Antagonists (Sanger and Andrews 2018)

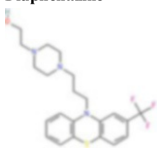
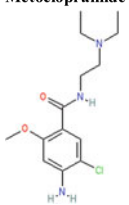
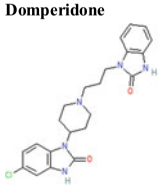
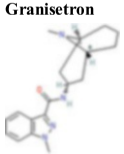
Dopamine D_2 receptor antagonists are presumed to be antiemetic because they block D_2 receptors within the AP. It is assumed, therefore, that they are effective against vomiting that is induced by

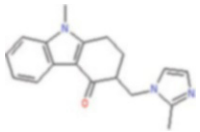
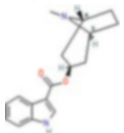
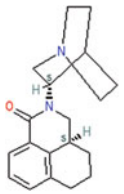
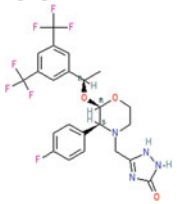
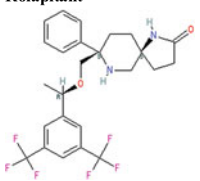
Emesis, Table 1 Receptor affinities of antiemetic drugs. Modified from Sanger and Andrews (2018). Data are from http://www.guidetopharmacology.org/ and are given as pK_i values for the human receptor; primary affinities are in bold. Additional information on drug affinity for human receptors (unless otherwise specified) is provided in italics

Receptors												
	D_1	D_2	D_3	D_4	α_1	α_2	H_1	M	5-HT_{2A}	5-HT_3A	NK_1	Other
Scopolamine 		*K_i > 10,000 nM (rat)*					*K_i > 10,000 nM (rat)*	M_1 **9.0** M_2 **8.7** M_3 **9.4** M_4 **9.5** Antagonist				
Diphenhydramine 		*K_i > 10,000 nM (rat)*					**7.9** antagonist	*pA_2 7.1 antagonist (rat)*				
Cyclizine 							**8.4** antagonist					*Pre-ganglionic cholinergic inhibition (animals)*
Mirtazapine 					α_{2A} **7.7** antagonist	α_{2C} **7.7** antagonist	*$p1C_{50}$ 9.6 inverse agonist* **8.9** antagonist		**7.2** antagonist			5-HT_{2C} **7.4** antagonist
Prochlorperazine 	7.1 antagonist	**8.4** antagonist	**8.4** antagonist	6.1 antagonist	*K_i 200 nM (rat)*		*$p1C_{50}$ 6.7 inverse agonist 8.2*	*K_i 2100 nM (rat)*	***8.2***	*Inactive (rat)*		
Chlorpromazine 	7.1 antagonist	7.0–7.6 antagonist	7.2–7.5 antagonist	7.8 antagonist	α_{1A} *K_i 028 nM*	α_{2A} 5.9–6.6 α_{2B} 7.2–8.3 α_{2C} 6.9–7.4 antagonist at each	**8.2** antagonist	*K_i 47 M_3 (rat)*	**8.1** inverse agonist	*Inactive (rat)*		D_5 6.9 antagonist 5-HT_{1A} 6.2 antagonist 5-HT_{2C} 7.6–8.2 antagonist 5-HT_6 7.7–7.8 Inverse agonist 5-HT_7 7.6 inverse agonist

(continued)

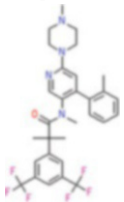
Emesis, Table 1 (continued)

Receptors												
	D_1	D_2	D_3	D_4	α_1	α_2	H_1	M	5-HT_{2A}	5-HT_3A	NK_1	Other
Fluphenazine 	7.7 antagonist	**8.8** antagonist			*K_i 8.1 nM (rat)*		7.7 antagonist	*K_i 340 nM (rat)*	7.5 antagonist	*Inactive (rat)*		D_5 7.9 antagonist 5-HT_7 7.9 inverse agonist 5-HT_6 7.3–7.4 inverse agonist
Metoclopramide 		**7.5** antagonist (mouse)			*K_i > 10,000 nM (rat)*		*K_i 1100 nM (rat)*	*K_i > 10,000 nM (rat)*		5-HT_3A **6.0–6.4** antagonist 5-HT_3AB **5.7** antagonist		5-HT_4 **6** agonist (mouse)
Domperidone 		**7.9–8.4** antagonist	**7.1–7.6** antagonist	*K_i 30.4 nM*	*K_i α_{1A}, 71; α_{1B}, 530; α_{1D}, 710 nM*					*Inactive (rat)*		
Olanzapine 	*K_i 31 nM (rat)*	**8.7** antagonist		*K_i 27 nM (rat)*	α_{1A} *K_i 115 nM*	*α_{2A} K_i 314; α_{2B} 81.6; α_{2C} 28.8 nM*	**8.7–9.2** antagonist	*K_i 105 nM at M_3*	**8.6–8.9** antagonist	*K_i 57 nM (rat)*		5-HT_{2C} **8.1–8.4** inverse agonist 5-HT_6 **8** inverse agonist 5-HT_7 6.5 antagonist
Granisetron 	*Inactive (>10,000 nM) (rat)*	*Inactive (>10,000 nM) (rat)*			*Inactive (>10,000 nM) (rat)*	*Inactive (>10,000 nM) (rat)*			*Ki > 6.3 μM*	5-HT_3A ~ **8.6–8.8** antagonist		*Ki 6.9 μM (5-HT_{1A}) > 10 μM (5-HT_{1B}) (rat)*

Ondansetron 										5-HT_3A ~ **7.8–8.3** 5-HT_{3A}B **7.8** antagonist		
Tropisetron 		*Inactive (>10,000 nM) (rat)10*								5-HT_3A **8.5–8.8** antagonist		5-HT_4 6.3–7.1 antagonist
Palonsetron 										5-HT_3A **10.5** antagonist		
Aprepitant 											**10.1** antagonist	
Rolapitant 											**9.2** antagonist	

(continued)

Emesis, Table 1 (continued)

Receptors												
	D_1	D_2	D_3	D_4	α_1	α_2	H_1	M	5-HT_{2A}	5-HT_3A	NK_1	Other
Netupitant 											**9** antagonist	

circulating agents which reach the AP and induce somehow induce D_2 receptor activation; surprisingly however, relatively little is known about the relationships between dopamine and D_2 receptors in the human AP (Koch and Hasler 2017). One class of receptor antagonist, the phenothiazines (first synthesized in 1883), arose from research into the role of dopamine in schizophrenia. These drugs include chlorpromazine, prochlorperazine, and fluphenazine. They are brain penetrant D_2 receptor antagonists but some have additional affinity for the H_1 receptor (prochlorperazine, fluphenazine) causing a degree of somnolence (Table 1). A disadvantage of brain penetrance is that D_2 receptors involved in motor functions may also be antagonized, leading to "extrapyramidal side-effects" such as akathisia, pseudo-Parkinsonism, and tardive dyskinesia.

A second, and important, class of drug that antagonizes the D_2 receptor are the substituted benzamide's, exemplified by the first of these, metoclopramide. In addition, metoclopramide is a 5-hydroxytryptamine$_4$ (5-HT$_4$) receptor agonist (leading to stimulation of gastric movements) and a 5-hydroxytryptamine$_3$ (5-HT$_3$) receptor antagonist (providing additional antiemetic activity; see below). In terms of receptor affinity, this is highest for the D_2 receptor. However, amplification of the response to 5-HT$_4$ receptor activation within the enteric cholinergic neurons (facilitating the release of acetylcholine to increase GI motility) means that metoclopramide is a 5-HT$_4$ receptor agonist (and gastric prokinetic agent) and D_2 receptor antagonist (antiemetic) at approximately similar doses in vivo (Table 1). Because of its ability to act as a gastric prokinetic, this action, together with the antiemetic activity, means that the drug is often provided for patients with upper GI disorders such as gastroparesis, a disorder characterized by delayed gastric emptying together with symptoms of nausea or vomiting, early satiety, pain, and abdominal or gastric bloating (see below). Extrapyramidal side-effects can occur with metoclopramide but are not usually serious. Of great significance is that higher doses of metoclopramide are able to antagonize the 5-HT$_3$ receptor, activity of fundamental importance in expanding the therapeutic utility of metoclopramide and in the later discovery that emesis caused by highly emetogenic anticancer therapy could be prevented by selective 5-HT$_3$ receptor antagonists (see below).

The third class of D_2 antagonist is represented by domperidone, designed not to cross the blood brain barrier. This drug is therefore normally free of extrapyramidal side-effects but by acting at the AP (not protected by the blood-brain barrier), retains antiemetic activity; some evidence exists to suggest that in patients with delayed gastric emptying, domperidone may increase gastric emptying. Domperidone is also a D_3 receptor antagonist and animal studies suggest that such activity enhances the antiemetic activity of D_2 antagonism. Domperidone has not been registered for use in the USA and the discovery that this drug is able to block the human ether-a-go-go potassium channel (*h*ERG) at clinically relevant concentrations (potentially leading to cardiac arrhythmia) has further restricted its use.

5-Hydroxytryptamine$_3$ (5-HT$_3$) Receptor Antagonists (Sanger and Andrews 2018)

The muscarinic, histamine, and dopamine receptor antagonists were largely discovered before it was possible to screen compounds for activity against a bank of recombinant human receptors (meaning that nonselectivity of action is not uncommon) and without being directed towards the nausea and vomiting associated with any particular clinical condition. This changed with the introduction of cisplatin-based anticancer drugs which cause chemotherapy-induced nausea and vomiting (CINV), in common with most cytotoxic anticancer drugs (Sanger and Andrews 2018). However, the severity of the vomiting induced by these drugs could not be prevented by any of the above antiemetic drugs, greatly limiting their use in cancer patients and seriously reducing quality of life during treatment; a single treatment can cause patients to experience severe nausea and vomiting during the first 24 h after administration ("acute phase") and then after some recovery, the symptoms return ("delayed phase") for a further 3–5 days. This type of severe vomiting was not prevented by high-dose D_2

receptor antagonists or cannabis (acting at CB_1 receptors). Metoclopramide also proved ineffective but surprisingly, when given at higher doses, successfully controlled cisplatin-evoked vomiting. Together with the knowledge that high concentrations of metoclopramide antagonize at the 5-HT_3 receptor (Table 1), this led to an understanding of the mechanisms of this type of severe vomiting and the development of selective 5-HT_3 receptor antagonists for treatment of chemo- and radiotherapy induced emesis. The drugs are effective when given before treatment or after emesis has begun, because they block the actions of 5-HT released from the enterochromaffin cells of the small intestine to both activate and sensitize the peripheral terminals of the abdominal vagal afferents. Projections from these afferents, largely to the NTS, initiate nausea and vomiting. The 5-HT_3 receptor antagonists are also registered for the treatment of post-operative nausea and vomiting but the causal mechanism is not fully known. Today, cancer patients receiving "moderately severe emetogenic treatments" receive a 5-HT_3 receptor antagonist together with the corticosteroid dexamethasone; the latter has anti-inflammatory and other actions which help to reduce the delayed emesis and when given with a 5-HT_3 receptor antagonist achieves good control of vomiting. It is recommended that cancer patients receiving "severe emetogenic treatments" (e.g., platinum-based therapies) are given a 5-HT_3 receptor antagonist, dexamethasone, and also an NK_1 receptor antagonist, the most recent novel class of antiemetic drug (see below). A recent addition to the family of selective 5-HT_3 receptor antagonists is the drug palonosetron, a compound with high affinity for the receptor and a long plasma half-life (42 h) in healthy volunteers (Sanger and Andrews 2018). Compared with the earlier 5-HT_3 receptor antagonists, palonosetron appears to have a superior ability to inhibit both acute and delayed phases when given alone. Further studies suggest that palonosetron binds allosterically to the receptor, in a manner which provides prolonged activity at the receptor and its internalization; the internalized complex is suggested to "cross-talk" with NK_1 receptor signaling pathways, inhibiting the functions of its major ligand, substance P, and thereby explaining the superior antiemetic activity.

Neurokinin$_1$ (NK_1) Receptor Antagonists (Sanger and Andrews 2018)

NK_1 receptor antagonists block the ability of the tachykinin peptides, most notably substance P, to activate the NK_1 receptor. Substance P is released from gut enteroendocrine cells to act on peripheral vagal afferent terminals (cf. 5-HT), and also from the central terminals of the vagal afferents and from NTS neurons, providing a greater ability to inhibit vomiting, compared with the 5-HT_3 receptor antagonists which inhibit only the activation of the vagal afferent nerve by 5-HT. When combined together with a 5-HT_3 receptor antagonist and dexamethasone, maximum efficacy is achieved in cancer patients receiving highly emetogenic chemotherapy, against both the "acute" (first 24 h) and delayed forms of CINV.

Treating Nausea: A Remaining Challenge

Despite great improvements in the control of nausea and vomiting in cancer patients, for many, the problem of persistent nausea remains with a major impact on quality of life. To try and achieve added nausea control, the benefits of the more recently developed antipsychotic medications are being explored. Most notable among these is olanzapine, an antagonist at D_1, D_2, D_3, D_4, 5-HT_{2A}, 5-HT_{2C}, 5-HT_3, 5-HT_6, α_1 adrenergic, H_1 and m_1, m_2, m_3, m_4 receptors. When given to cancer patients together with a 5-HT_3 receptor antagonist, dexamethasone, and an NK_1 receptor antagonist, some further reduction in nausea has been reported (in addition to a degree of somnolence). The enhanced efficacy of these multidrug combinations antagonizing multiple receptors (up to 17!) may reflect the activation of multiple pathways with differing receptor combinations (see above).

Nausea is also a common symptom of many diseases. For example, among the "functional bowel disorders" nausea is particularly present in gastroparesis (idiopathic and diabetic), chronic unexplained nausea and vomiting and functional

dyspepsia (Yu et al. 2017). At present only metoclopramide is registered for the treatment of gastroparesis within the USA and a recent analysis is consistent with a beneficial action of this drug, despite extrapyramidal side-effects. In these patients, other drugs may have beneficial activity but have restricted use (e.g., domperidone) or as yet, have not demonstrated a consistent benefit (e.g., 5-HT_3 or NK_1 receptor antagonists). New approaches (e.g., ghrelin receptor agonism, to achieve a gastric prokinetic activity together with an ability to inhibit vomiting) are currently under examination. New causal mechanisms of nausea are also being explored (Grover et al. 2019). In particular, it is thought that the activity of the pacemaker cells within the wall of the stomach (e.g., the interstitial cells of Cajal) may become disrupted during gastroparesis. These cells spontaneously depolarize to generate slow waves of electrical activity that pass circumferentially through the stomach towards to the pyloric sphincter; it is believed that such activity is communicated (via gap junctions) to the muscle to promote gastric contractions which empty contents into the intestine. Disruptions in slow wave activity and the consequential contractile changes are thought to be detected by vagal mechanoreceptors for transmission to the NTS and interpretation by higher brain centers as nausea. These disruptions may follow damage to the pacemaker cells caused by macrophage invasion in certain patients with gastroparesis or perhaps in other patients', changes are secondary to the induction of nausea by a stimulus acting away from the stomach (e.g., motion sickness). Apart from gastroparesis, dysrhythmic gastric electrical activity has been observed in many patients with nausea (e.g., motion sickness, chronic renal failure, cyclical vomiting syndrome, pregnancy sickness). Drugs which can modulate pacemaker cell disturbances and hence alleviate nausea have not yet been identified.

Genomics and Emesis

Only limited pharmacogenomics studies have been applied to nausea and vomiting. In addition to those which apply to drug metabolism and transport (e.g., ondansetron metabolized mostly by the cytochrome P450 CYP2D6 which exhibits genetic polymorphism; *ABCB1* polymorphisms and granisetron efficacy), genetic variations are known to exist for the genes encoding the opioid mu-1 receptor and the different subunits comprising the 5-HT_3 receptor (a ligand-gated cation channel assembled as a pentamer) (Aroke and Hicks 2019).

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Endocannabinoids

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Synonyms

Endogenous agonists of cannabinoid receptors

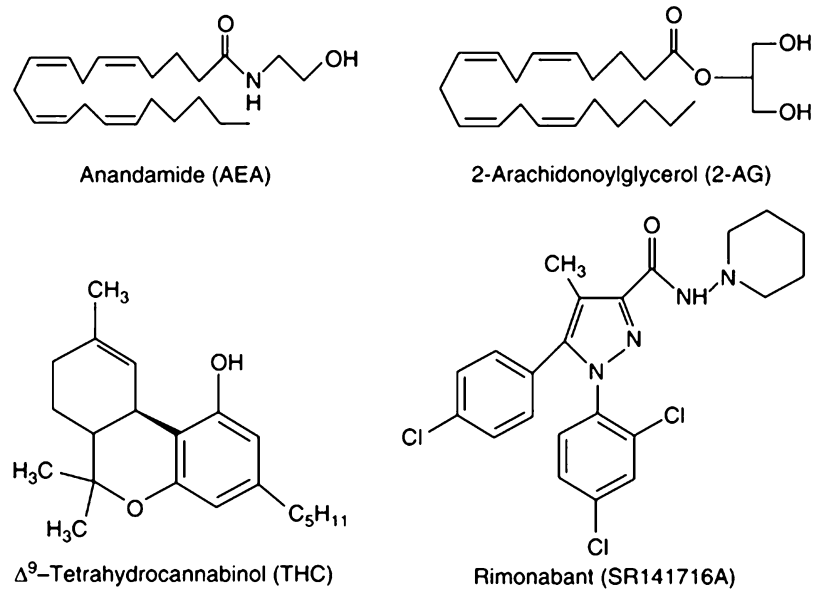
Definition

Endocannabinoids are endogenous mediators acting via the binding to, and activation of, cannabinoid receptors, CB_1 and CB_2 (Pertwee 2006). *N*-arachidonoyl-ethanolamine (AEA, anandamide) and 2-arachidonoyl-glycerol (2-AG) (Fig. 1) are the two most potent and efficacious endocannabinoids. In the nervous system, endocannabinoids act as neuromodulators. In peripheral non-nervous tissues, they act as autocrine or paracrine regulators of the homeostasis of other chemical mediators.

Basic Characteristics

Endocannabinoid Biosynthesis

It is commonly accepted that both AEA and 2-AG are biosynthesized "on demand" and released from cells immediately after their production. These events are usually triggered by the enhancement of intracellular Ca^{2+} concentrations that follows cell depolarization or the mobilization of intracellular Ca^{2+} stores subsequent to stimulation of $G_{q/11}$ protein-coupled receptors (Howlett 2005). However, some preliminary evidence is emerging to suggest that endocannabinoids might be secreted in extracellular vesicles (Ligresti et al. 2016). At any rate, in many cases, the levels of the two main endocannabinoids are regulated in different and sometimes even opposing ways via the modulation of the activity and/or expression of either their different biosynthetic or degrading enzymes. AEA is produced from the processing of *N*-arachidonoyl-phosphatidylethanolamine (NArPE), which in turn is obtained from the enzymatic transfer of arachidonic acid esterified on the *sn*-1 position of phospholipids to the nitrogen atom of phosphatidylethanolamine, via Ca^{2+}-independent and, more often, -dependent *N*-acyltransferases (Ligresti et al. 2016). The enzyme catalyzing the one-step conversion of NArPEs and other *N*-acylphosphatidylethanolamines (NAPEs) into AEA and other *N*-acylethanolamines, respectively, is the same and is termed NAPE-selective phospholipase D (NAPE-PLD). It is: (i) enzymatically distinct from other PLD enzymes, (ii) stimulated by Ca^{2+}, and (iii) a member of the β-lactamase fold of the zinc-metallo-hydrolase family of enzymes. Its over-expression in cells leads to higher cellular levels of AEA and correspondingly lower levels of NArPE, thus supporting its role in the biosynthesis of this endocannabinoid. However, three more pathways have been identified that can transform NArPE into AEA. In macrophages, a phospholipase C (PLC) converts NArPE into AEA via phospho-AEA. PTPN22, a protein tyrosine phosphatase, is the



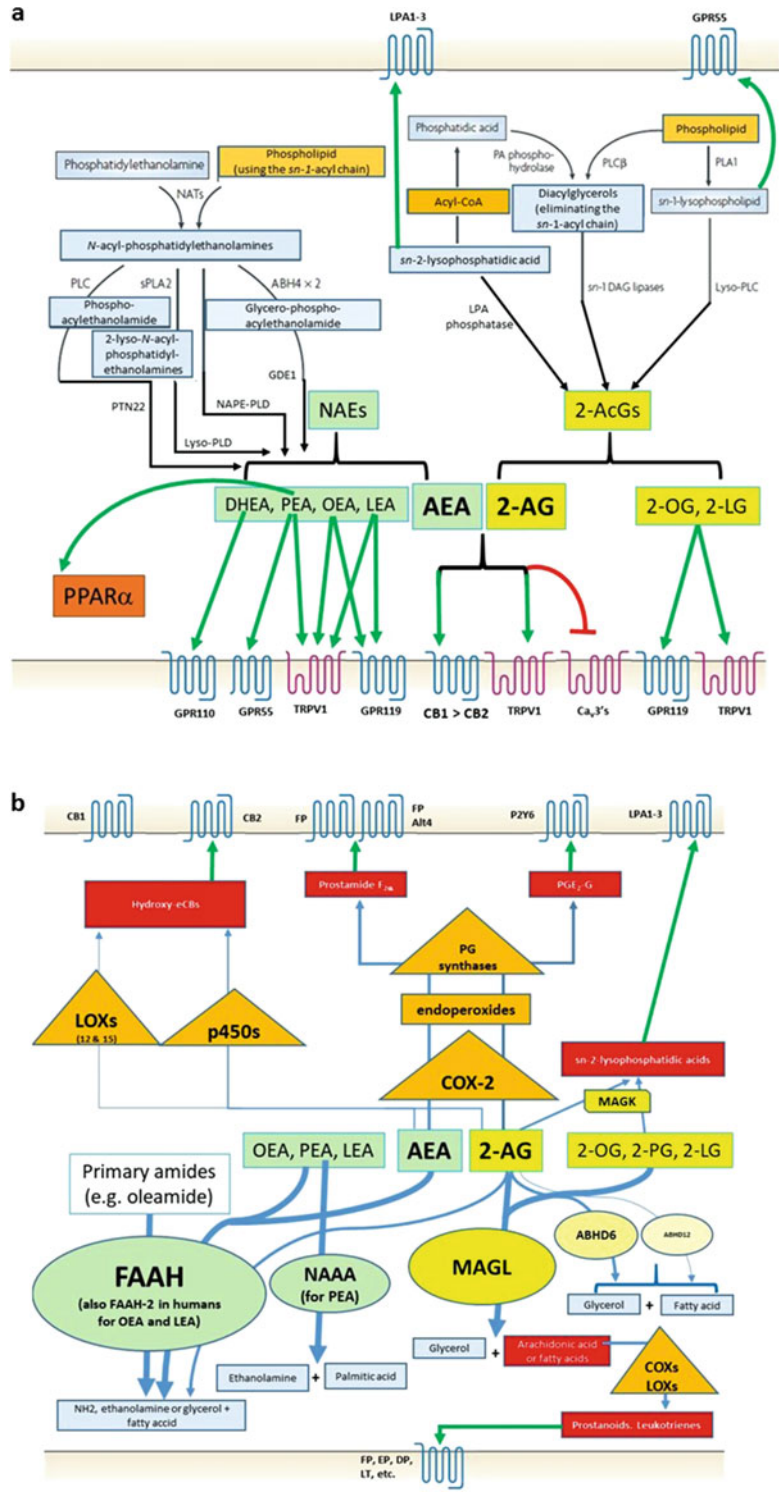
Endocannabinoids, Fig. 1 Chemical structures of the two most studied endocannabinoids, anandamide and 2-arachidonoylglycerol, of *Cannabis sativa* psychoactive principle, Δ^9-tetrahydrocannabinol, and of the CB_1 receptor antagonist/inverse agonist, rimonabant

enzyme catalyzing phospho-AEA hydrolysis to AEA. In brain homogenates, NArPE can be converted into 2-lyso-NArPE, via the action of a groupIB soluble phospholipase A_2, or into glycerophospho-AEA, via alpha/beta-hydrolase 4. AEA is then formed via the hydrolysis of 2-lyso-NArPE by a selective lysophospholipase D, or of glycerophospho-AEA by glycerophosphodiester phosphodiesterase 1 (GDE1), respectively (Fig. 2). Studies using NAPE-PLD/GDE1 double knockout mice have confirmed that these two enzymes can concur to the production of at least part of AEA and other *N*-acylethanolamines in the brain.

2-Arachidonate-containing DAGs are the most frequent biosynthetic precursors of 2-AG. They are produced from the hydrolysis of phosphoinositol-bis-phosphate ($P1P_2$), catalyzed by the PIP_2-selective PLC, or, in some cases, from the hydrolysis of phosphatidic acid, catalyzed by a phosphohydrolase. DAGs are then converted into 2-AG by the action of two plasma membrane *sn*-1-selective DAG lipases, DAGLα and DAGLβ (Fig. 2). These two enzymes (i) contain the typical lipase-3 and Ser-lipase signature sequences, in which two highly conserved amino acid residues, Ser443 and Asp495, are necessary for enzymatic activity, (ii) exhibit also four hydrophobic, possibly *trans*-membrane, domains near their N terminus, (iii) are stimulated by Ca^{2+}, and (iv) do not prefer DAGs with any particular fatty acyl chain in the *sn*-2 or *sn*-1 position, although they exhibit strong selectivity for DAGs over phospholipids, monoacyloglycerides, triacylglycerols, and fatty acid amides. DAGLα is more abundant in the adult brain and DAGLβ in the developing brain. Both enzymes are co-localized with CB_1 receptors in neuronal axons of the perinatal nervous system, and "move" to postsynaptic neurons in the adult brain. DAGLα is found in postsynaptic dendritic spines establishing synapses with CB_1 expressing axons, thus supporting the proposed roles for 2-AG as a retrograde messenger of synaptic plasticity in the adult brain. In adult rodents, DAGLβ is often more abundant than DAGLα in non-neuronal cells in both central and peripheral tissues, for example, microglia, hepatic stellate cells, dendritic cells, and macrophages. Importantly, the two DAGL isoforms are responsible for the production of other long chain 2-acy-glycerols, which, like the non-endocannabinoid *N*-acyl-ethanolamines, are no longer considered devoid of biological actions (Di Marzo 2018).

Endocannabinoid Action

Once released from cells, endocannabinoids bind to cannabinoid receptors and elicit typical CB_1- and CB_2-mediated intracellular signaling events, thereby leading to biological responses typical of cannabinoid receptor agonists (Pertwee 2006; Howlett 2005). However, the functional role of endocannabinoids cannot be inferred only from the knowledge of the pharmacological effects of Δ^9- tetrahydrocannabinol (THC) and synthetic cannabinoids for several reasons: (i) because of the mechanisms underlying their biosynthesis and because of their rapid metabolism, endocannabinoids appear to be produced only "when and where" needed. Thus, for example, if endocannabinoids are being made and released to protect a neuron from glutamate-induced excitotoxicity, they will be released only from the postsynaptic neuron undergoing excessive stimulation by glutamate, to inhibit only glutamate release from the presynaptic glutamatergic neuron, without inhibiting the activity of GABA-ergic interneurons, which, by releasing GABA, also tone down excitotoxicity. This specificity of endocannabinoid action is not always maintained when CB_1 receptors are activated by exogenous agonists, (ii) in some cases endocannabinoids are biosynthesized together with congeners that are inactive per se at cannabinoid receptors, but can nevertheless modulate the activity of AEA and 2-AG or act at different receptors (see below), (iii) endocannabinoids can act also at non-cannabinoid receptors, including ion channels and receptors for neurotransmitters or other neuromodulators, (iv) both AEA and 2-AG can be oxidized by enzymes of the arachidonic acid (AA) cascade, thereby producing metabolites that are not necessarily inactive at cannabinoid receptors (as in the case of lipoxygenase or epoxygenase metabolites) or act on other targets (as in the case of the cyclooxygenase-2 metabolites) (see below), and finally (v) AA produced from the degradation of 2-AG can be used in some tissues, including the brain under inflammatory conditions, to generate



Endocannabinoids, Fig. 2 The endocannabinoid system. Proteins and pathways for the biosynthesis (**a**) and degradation (**b**) of endocannabinoids and their congeners. Receptors for endocannabinoids and endocannabinoid

eicosanoids acting at their own targets (Di Marzo 2018) (Fig. 2). Therefore, to understand endocannabinoid action at cannabinoid receptors, pharmacological studies using selective cannabinoid CB_1 and CB_2 receptor agonists and antagonists/inverse agonists, or recently emerging allosteric modulators of these two receptors, must be used. The study of the pharmacological effects of inhibitors of endocannabinoid biosynthesis or catabolism under physiological and pathological conditions (see below) must be accompanied by observations on the phenotype of CB_1, CB_2, and metabolic enzyme (preferably tissue-specific and conditional) "knock-out" mice and, most importantly, by the quantification of anandamide and 2-AG levels in various tissues following treatment (Ligresti et al. 2016; Di Marzo 2018).

It was established that, in the brain, endocannabinoids act as retrograde messengers acting on presynaptic CB_1 receptors in both short-term and long-term forms of synaptic plasticity. These retrograde actions underlie at least part of CB_1-mediated regulation of cognitive and emotional functions in the brain, reinforcement of substances of abuse in the mesolimbic system, induction of appetite or reduction of satiety in the hypothalamus and brainstem, and control of movement and posture in the basal ganglia and cerebellum. Neuromodulatory, CB_1-mediated actions of endocannabinoids in the sensory and autonomic nervous systems result in the regulation of, among others, circulatory and gastrointestinal functions and the hypothalamic-pituitary-adrenal axis. Direct autocrine or paracrine actions in nonneuronal cells underlie the several CB_1- and CB_2-mediated effects of these compounds on, among others, female and male reproduction, bone formation, immune response and adipocyte and β-cell function (Ligresti et al. 2016).

Endocannabinoid Inactivation

AEA is inactivated through intracellular enzymatic hydrolysis to arachidonic acid and ethanolamine (Fig. 2). The enzyme catalyzing this reaction was named "fatty acid amide hydrolase" (FAAH) as it recognizes as substrates also other long chain fatty acid amides, including *N*-acylethanolamines, primary amides, some *N*-acyl-aminoacids, and *N*-acyl-taurines. FAAH catalyzes efficiently also the hydrolysis of long chain fatty acid esters, including 2-AG. The physiopathological role of FAAH as a major endocannabinoid-inactivating enzyme has been assessed through: (i) the study of the phenotype of transgenic mice lacking the enzyme, that is, the FAAH "knockout" mice, (ii) the design and pharmacological testing in vivo of specific FAAH inhibitors, (iii) immunohistochemical studies describing the tissue and cellular distribution of the enzyme and its relationship with cannabinoid receptor distribution, and (iv) the identification of *Faah* gene polymorphisms associated with disorders such as obesity, propensity to drug addiction and schizophrenia. A second FAAH,

Endocannabinoids, Fig. 2 (continued) congeners, biosynthetic precursors, and metabolic products are also shown. Abbreviations used: 2-AcGs, 2-acyl-glycerols; 2-AG, 2-arachidonoyl glycerol; Abh4 or 6 or 12, alpha/beta-hydrolase 4 or 6 or 12; Ca_{v3s}, T-type calcium channels; COX, cyclooxygenase; DAGL, *sn*-1-selective diacylglycerol lipase; DHEA, *N*-docosahexaenoyl-ethanolamine; DP, EP, FP, receptors for prostaglandins of D, E and F type; FAAH, fatty acid amide hydrolase; GDE1, glycerophosphodiester phosphodiesterase 1; GPR, orphan G-protein-coupled receptor; FP Alt4, alternative splicing variant 4 of the FP receptor making an heterodimer with the FP receptor; LEA, *N*-linoleoyl-ethanolamine; 2-LG, 2-linoleoyl-glycerol; LOX, lipoxygenase; LPA, sn-1-lyso-2-acyl-phosphatidic acid; LPA1-3, lysophosphatidic acid receptors 1–4; MAGK, monoacylglycerol kinase; MAGL, monoacylglycerol lipase; NAAA, *N*-acyl-ethanolamine acid amidohydrolase; NAEs, *N*-acyl-ethanolamines; NAPE-PLD, *N*-acylphosphatidyl-ethanolamine-selective phospholipase D; NATs, *N*-acyl-transferases; lyso-PLD, lysophospholipase D; 2-OG, 2-oleoyl-glycerol; OEA, *N*-oleoyl-ethanolamine; p450, cytochrome p450 monoxygenase; P2Y6, purinergic receptor 2Y6; PEA, *N*-palmitoyl-ethanolamine; 2-PG, 2-palmitoyl-glycerol; PG, prostaglandin; PLA_2, phospholipase A_2; PLC, phospholipase C; PPARα, peroxisome proliferator-activated receptor-α; PTPN22, protein tyrosine phosphatase N22; $sPLA_2$, soluble phospholipase A_2; TRPV1, transient receptor potential vanilloid type-1 channel. Arrows denote activation, transport, or transformation. Thicker arrows denote a more important role of the enzyme in the process. Adapted from: Veilleux et al. (2019) Curr Diab Rep. 19(11):117

named FAAH-2, has also been identified, although so far only in human tissues, and shown to prefer non-AEA long chain *N*-acylethanolamines as substrates.

In addition, a monoacylglycerol lipase (MAGL) was cloned from the rat and evidence for its role in 2-AG degradation in isolated cells was provided by the use of silencing RNA techniques (Fig. 2). The cloned MAGL seems to account for the vast majority of 2-AG-hydrolyzing activity in soluble fractions of rat brain, and other enzymes, such as alpha/beta-hydrolase 6 and 12, have been suggested to play a role in 2-AG inactivation under particular circumstances (Fig. 2). The cloned MAGL recognizes as substrates both *sn*-1 and -2-acylglycerols with almost any unsaturated long chain fatty acid esterified to the glycerol backbone, but is inactive with fatty acid amides. It is distributed in the CNS in the same brain regions as CB_1 receptors and is a presynaptic enzyme, in agreement with the necessity of inactivating 2-AG acting as a retrograde signal.

Enzymes of the arachidonate cascade can also recognize AEA and 2-AG as substrates, thereby producing bioactive metabolites that, in the case of cyclooxygenase-2, have been isolated in tissues, and named prostaglandin-ethanolamides (or prostamides) and prostaglandin-glycerol esters (Fig. 2). Neither of these types of metabolites act on cannabinoid or prostaglandin receptors, and both seem to produce biological effects via other yet-to-be-fully-characterized receptors (Di Marzo 2018).

Endocannabinoid Membrane Transport

AEA and 2-AG need to be transported across the membrane in order to interact with intracellular hydrolyzing enzymes (Ligresti et al. 2016). It is still being debated whether or not endocannabinoid uptake by, and release from, cells occurs via a membrane transporter, and not via simple passive diffusion. Intracellular degradation of AEA by FAAH was suggested to be the only drive behind its cellular uptake. However, strong indirect evidence exists for specific proteins facilitating the membrane transport of both AEA and 2-AG. Experiments carried out using cells from FAAH knockout mice, confocal microscopy to assess the spatial and functional separation between anandamide uptake and hydrolysis, and synthetic inhibitors capable to distinguish between FAAH and proteins responsible for the cell membrane binding/cellular uptake of AEA favor the existence of one or more specific proteins for AEA transport. Nevertheless, no such protein has been cloned or identified to date.

The Endocannabinoidome

The ever increasing realization that AEA and 2-AG: (i) modulate the activity of receptors other than CB_1 and CB_2, (ii) are produced and metabolized by concurrent and redundant pathways, and often converted to other bioactive lipids, and (iii) are accompanied in tissues by other long chain fatty acid derivatives that, like the congener *N*-acylethanolamines and 2-acylglycerols, share with endocannabinoids both biosynthetic and catabolic enzymes and pathways, as well some of their non-cannabinoid receptors (Fig. 2), or, like the *N*-acyl-aminoacids (or l*ipoaminoacids*), *N*-acyl-serotonins and *N*-acyl-dopamines, may share with endocannabinoids non-cannabinoid receptors and only in some cases catabolic enzymes, led to the notion of the "expanded endocannabinoid system" or "endocannabinoidome" (Di Marzo 2018). The endocannabinoidome is now thought to include hundreds of endocannabinoid-like molecules and tens of receptors and metabolic enzymes. Its existence complicates the development of therapeutic drugs from inhibitors of endocannabinoid biosynthetic or degrading enzymes, in as much as these substances may end up indirectly modifying the activity also of non-cannabinoid receptors.

Drugs

Pathological Conditions in Which Endocannabinoids Are Involved

Endocannabinoid levels often undergo dramatic tissue-specific changes in both animal models of disorders and in human diseases (Table 1) (Ligresti et al. 2016). During some acute pathological states or transient near-physiological perturbations of the normal homeostasis of the

organism, the levels of at least one endocannabinoid are elevated only in the tissues specifically involved in the disorder to help re-establishing the levels of other endogenous mediators. This occurs, for example, following insults or stressful stimuli ranging from brief food deprivation, restriction-induced stress, and retrieving of aversive memories to administration of acute painful stimuli, head injury, and ischemia. In the case of progressive/chronic disorders, endocannabinoid levels can become permanently elevated, although again in a tissue-selective way. This might lead again to homeostatic (e.g., anti-inflammatory or cell protective) effects in ways mediated by CB_1- or CB_2 receptors (or also by non-cannabinoid receptors), as in the case of some types of cancer (breast and colorectal carcinoma, glioma, etc.) or inflammatory gastrointestinal disorders (colitis, diverticular disease, celiac disease). However, a chronic over-activity of CB_1 receptors might eventually contribute to the development of also some of the symptoms typical of the disorder. This situation has been described for some experimental models of Parkinson's disease (PD) and Alzheimer's disease (AD), where the protective or disease exacerbating action of CB_1 receptor might depend on the phase and severity of disorder, and in models of genetic and diet-induced obesity, and of hepatic fibrosis, osteoporosis, paralytic ileus, and noxious hypotension. In the female reproductive system, a critical balance between anandamide biosynthesis and degradation in mouse embryos and oviducts creates the appropriate conditions for normal development of embryos and their oviductal transport, the malfunctioning of which possibly explains the association observed between lower FAAH/higher AEA levels in the blood of pregnant women and preterm abortion. Finally, a reduction of endocannabinoid levels is found in the brain of mice with experimental allergic encephalomyelitis, a mouse model of multiple sclerosis, in animal models of Huntington's chorea, and in patients with migraine. The subsequent impaired CB_1 signaling might explain some of the clinical hallmarks of these disorders (Table 1).

Endocannabinoids, Table 1 Select examples of observed alterations of endocannabinoid levels during pathological conditions and subsequent possible pharmacological interventions

Disorder	Effects on endocannabinoid levels	Potential drugs
Neurodegenerative/neuromotor disorders:		
1. Parkinson's disease (PD)	1. In a nonhuman primate model of PD endocannabinoid levels are elevated in the basal ganglia and may contribute to the generation of parkinsonian symptoms and/or to expression of levodopa-induced dyskinesia. The cerebrospinal fluid of untreated PD patients contains elevated levels of AEA	1. CB_1 antagonists or biosynthesis inhibitors
2. Alzheimer's disease (AD)	2. In the hippocampus of β-amyloid-treated rats, an animal model of AD, 2-AG levels are elevated and exert neuroprotection but also participate in memory retention loss	2. Inhibitors of degradation or CB_1 antagonists, possibly depending on the phase of the disorder
3. Amyotropic lateral sclerosis (ALS)	3. AEA and 2-AG increase in the spinal cord of SOD1 transgenic mice, a model of ALS, to inhibit disease progress	3. CB_2 receptor agonists or inhibitors of degradation
4. Multiple sclerosis (MS)	4. In rats with EAE, an animal model of multiple sclerosis, AEA and 2-AG levels are decreased in the striatum and midbrain. This might be associated with motor impairment. However, in mice with chronic relapsing EAE, endocannabinoid levels are increased in the brain and spinal cord concomitantly with the spasticity phase of the disorder, and this could represent an adaptive protective mechanism	4. Inhibitors of enzymatic hydrolysis and cellular re-uptake

(continued)

Endocannabinoids, Table 1 (continued)

Disorder	Effects on endocannabinoid levels	Potential drugs
Neuronal excitotoxicity	AEA levels are elevated in the hippocampus of mice treated with kainic acid. 2-AG levels are elevated in rats treated with pilocarpine. These are two animal models of epileptic seizures, where the endocannabinoids play an anticonvulsant and protective function	Inhibitors of enzymatic hydrolysis and cellular re-uptake
Neuropathic pain	Endocannabinoid levels are elevated in periaqueductal grey, rostral ventral medulla, and spinal cord during chronic constriction injury of the sciatic nerve in the rat, probably to inhibit pain	Inhibitors of enzymatic hydrolysis and cellular re-uptake
Gastrointestinal disorders:		
1. Colon inflammation	1. Endocannabinoid levels are elevated in the colon of DNBS-treated mice and in the colon submucosa of TNBS-treated rats, two animal models of inflammatory bowel diseases, and in the biopsies of patients with ulcerative colitis, possibly to control inflammation	1. Inhibitors of enzymatic hydrolysis and cellular re-uptake
2. Cholera toxin-induced diarrhea	2. Increased AEA levels after administration of cholera toxin to mice, a model of diarrhea, exert antisecretory action in the small intestine	2. Inhibitors of FAAH or AEA cellular reuptake
3. Diverticular disease	3. Increased AEA levels in colon strips from patients with diverticular disease participate in alterations of neural control of colon motility	3. None tested
4. Paralytic ileus	4. Increased AEA levels participate in inhibition of small intestine motility	4. CB_1 antagonists
5. Colorectal cancer	Endocannabinoid levels are elevated in the colon of azoxymethane-treated mice and the biopsies of patients colorectal cancer, possibly to counteract cancer cell proliferation	5. Inhibitors of enzymatic hydrolysis and cellular re-uptake
Eating and metabolic disorders:		
1. Anorexia nervosa (AN) and binge-eating disorder (BED)	1. Increased blood levels of AEA in patients with AN and BED may participate in reward aspects of aberrant eating behaviors	1. None tested
2. Obesity and hyperglycemia	2. 2-AG levels are elevated in mouse adipocytes and epididymal of mice with DIO. AEA and 2-AG levels are elevated in rat insulinoma β-cells, in pancreas of mice with DIO, and in obese women. Patients with obesity or hyperglycaemia caused by type 2 diabetes exhibit elevated levels of 2-AG or of both endocannabinoids in visceral fat or blood, respectively. AEA levels are elevated in the liver of DIO mice	2. CB_1 antagonists
Cancer	Elevated levels of AEA in glioblastomas, increased levels of 2-AG in meningiomas, with possible antitumor action	Inhibitors of degradation (both FAAH and cellular re-uptake)

Selected data are shown on those pathological conditions in which endocannabinoid levels are found to be altered in the tissues or organs affected by the disorder. Depending on the possible functional significance of these changes, pharmacological intervention with degradation inhibitors/agonists or with biosynthesis inhibitors/antagonists is suggested as a possible therapeutic strategy.
AEA anandamide, *2-AG* 2-arachidonoyl glycerol, *EAE* experimental autoimmune encephalomyelitis, *DIO* diet-induced obesity

Endocannabinoid-Based Drugs

Based on the role of endocannabinoids and cannabinoid receptors in several pathological conditions, the pharmacological manipulation of their levels or action is being developed as a therapeutic strategy. Enhancement of endocannabinoid signaling would be useful when this plays uniquely a protective role, whereas inhibition of endocannabinoid signaling would be beneficial in those conditions in which tissue endocannabinoids have lost the specificity of their pro-homeostatic action. These therapeutic strategies may be effected in a safer way using: (i) cannabis extracts in which the presence of nonpsychotropic cannabinoids with therapeutic activity per se mitigates the unwanted CB_1-mediated effects of THC and potentiates those of more therapeutic value, acting also through the modulation of proteins of the endocannabinoidome, (ii) synthetic compounds specific for CB_2 receptors, which seem to have less potential for addiction and unwanted central side effects than CB_1 receptors, (iii) inhibitors of endocannabinoid degradation or biosynthesis, which are likely to act more selectively in those tissues where there is an ongoing defective or excessive "tone" of endocannabinoids, but can also interfere with other endocannabinoidome mediators, and hence produce effects at other receptors, and (iv) positive or negative allosteric modulators of CB_1 and CB_2 receptors that, because acting preferentially in the presence of defective or excessive endocannabinoid levels, respectively, should preserve or restore the space- and time-selectivity of endocannabinoid pro-homeostatic action (Ligresti et al. 2016; Di Marzo 2018). The marketing in Europe of a CB_1 receptor antagonist/inverse agonist (rimonabant, Acomplia®) (Fig. 1), which proved to be very effective against obesity and the metabolic syndrome in several clinical trials, was interrupted in 2008 due to adverse psychiatric effects. These latter were likely due to the important pro-homestatic role played by brain CB1 receptors, which is also the reason why THC or its synthetic analogue, nabilone, have found so far limited use as therapeutic drugs. The current marketing in several countries of nabiximols, a standardized cannabis extract in which the psychotropic effects of THC are somehow tamed by approximately equal amounts of the non-psychotropic cannabinoid, cannabidiol, against spasticity and neuropathic pain in multiple sclerosis, and the recent approval in the USA and Europe of purified botanical cannabidiol, which has been suggested to act, among others, on several endocannabinoidome targets, against rare forms of untreatable pediatric epilepsies, demonstrate that it is possible to interact safely with the "expanded" endocannabinoid system (Pacher et al. 2019). Clinical studies are ongoing with more selective, synthetic drugs targeting receptors and metabolic enzymes of the endocannabinoid system in several disorders.

Cross-References

- ▶ Anti-obesity Drugs
- ▶ Appetite Control
- ▶ Inflammation

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Endogenous Agonists of Cannabinoid Receptors

▶ Endocannabinoids

Endoplasmic Reticulum Stress

► Unfolded Protein Response

Endothelins

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Definition

Endothelins comprise a family of three vasoactive isopeptides of 21 amino acids acting via two subtypes of endothelin receptors (ET_A and ET_B). Endothelins have an essential role in the regulation of the vascular tone, control of renal natriuresis, and neural crest development. Endothelins are also involved in nociception and may have a critical role in the progression of prostate and ovarian cancer. Several endothelin receptor antagonists are registered for the treatment of primary pulmonary arterial hypertension (PAH).

Basic Characteristics

Endothelin and Endothelin-Converting Enzymes

In 1985 a peptide was described in the supernatants of endothelial cells that mediated vasoconstriction. This peptide was isolated, sequenced, and the cDNA was cloned. According to its origin from endothelial cells, it was named endothelin (Yanagisawa et al. 1988).

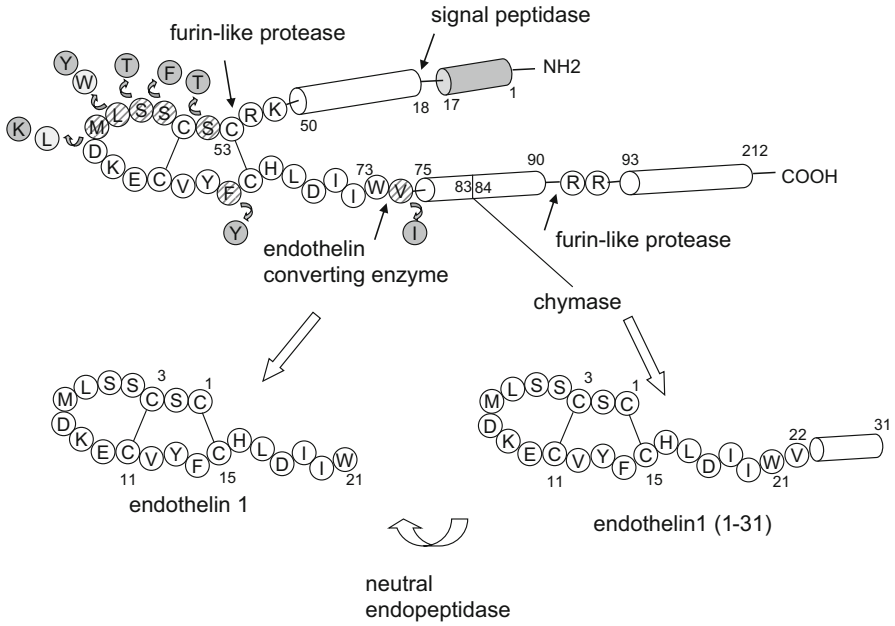
Three endothelin isoforms are known (ET-1, ET-2, ET-3), encoded by different genes, of which ET-1 is the most abundant in humans (Warner 2001). Endothelins are synthesized as prepropolypeptides of approximately 200 amino acids (Table 1). The biological active endothelins are generated in a two-step proteolytic process (Fig. 1). In the first step, furin-like proteases generate big-endothelins (big-ETs) of 38–41 amino acids that are biologically inactive. In a second step, specific endothelin-converting enzymes (ECEs) specifically cleave big-ETs between tryptophan 21 and valine/isoleucine 22, thereby producing the mature endothelins. Big-ET-1 was also found to be cleaved by mast cell chymase resulting in ET-1(1–31). While ET-1(1-31) is not a substrate of ECEs, it is further processed by neutral endopeptidase (NEP) to the biological active ET-1. Matrix metalloproteinase 2 (gelatinase A) also cleaves big ET-1 and yields ET-1(1–32) (Warner 2001). Whether ET-1(1-32) is also a substrate of NEP remains to be shown.

ECEs are metalloproteinases that are homologous to the neutral endopeptidase (NEP, E-24.11, neprilysin); unlike NEP, however, they form disulfide-bonded homodimers. In man, with ECE-1 and ECE-2, two isoforms are known, which are encoded by two separate genes (Warner 2001). For ECE-1, four different variants have been identified (ECE-1a–d), which are generated by alternative promoters. The ECE-1 isoforms only differ in their N-terminal amino acid sequence. For ECE-2, a single gene product has been described in man. In calf, an ECE-3 isoform has been isolated from iris microsomes and the choroid plexus, which is specifically involved in the conversion of big-ET-3 to ET-3. A human homologue of bovine ECE-3 has not been identified.

The ECE isoforms show different subcellular distributions and enzymatic characteristics. While ECE-1 activity is highest at neutral pH at 6.8–7.2, ECE-2 requires an acidic environment of pH = 5.0–5.5. ECE-1a and ECE-1c are mainly expressed at the cell surface, whereas ECE-1b, ECE-1d, and ECE-2 are expressed intracellularly. ECE-1b is found in the transGolgi network, late endosomes, multivesicular bodies, and secretory vesicles, ECE-1d in recycling endosomes, and ECE-2 in endosomes/lysosomes and autophagic vesicles. Plasma membrane-bound ECE cleaves bigET-1 circulating in the blood, whereas intracellular ECE isoforms are involved in the

Endothelins, Table 1 Human endothelin isoforms

	Number of amino acids in		Chromosome	Diseases
	prepro-ET-1	big ET-1		
hET1	212	38	6p23-24	The G allele at rs9349379 is associated with increased risk of CAD/MI but lower risk of migraine headache, cervical artery dissection, fibromuscular dysplasia, and hypertension. G allele at rs9349379 is associated with higher EDN1 expression and higher ET-1 secretion
hET2	178	38	1p34	
hET3	238	41	20q13.2-13.3	Hirschsprung's disease Waardenberg syndrome, type 4



Endothelins, Fig. 1 Processing of prepro-ET-1. ET-1 is generated as a preprohormone consisting of 212 amino acids. Amino acids 53 to 90 represent big-ET-1, amino acids 53–73 mature ET-1. Amino acids that vary between the three endothelin isoforms are depicted by hatched circles (only variant amino acids of the mature ET-1 moiety and of the ECE-cleavage site are indicated). The amino acids present in ET-2 and ET-3 are depicted by light or dark grey circles, respectively. The signal peptide (grey cylinder) is cleaved off in the endoplasmic reticulum by the signal peptidase. Big-ET-1 is generated by proteolytical processing (furin-like proteases), which recognize dibasic amino acids motifs. Mature ET-1 is formed after processing through the endothelin converting enzyme. Mast cell chymase generates ET-1(1-31) which is further processed by NEP to yield ET-1

generation of mature endothelins. In addition, ECEs (as well as NEP and the insulin-degrading enzyme) contribute to the degradation of amyloid β (Aβ) protein. The relevance of ECE-1, ECE-2, and NEP-mediated amyloid β breakdown and in the development or progression of Alzheimer's disease still remains elusive.

Human umbilical vein endothelial cells (HUVEC) express the isoforms ECE-1a, -1b, -1d, and ECE-2. In these cells, ET-1 is secreted *via* both a constitutive and a regulated pathway. The ratio of released ET-1:big ET-1 is 4:1. About 80% of the ET-1 is secreted at the abluminal cell surface of endothelial cells. ECE-isoforms are

abundantly expressed on the cell surface of endothelial cells and to a lower level also on vascular smooth muscle cells. In atherosclerotic lesions of vessels, however, ECE expression in smooth muscle cells is upregulated. ECE isoforms expressed in smooth muscle cells contribute significantly to the generation of mature ET in normal and in particular atherosclerotic vessels (Warner 2001).

Endothelial cells are the major source of ET-1-synthesis. ET-1 is also produced by astrocytes, neurons, hepatocytes, bronchial epithelial cells, and renal epithelial and mesangial cells. Physiological stimuli of ET-1-synthesis in endothelial cells are angiotensin II, catecholamines, thrombin, growth factors, insulin, hypoxia, and shear stress. Inhibitors of ET-1 synthesis are atrial natriuretic peptide, prostaglandin E2, and prostacyclin. ET-2 is mainly synthesized in kidney, intestine, myocardium, and placenta, and ET-3 is predominantly produced by neurons, astrocytes, and renal epithelial cells (Warner 2001).

Endothelin Receptors

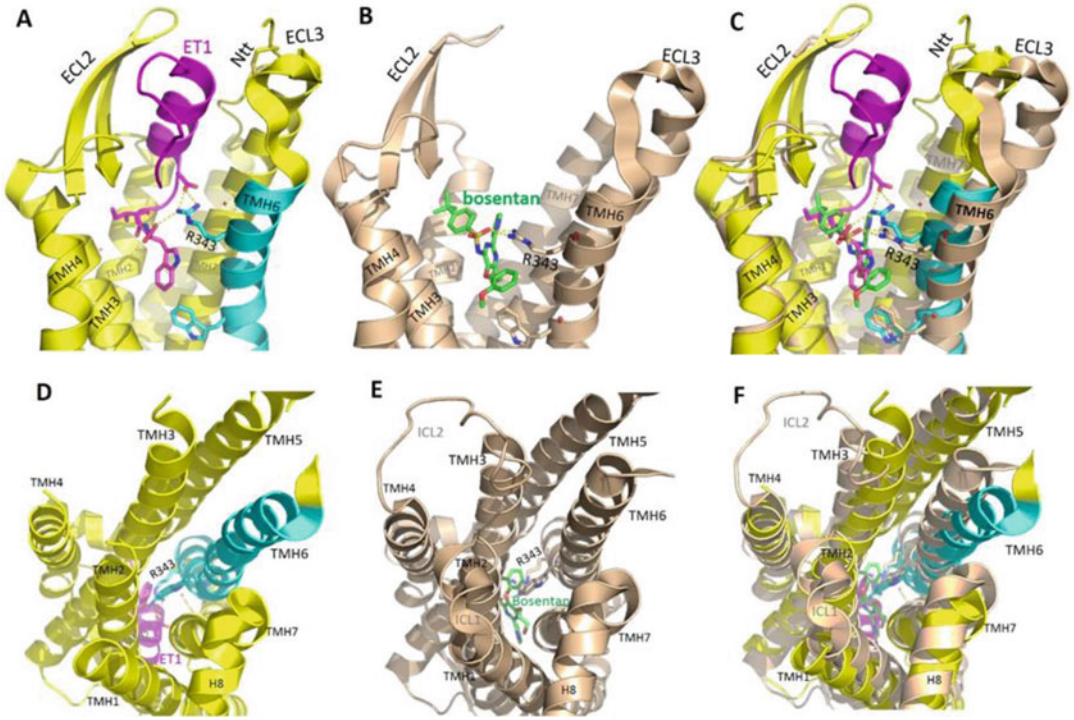
Endothelins exert their diverse actions *via* the two G protein-coupled receptors endothelin A (ET_A) and endothelin B (ET_B), which share an identity of about 64% in their amino acid sequences. Both receptors display a signal peptide that is required for the correct biogenesis. After the N terminus is accessible in the ER lumen, the signal peptide is cleaved off (comprises 20 and 26 amino acids in ET_A and ET_B receptors, respectively). Further posttranslational modifications are Asn-linked glycosylation of the extracellular N terminus (hET_A: Asn29, Asn 62; hET_B Asn 59) and palmitoylation of cysteine residues in the intracellular C terminus. Mass spectometry of the bovine ET_B receptor revealed that cysteine residues 402 and 404 are palmitoylated. The palmitoylation of endothelin receptors is essential for the activation of G proteins, since palmitoylation-deficient ET_A and ET_B receptors fail to stimulate $G_{q/11}$ and G_i proteins, respectively. Crystal structure of the ET_B receptor has demonstrated two disulfide bonds between cysteine residues of the 1st (ET_B: Cys174) and the 2nd extracellular loop (ET_B: Cys255) as well as between cysteine residues in the extracellular N terminus (ET_B: Cys90) and the 3rd extracellular loop (ET_B: Cys359) (Shihoya et al. 2016, 2017). All four cysteine residues are conserved in the ET_A receptor suggesting that two disulfide bonds are formed between the N terminus and the 3rd extracellular loop (Cys69 and Cys341, respectively) and the 1st and 2nd extracellular loop (Cys158 and Cys239, respectively). The extracellular N terminus of the ET_B receptor undergoes agonist-induced limited proteolysis. Upon binding of ET-1 the N terminal 38 amino acids are cleaved off by a metalloprotease. The cleavage involves the single Asn-linked glycosylation site, resulting in a nonglycosylated receptor. The functional role for the N-terminal cleavage is not known, yet. However, genetic deletion of the N terminus or proteolytic removal results in an ET_B receptor with an altered signal transduction.

The endothelin receptor subtypes show differences in their signal transduction, ligand binding, and tissue distribution. The ET_A receptor is isopeptide-selective and binds ET-1 and ET-2 with the same and ET-3 with 70–100 fold lower affinity. The ET_B receptor binds all three isoforms with the same affinity (Warner 2001). Pharmacological studies provided evidence for two subtypes of ET_A (ET_{A1}, ET_{A2}) and ET_B receptors (ET_{B1}, ET_{B2}), although genetic studies revealed only two different genes. Thus, the additional receptor subtypes may be derived from (i) alternative splicing, (ii) differences in posttranslational processing, or (iii) protein-protein interactions. For the ET_B receptor, splice variants have been described. However, these isoforms most likely do not account for the postulated second receptor subtype. One splice variant harbors 10 additional amino acids in the 3rd intracellular loop and has normal binding characteristics and functional activity (IP and cAMP formation). The second splice variant, which carries a completely altered intracellular C terminus, has normal binding properties but lacks functional activity. In vitro, homo- and heterodimerization of endothelin receptor subtypes has been demonstrated, but these receptor-receptor interactions are unlikely to account for the observed subtypes of ET_A and ET_B receptors: Pharmacological binding profiles of ET_A/ET_B receptor heterodimers

were essentially similar to those of individually expressed ET_A and ET_B receptors.

The identification of the crystal structures of the ET_B receptor bound to ET-1 and of the receptor bound to the antagonist bosentan allows conclusions about the molecular mechanisms of ET_B receptor activation and inhibition (Shihoya et al. 2016, 2017). The N-terminal tail, the three extracellular loops (ECL1–ECL3), and the six TM helices (TM2–TM7) form the binding pocket for ET-1 (Fig. 2a). The C-terminal tail of ET-1 binds to the ET_B receptor between the transmembrane helices (TMH) interacting tightly with Arg343 (6.55) in TM6, thereby causing a strong attraction of TM6 at the extracellular side. At the intracellular side, an outward movement occurs due to a helix-kink in of TM6 (Fig. 2d) allowing the interaction with the G protein. The antagonist bosentan (Fig. 2b) occupies the site similar to the C-terminal tail of ET-1, but prevents the close attraction of TMH6 and thereby the intracellular outward movement of TM6 (Fig. 2e) restraining the ET_B receptor in its inactive state. The relative movement of TM6 at the extra- and intracellular areas is visualized by overlaying ET-1 and bosentan-bound structures (Fig. 2c, f).

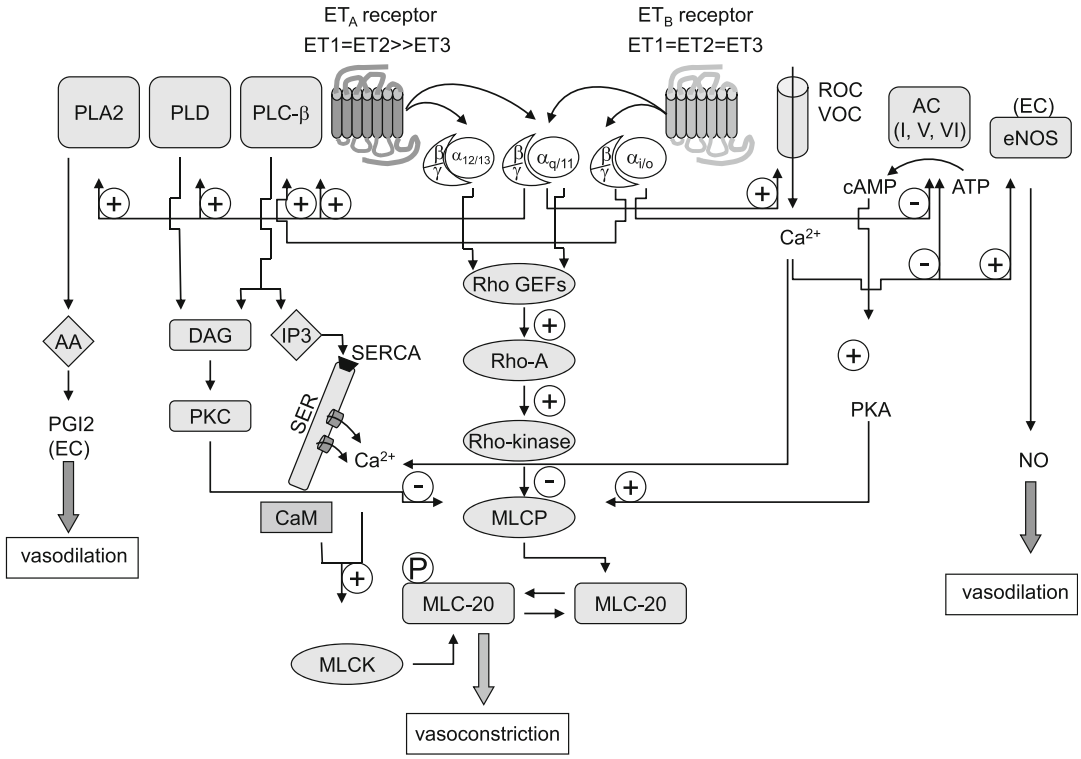
The ET_A receptor activates G proteins of the $G_{q/11}$ and $G_{12/13}$ family. The ET_B receptor stimulates G proteins of the G_i and $G_{q/11}$ family (Fig. 3). In endothelial cells, activation of the ET_B receptor



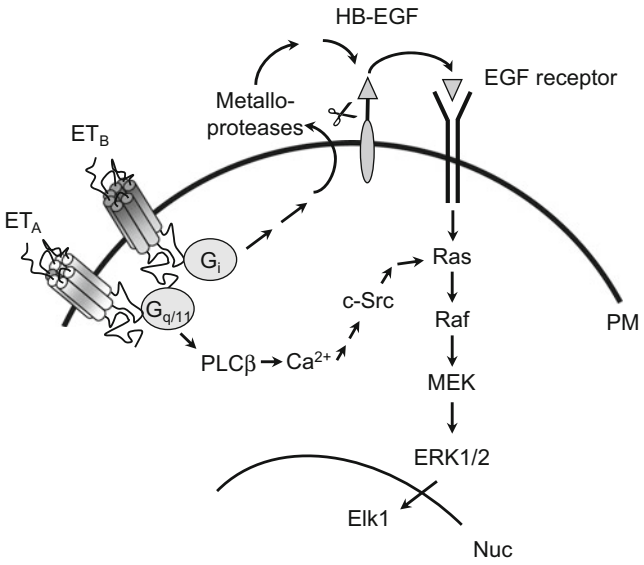
Endothelins, Fig. 2 Crystal structures of ET-1-bound and bosentan-bound ET_B-receptors. (**a–c**) Side views onto the extracellular and upper transmembrane (TM) region of the ligand-bound ET_B receptors. TM helix 5 is not depicted for clarity. (**a**) PDB: 5GLH Active conformation of the ET_B receptor (yellow with TM6 depicted in cyan) with agonist ET-1 (magenta). (**b**) PDB: 5XPR Inactive conformation of the ET_B receptor (beige) with antagonist bosentan (green). (**c**) Overlay of both crystal structures to visualize relative movement of TM6 by the agonist-bound state: ET-1 attracts transmembrane helix TM6 inwards (cyan) via R343 compared to antagonist bosentan (TM6 depicted in beige). (**d–f**) Respective views of the ligand-bound ET_B receptors from the intracellular side. (**d**) ET-1-bound state results in an intracellular outward movement of TM6 due to the kinked TM6 (cyan) opening a crevice that enables G-Protein binding. (**f**) Overlay of both crystal structures to visualize the outward movement of TM6 (cyan) in the ET-1 bound state compared to the bosentan-bound conformation (beige)

stimulates the release of NO and prostacyclin (PGI_2) *via* pertussis toxin-sensitive G proteins. In smooth muscle cells, the activation of ET_A receptors leads to an increase of intracellular calcium *via* pertussis toxin-insensitive G proteins of the $G_{q/11}$ family and to an activation of Rho proteins most likely *via* G proteins of the $G_{12/13}$ family. Increase of intracellular calcium results in a calmodulin-dependent activation of the myosin light chain kinase (MLCK, Fig. 3). MLCK phosphorylates the 20 kD myosin light chain (MLC-20), which then stimulates actin-myosin interaction of vascular smooth muscle cells resulting in vasoconstriction. Since activated Rho inhibits the myosin light chain phosphatase *via* Rho-kinase, the dephosphorylation of the MLC-20 is blocked. The dual action of the ET_A receptor signaling on MLC-20 results in a robust vasoconstriction of vessels. Beside the short-term effects such as vasodilation and vasoconstriction, endothelin receptors also stimulate long-term events (cell growth and differentiation). While stimulation of the ET_A receptor results in a transient, monophasic activation of ERK1/2 via a $G_{q/11}$-proteins, activation of the ET_B receptor elicits a long-lasting, bisphasic ERK1/2 activation involving a $G_{q/11}$- and a G_i-mediated pathway (Fig. 4) (Table 2).

ET-1 also stimulates antiapoptotic signal cascades in fibroblasts, vascular smooth muscle, and



Endothelins, Fig. 3 Summary of short-term signaling events mediated by endothelin. In endothelial cells (EC), the activation of the ET_B receptor leads to the formation of prostacyclin (PGI_2) and NO, thereby eliciting vasodilation. In vascular smooth muscle cells, activation of the ET_A receptor results in a robust vasoconstriction by a dual regulation of the 20 kD myosin light chain (MLC-20). *AA* arachidonic acid, *AC* adenylyl cyclase, *ATP* adenosine triphosphate, *CaM* Calmodulin, *cAMP* cyclic adenosine monophosphate, *DAG* diacyl glycerol, *eNOS* endothelial NO synthase, *IP3* inositol-3,4,5-phosphate, *MLC-20* 20 kD myosin light chain, *MLCP* myosin light chain phosphatase, *Rho-GEF* Rho-guanine nucleotide exchange factor, *SER* smooth endoplasmic reticulum, *IP3R* IP3 receptor, *PKA* protein kinase A, *PKC* protein kinase C, *PLA* phospholipase A, *PLC* phospholipase C, *PLD* phospholipase D



Endothelins, Fig. 4 Diagram of ET_A and ET_B receptor-mediated ERK1/2 activation. ET_A and ET_B receptor stimulate a transient ERK1/2 activation via G proteins of the $G_{q/11}$/PLCβ/Ca^{2+}/c-Src–dependent pathway. In addition, the ET_B receptor stimulates a second, long-lasting phase of ERK1/2 activation via a G_i protein/metalloprotease/HB-EGF/EGF receptor-dependent pathway. *ERK2* extracellular signal-regulated kinase, *HB-EGF* heparin-binding epidermal growth factor, *Nuc* nucleus, *PM* plasma membrane

endothelial cells (*via* phosphatidylinositol-3-kinase and Akt/protein kinase B). In prostate and ovarian cancer, upregulation of endothelin synthesis and ET_A receptors has been associated with a progression of the disease. The inhibition of ET_A receptors results in a reduced tumor growth (Nelson et al. 1995). In malignant melanoma, ET_B receptors are associated with tumor progression. Endothelins can also stimulate apoptosis in stretch-activated vessels *via* the ET_B receptor, which contrasts the above-mentioned effects. The molecular basis for these differential anti- and pro-apoptotic reactions mediated by endothelins remains elusive.

Activation of matrix metalloproteinases (MMP) is also involved in vascular and cardiac remodeling. For example, the fibrillar collagen matrix of the heart maintains the shape of the left ventricle. If the delicate balance between matrix deposition and degradation is altered, cardiac fibrosis (increase of collagen synthesis) or left ventricular remodeling (increase of degradation) occurs. Activation of ET_A receptors leads to a stimulation of MMP-1, -2, and -9 in isolated myocytes and in the myocardium and thereby contributes to ventricular remodeling after myocardial infarction.

Sites of Endothelin Receptor Expression

ET_A receptors are expressed in the smooth muscle cells of the vascular medial layer and the airways, in cardiac myocytes, lung parenchyma, bronchiolar epithelial cells, prostate epithelial cells, astrocytes, and sensory neurons. ET_B receptors are expressed in endothelial cells, in bronchiolar smooth muscle cells, vascular smooth muscle cells of certain vessels (e.g., saphenous vein, internal mammary artery), keratinocytes, in epithelial cells of renal proximal and distal tubules, the renal collecting duct, in the cells of the atrioventricular conducting system and in astrocytes.

In addition, ET_B receptors are upregulated in vessels with atherosclerotic lesions and in pulmonary vessels of patients with severe pulmonary hypertension. The upregulation can be attributed

Endothelins, Table 2 Summary of endothelin receptor antagonists or monoclonal antibodies directed against endothelin receptors registered or in clinical development (as of November 2019)

Drug	Stage of development	Antagonist/inhibitor of	Indication(s)
Bosentan	Registered	ET_A/ET_B receptor	Approved: PAH, Eisenmenger syndrome, Digital ulcers in Scleroderma
Ambrisentan	Registered	ET_A receptor	Approved: PAH also in combination with tadalafil for PAH. Ongoing: treatment of acute kidney injury after renal transplantation, hepatorenal disorders in patients with severe liver failure, portal hypertension in patients with liver cirrhosis
Macitentan	Registered	ET_A/ET_B receptor	Approved: PAH, Pre-registration (EU): CTEPH. Ongoing: Congenital heart defect; Cardiac failure
Sparsentan	Phase 3	dual AT_1 and ET_A receptor antagonist (DARA)	Ongoing: IgA nephropathy; Focal segmental glomerulosclerosis
Clazosentan	Phase 3	ET_A receptor	Ongoing: Intracranial vasospasm following aneurysmal subarachnoid hemorrhage
Aprocitentan	Phase 3	ET_A/ET_B receptor	Ongoing: Essential hypertension; Resistant hypertension
Zibotentan	Phase 2	ET_A receptor	Ongoing: Alzheimer's disease; Intermittent claudication; Scleroderma
GMA-301	Phase 1	ET_A receptor-targeted humanized IgG4 mAb	Ongoing: PAH
PMZ-2123	Clinical	ET_A receptor	Ongoing: Diabetic ketoacidosis, pain and opiate dependence
Sitaxsentan	Withdrawn	ET_A receptor	Withdrawn in 2010 due to risk of fatal liver injury (was approved in 2006 for PAH)

CTEPH chronic thromboembolic pulmonary hypertension, *mAb* monoclonal antibody, *PAH* pulmonary arterial hypertension

to increased ET_B receptor expression in smooth muscle cells and to ET_B receptors expressed on infiltrating macrophages.

In the vascular system, endothelial ET_B receptors mediate a transient vasodilation, whereas ET_A receptors cause a long-lasting vasoconstriction (Fig. 3). The role of ET_B receptors expressed on smooth muscle cells remains elusive. In some vessels, ET_B receptor stimulation causes vasoconstriction which is, however, only of transient nature and quantitatively much lower than that following ET_A receptor activation. In the kidney, ET_A receptors, which are almost exclusively expressed in vessels, regulate renal circulation, whereas ET_B receptors, expressed in the proximal and distal tubule and in the collecting duct, are involved in natriuresis and diuresis (Gupta et al. 2017). The main natriuretic action occurs most likely *via* the inhibition of the amiloride-sensitive sodium channel (ENaC). In the lung, endothelin causes long-lasting vaso- and bronchoconstriction. The contribution of each receptor subtype to the endothelin-evoked pulmonary responses is still controversial. The current data suggest that in the healthy lung ET_A receptors are involved primarily in pulmonary vasoconstriction and ET_B receptors in bronchoconstriction. In the central nervous system, ET_A receptors are expressed on smooth muscle cells of large and small cerebral arteries. In addition, ET_A receptors were also found to be expressed on endothelial cells isolated from capillaries and larger microvessels of the brain, but the physiological role of ET_A receptor expression in endothelial cells in the brain remains elusive. Further, ET_A receptors are expressed in small sensory C fibers. Activation of ET_A receptors in C fibers causes sensitization of tetrodotoxin-resistant sodium channels as well as of the polymodal receptor TRPV1. Interestingly, stimulation of ET_B receptors expressed in keratinocytes leads to the release of β-endorphines, thereby counter-acting ET_A receptor-mediated pain (Khodorova et al. 2003). Neurons, in particular those of the level III and IV of the cortex predominately express ET_B receptors. In isolated astrocytes, both receptor subtypes were found to be expressed at the cell surface.

Genetic Studies and Human Diseases

Mice homozygous for an ET_A receptor gene disruption show craniofacial malformations, such as cleft palate, micrognathia, microtia, and microglossia. ET_A (-/-) mice die shortly after birth due to respiratory failure. Mice with an ET-1-null mutation show the same cranciofacial malformations and, in addition, cardiovascular disorders (e.g., septal defects, abnormal cardial outflow tract, aortic arch, and subclavian arteries).

Mice with a disruption of the ET-3 or the ET_B receptor gene display pigment disorder and a megacolon. These disorders resemble isolated congenital megacolon (Hirschsprung's disease) or congenital megacolon associated with pigment disorders and cochlear hearing problems (Type 4 Waardenberg syndrome) can be caused by inactivating mutations in the ET-3 and the ET_B receptor gene (about 5% of patients with Hirschsprung's disease have ET_B receptor gene mutations). The lack of ET-3/ET_B receptor results in the absence of parasympathic ganglionic neurons in the myenteric plexus (Auerbach). Mice with an ET-3/ET_B receptor disruption die within two weeks after birth. In transgenic mice, in which the expression of the ET_B receptor is driven by the dopamine-β-hydroxylase promoter, normal myenteric plexus are present and no enteric disorder develops. These mice, however, show a salt-sensitive hypertension, which can be efficiently treated with amiloride, indicating that ET_B receptors are involved in the regulation of natriuresis *via* the amilorid-sensitive sodium channel ENaC.

Genome-wide association studies identified a significant association of intracranial aneurysm with rs6841581 immediately upstream of the EDNRA gene (encoding the ET_A receptor), linking endothelins to intracranial aneurysm pathogenesis. Other studies identified the gene encoding phosphatase and actin regulatory protein 1 (PHACTR1; 6p24) as a risk for coronary artery disease (CAD, increased risk) as well as migraine headache, cervical artery dissection,

fibromuscular dysplasia, and hypertension (reduced risk). By genetic fine mapping, epigenomic profiling, and CRISPR/Cas9 genome editing, G allele at rs9349379 was identified as the causal variant, resulting in higher EDN1 expression (Gupta et al. 2017). The increase in CAD risk, but reduced risk for hypertension, may be attributable to the different actions via ET_A and ET_B receptors, for example, ET_A receptor mediated vasoconstriction and vascular smooth muscle cell proliferation and ET_B receptor-mediated vasodilation and natriuresis and diuresis.

The genetically engineered disruption of the ECE1 gene causes craniofacial and cardiovascular malformations (ET-1/ET_A receptor phenotype), congenital megacolon and pigment disorders (ET-3/ET_B receptor phenotype). The ECE-2 (–/–) mice do not display any abnormality, indicating that ECE-1 is of crucial importance in embryonic development. Strikingly, ECE-1 (–/–) mice and ECE-1 (–/–)/ECE-2 (–/–) mice still have about 60% of wild-type ET-1 levels. This result indicates that alternative pathways in the generation of mature ET-1 exist (e.g., NEP). This alternatively generated ET-1, however, cannot compensate for the embryogenic defects (Yanagisawa et al. 2000).

ECE isoforms are also involved in the degradation of amyloid Aβ protein. A genetic variant of ECE-1 with an increased transcriptional activity is associated with a decreased risk for AD. Thus, the inhibition of ECE in the CNS may increase the risk for the development of Alzheimer's disease (AD).

In malignant prostate epithelial cells, auto- and paracrine release of ET-1 is a critical factor in ET_A receptor-mediated proliferation (Nelson et al. 1995). In addition, the ET-1/ET_A receptor axis has emerged as a potential target in prostate cancer bone metastasis.

Drugs

Clinical Use

Endothelins mediate potent vasoconstrictor effects. A great number of ECE-inhibitors and mixed and selective ET_A and ET_B receptor antagonists have been developed in the past (e.g., acute and chronic heart failure, pulmonary hypertension, subarachnoid hemorrhage, chronic kidney disease, and prostate and ovarian cancer).

For specific inhibitors of ECE, however, only very limited effects on the endothelin system were found and there is no ongoing development reported. The limited potency of ECE inhibition might be due to the generation of mature ET-1 from big-ET-1 by other proteases such as neutral endopeptidase or other currently unidentified proteases. Dual inhibition of ECE and NEP might inhibit ET-1 generation more efficiently; however, dual inhibition of ECE and NEP could also increase the risk for the development of AD, as both enzyme classes are involved in the degradation of Aβ protein.

In the case of receptor antagonists, it is still unknown whether mixed antagonism of endothelin receptors or selective blockade of ET_A receptors is of greater benefit in the treatment of diseases. At present three endothelin receptors have been approved for treatment of pulmonary arterial hypertension (PAH) of which ambrisentan is a selective ET_A receptor antagonists and bosentan and macitentan are mixed ET_A/ET_B receptor antagonists (Table 2). The current data suggest that ET_A receptor is critical for the treatment effect and inhibition of the ET_B receptor may have some additional benefit (due to upregulation of ET_B receptors in pulmonary vascular smooth muscle). However, no direct comparisons of ET_A receptor vs mixed ET_A/ET_B receptor antagonists have been done to explore this question. Several clinical trials with selective ET_A receptor and mixed ET_A/ET_B receptor antagonists are ongoing involving Alzheimer's disease, intermittent claudication, scleroderma, and subarachnoid hemorrhage. Further notable developments are the first dual AT1 and ET_A receptor antagonist (DARA) in IgA nephropathy and focal segmental glomerulosclerosis and the first monoclonal antibody directed against the ET_A receptor in PAH (Table 2). Oncological studies in ovarian and prostate

cancer as well as nonsmall cell lung cancer were investigated in phase 2 and 3 clinical studies but have been discontinued following failures to meet the primary endpoints.

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Enoxaparin

▶ Heparin and Related Drugs

Eosinophils

▶ Interleukin-5

Eph Receptors and Ephrins

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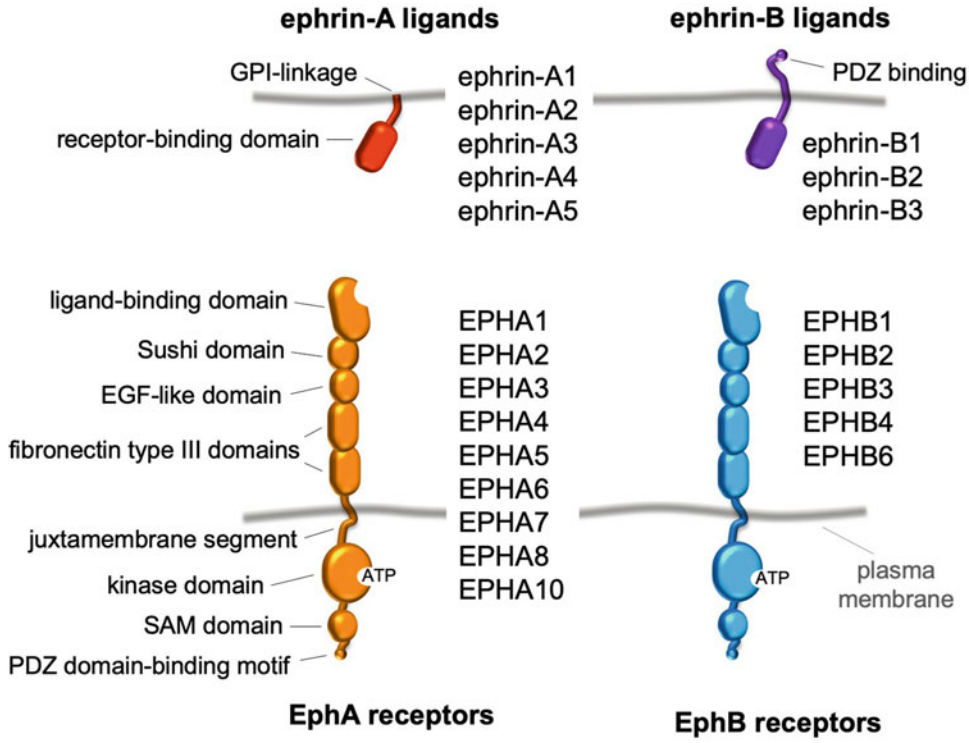
E

Synonyms

EFNA1, ephrinA1, ephrin-A1; EFNA2, ephrinA2, ephrin-A2; EFNA3, ephrinA3, ephrin-A3; EFNA4, ephrinA4, ephrin-A4; EFNA5, ephrinA5, ephrin-A5; EFNB1, ephrinB1, ephrin-B1; EFNB2, ephrinB2, ephrin-B2; EFNB3, ephrinB3, ephrin-B3; EPHA1; EPHA2; EPHA3; EPHA4; EPHA5; EPHA6; EPHA7; EPHA8; EPHA10; EPHB1; EPHB2; EPHB3; EPHB4; EPHB6

Definition

The Eph receptors are the largest of the tyrosine kinase families. There are nine EphA and five EphB receptors, subdivided based on sequence similarities in their kinase domains (Fig. 1) (Pasquale 2005; Kania and Klein 2016). The subdivision also reflects ligand preferences. The activating ligands for the Eph receptors are the ephrins (*Eph* *r*eceptor-*in*teracting proteins), which include five ephrin-As and three ephrin-Bs (Fig. 1). The EphA receptors preferentially bind the ephrin-A ligands, and the EphB receptors preferentially bind the ephrin-B ligands, but some interactions can also occur between the A and B subfamilies of receptors and ligands. Eph receptors and/or ephrins are expressed in most, if not all, cell types and represent an important cell contact-dependent communication system that regulates many physiological and pathological



Eph Receptors and Ephrins, Fig. 1 Schematic illustrating the domain structure of Eph receptors and ephrins. Both ephrins and Eph receptors are subdivided into A and B subfamilies, whose members are indicated. The ephrin-binding pocket is shown as a cavity in the ligand-binding domain of the Eph receptors, and the ATP-binding pocket in the kinase domain is also indicated (ATP)

processes. Therefore, there is strong interest in developing pharmacological agents capable of targeting them for medical applications.

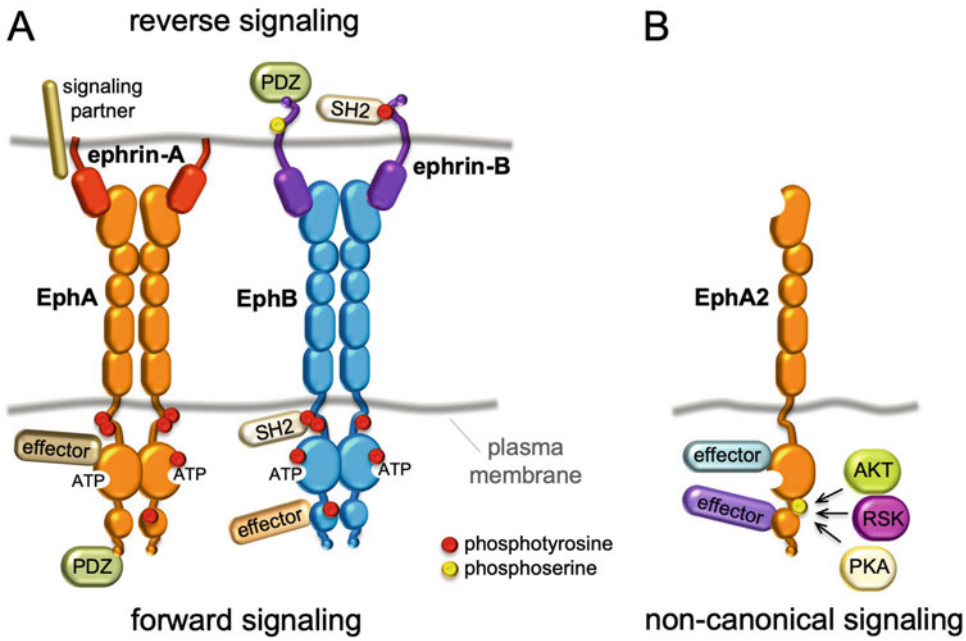
Basic Characteristics

Domain Structure

All Eph receptors have the same domain structure, including an extracellular region containing multiple domains, a single transmembrane helix, and a cytoplasmic region containing the tyrosine kinase domain (Fig. 1). The kinase domains of the EPHA10 and EPHB6 receptors lack residues needed for catalytic activity and are therefore inactive. The ligand-binding domains of the Eph receptors contain a pocket that interacts with a loop from the receptor-binding domain of the ephrins. In the ephrin-As, this domain is followed by a linker and a glycosylphosphatidylinositol (GPI) group mediating attachment to the plasma membrane (Fig. 1). The ephrin-Bs contain a transmembrane helix and a short cytoplasmic region. Alternatively spliced variants and proteolytically cleaved forms of Eph receptors and ephrins increase structural and functional diversity (Lisabeth et al. 2013; Barquilla and Pasquale 2015).

Signal Transduction

Eph receptors and ephrins interact with each other *in trans* across intercellular junctions, which leads to the lateral dimerization and oligomerization of multiple Eph receptor/ephrin complexes and downstream signaling (Pasquale 2005; Kania and Klein 2016). Complexes assembled at sites of cell-cell contact mediate bidirectional signals that affect the behavior of both interacting cells



Eph Receptors and Ephrins, Fig. 2 Eph receptor and ephrin signaling. (**a**) Bidirectional signaling. Eph receptors interact with ephrins across cell-cell junctions. The interaction leads to dimerization of Eph receptor/ephrin complexes, which then can assemble into larger oligomers (not shown). Dimerization and oligomerization cause receptor autophosphorylation on tyrosine residues and increase in kinase activity and binding of signaling effectors resulting in forward signaling. The ephrin-B ligands can also be phosphorylated, for example, on tyrosine residues by associated SRC family kinases and on serine residues by serine/threonine kinases, resulting in reverse signaling. Ephrin-A ligands can also mediate reverse signaling through association with transmembrane signaling partners. (**b**) EPHA2 non-canonical signaling through S897 phosphorylation. The AKT, RSK, and PKA serine/threonine kinases trigger EPHA2 non-canonical signaling through phosphorylation of S897 in the linker connecting the kinase and SAM domains of the receptor. SH2, protein containing an SH2 domain that interacts with an Eph receptor/ephrin-B tyrosine-phosphorylated motif; PDZ, protein containing a PDZ domain that interacts with a binding motif in the C-terminus of an Eph receptor or ephrin-B; effector, protein that transduces downstream signals

(known as "forward signals" for the Eph receptors and "reverse signals" for the ephrins; Fig. 2A). Eph/ephrin complexes can also mediate unidirectional signaling, for example, when proteolytically cleaved ephrin-As function as soluble ligands or when Eph receptors or ephrins are deployed on extracellular vesicles (such as exosomes) to interact with distant cells.

Eph Receptor Signaling

Eph receptor dimerization/oligomerization induced by ephrin binding enables cross-phosphorylation of neighboring receptor molecules (Fig. 2A) (Boyd et al. 2014; Barquilla and Pasquale 2015; Kania and Klein 2016). Three of the resulting tyrosine autophosphorylation sites, two in the juxtamembrane segment and one in the activation loop of the kinase domain (Figs. 1 and 2A), are important for Eph receptor kinase activity. In addition, the phosphorylated tyrosines create binding sites for a variety of effectors, including kinases, phosphatases, guanine nucleotide exchange factors, GTPase-activating proteins, adaptors, and scaffolding proteins (Fig. 2A). A carboxy-terminal PDZ domain-binding motif present in most Eph receptors also mediates association with binding partners that regulate Eph receptor localization and function (Figs. 1 and 2A). In some cases, the Eph receptors regulate the activity of downstream effectors by phosphorylating them.

Like other receptor tyrosine kinase families, the Eph receptors control the activity of central cellular signaling networks, such as those involving the RAS-ERK MAP kinase cascade, PI3-kinase-AKT-mTORC1, ABL-CRK, NF-kB, and the RHO small GTPase family (Pasquale 2010; Boyd et al. 2014; Kania and Klein 2016). However, Eph receptor forward signaling has distinctive characteristics. For example, in many cell types, the Eph receptors inhibit ERK and AKT, whereas other receptor tyrosine kinases typically activate them. Furthermore, while ephrins expressed by neighboring cells promote Eph receptor forward signaling, ephrins co-expressed on the same cell surface can attenuate forward signaling through lateral *cis* interactions with the Eph receptors (Lisabeth et al. 2013).

Besides their "canonical" forward signaling activities, the Eph receptors are capable of additional "non-canonical" forms of signaling. For example, the linker connecting the kinase and SAM domains of EPHA1 and EPHA2 contains a serine (S897 in EPHA2), which is not present in other Eph receptors. The phosphorylation of this serine by kinases such as RSK, AKT, and PKA is responsible for a "non-canonical" form of EPHA2 signaling that does not require ligand binding or kinase activity (Barquilla and Pasquale 2015; Zhou and Sakurai 2017) (Fig. 2B). However, it is not well understood how S897 phosphorylation regulates EPHA2 downstream signaling pathways, such as those involving ephexin4 and leading to the activation of the RAC RHO family GTPase and the PI3-kinase-AKT pathway (Lisabeth et al. 2013).

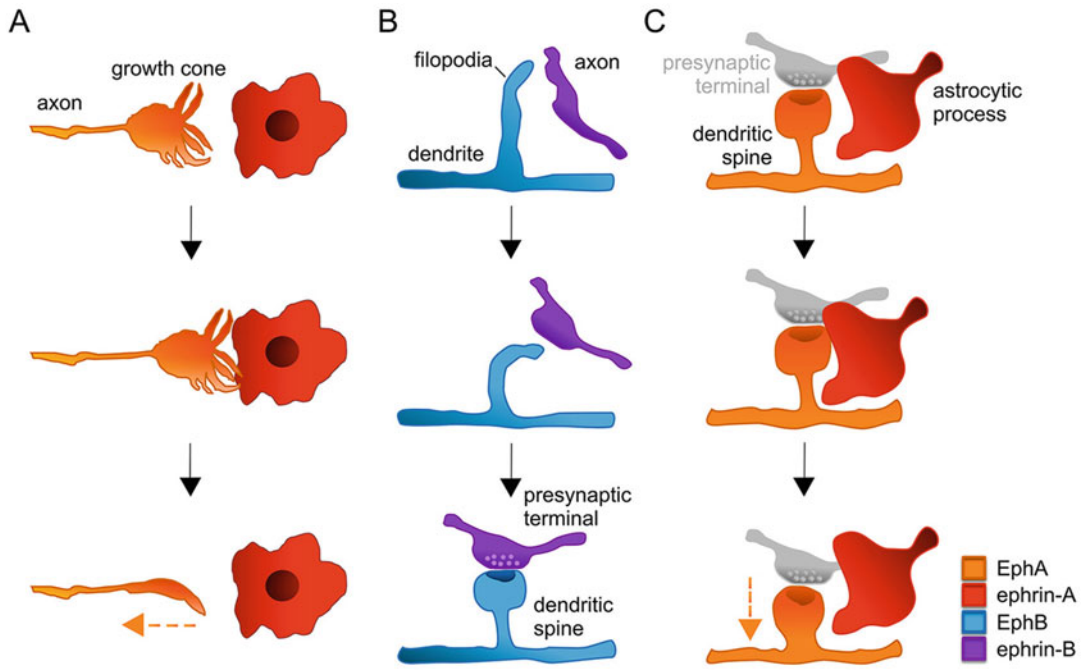
Other Eph receptors are endowed with ligand- and/or kinase-independent signaling ability, which can cause different effects than forward signaling (Pasquale 2010; Boyd et al. 2014). For example, EPHA10 and EPHB6 can signal despite lacking kinase activity (Liang et al. 2019). Their kinase domains can still bind ATP, which regulates folding of the domain and thus the non-catalytic signaling functions of these two pseudokinases. EPHA10 and EPHB6 can also be phosphorylated by other tyrosine kinases, such as other Eph receptors or SRC family kinases, to serve as scaffolds for downstream signaling effectors. In addition, association with EPHA10 and EPHB6 changes the signaling output of other Eph receptors (Liang et al. 2019).

Ephrin Signaling

The ephrin-As do not have a cytoplasmic portion, but can nevertheless transmit reverse signals through association with transmembrane co-receptors such as the RET and TRKB receptor tyrosine kinases and the p75 neurotrophin receptor (Fig. 2A) (Barquilla and Pasquale 2015; Kania and Klein 2016). Upon interaction with EphB receptors, the ephrin-B cytoplasmic region can be phosphorylated on tyrosine and serine residues by various kinases (Fig. 2A), including SRC family kinases. The resulting phosphorylated motifs regulate interactions with effector molecules such as NCK2, STAT3, and RHO family GTPases (Coulthard et al. 2012; Lisabeth et al. 2013; Boyd et al. 2014). Interactions mediated by their carboxy-terminal PDZ domain-binding motif also contribute to ephrin-B reverse signaling (Fig. 2A). Forms of ephrin-B signaling that do not depend on EphB receptor binding have also been reported and involve phosphorylation by several receptor tyrosine kinases or physical association with other transmembrane proteins such as Claudin-5 (Pasquale 2005; Barquilla and Pasquale 2015).

Cellular Effects of Eph Receptor/Ephrin Signaling

Eph receptors and ephrins regulate cell morphology and motility through their effects on the actin cytoskeleton, cell-substrate adhesion, and cell-cell interaction (Pasquale 2005, 2010; Boyd et al. 2014; Kania and Klein 2016). Cell repulsion is the most characteristic response observed after encounters of two cells in which Eph receptors and ephrins engage across adjacent plasma membranes, initiating forward and reverse signaling. These repulsive effects cause one or both of the cells to locally retract, leading to the separation of the two plasma membranes that were initially in contact (Fig. 3). They play a role, for example, in "contact-inhibition of locomotion," cell sorting and growth cone collapse. The repulsive effects are counterintuitive, because Eph receptor-ephrin complexes can mediate a strong adhesion between two cells. Two mechanisms have been described



Eph Receptors and Ephrins, Fig. 3 Eph receptor/ephrin signaling regulates the formation and remodeling of neuronal connections. (**a**) Growth cone collapse. When an Eph receptor-expressing growth cone at the tip of a growing axon encounters an ephrin-expressing cell, the growth cone collapses and the axon may retract before resuming growth in a different direction. (**b**) Dendritic spine formation. When an EphB-expressing filopodial protrusion on a dendrite comes in contact with an ephrin-B-expressing axon, it may form a synaptic connection. In the case of excitatory synapses, EphB forward signaling in the postsynaptic terminal leads to shortening of the filopodium and its maturation into a dendritic spine, characterized by an enlarged head and a narrow neck. (**c**) Dendritic spine remodeling through neuron-astrocyte interaction. When a dendritic spine expressing EPHA4 comes in contact with a perisynaptic astrocyte process expressing ephrin-A3, it locally retracts and may shorten or collapse

that enable cell separation. The first involves extracellular proteolytic cleavage of Eph receptor/ephrin complexes, abrogating their ability to connect two cells. In the second, a cell internalizes vesicles containing intact Eph receptor/ephrin complexes by removing a portion of the plasma membrane from the adjacent cell. In certain cellular contexts, however, Eph receptors and ephrins can also promote cell-cell adhesion (e.g., when repulsive signaling is weak). Other activities of forward and/or reverse signaling include regulating cell proliferation, survival, and metabolism. EPHA2 non-canonical signaling through S897 phosphorylation elicits very different responses, for example, promoting epithelial-mesenchymal transition and cell migration (Barquilla and Pasquale 2015; Zhou and Sakurai 2017).

Physiological and Pathological Functions

Nervous System

Eph receptors and ephrins are crucial mediators of cell-cell communication in the developing and adult nervous system, where many of them are highly expressed. During neural development, the Eph/ephrin system controls neurogenesis, cell sorting and the formation of tissue boundaries, neuronal migration, axon guidance, and the establishment and pruning of neuronal connections (synapses) (Kania and Klein 2016; Henderson and Dalva 2018). A well-known role is to guide the development of highly ordered neuronal circuits by regulating both the position of neurons and the formation of precisely organized connections between them (Pasquale 2005; Kania and

Klein 2016). One of the first recognized roles of Eph receptors and ephrins is as positional labels that regulate the establishment of topographic neuronal connections, such as those in the visual system. Neurons in the retina extend long axons to reach their synaptic partners in the visual centers of the brain. The spatial organization of the retinal neurons is maintained in their connections, so that axons from neighboring retinal neurons form synapses with neighboring brain neurons. This is in part controlled by gradients of Eph receptor and ephrin expression in the retina and the visual centers of the brain. For example, retinal growth cones with high EphA receptor expression avoid regions with high ephrin expression due to the repulsive effects mediated by forward signaling (Fig. 3A).

The Eph/ephrin system also controls the ability of axons to cross the body midline and form connections in the contralateral side. For instance, EPHA4 knockout mice display a hopping gait caused by the inappropriate midline crossing of spinal cord axons, due to their inability to sense the repulsive effects of ephrins expressed at the midline (Kania and Klein 2016). In addition, inactivation of a number of Eph receptor and ephrin genes in the mouse results in anatomical abnormalities in the axonal structures that connect the left and right sides of the brain and spinal cord (commissures). Eph receptor/ephrin repulsive effects also play an important role in the proper development of other long-distance axonal projections, such as the motor axons connecting the brain with the spinal cord or the spinal cord with muscles.

Eph receptors and ephrins also play an important role in the formation of synaptic connections between axons and dendrites. EphB signaling increases the motility of dendritic filopodial protrusions, facilitating contact with ephrin-B-expressing axons (Pasquale 2008; Henderson and Dalva 2018) (Fig. 3B). The contacts may then be stabilized, or lost due to retraction of the filopodium, depending on the kinetics of EphB forward signaling. The interaction between EphB receptors and ephrin-Bs, which can both be expressed either pre- or postsynaptically depending on the type of synapse, also promotes the maturation of functional presynaptic terminals and of the knob-like postsynaptic structures known as dendritic spines (Pasquale 2008; Lisabeth et al. 2013; Kania and Klein 2016; Henderson and Dalva 2018) (Fig. 3B). Dendritic spines are characteristic of excitatory synapses, and their shape is functionally important in the regulation of synaptic transmission by compartmentalizing the postsynaptic space from the dendritic shaft.

Once dendritic spines mature, the repulsive action of the EphA/ephrin-A system regulates their morphology (Fig. 3C). EPHA4 is expressed in mature dendritic spines and interacts with ephrin-A3 in perisynaptic astrocytic processes (Lisabeth et al. 2013; Kania and Klein 2016; Henderson and Dalva 2018). This leads to localized repulsive effects that regulate dendritic spine remodeling. Interestingly, reverse signaling through ephrin-A3 in the astrocyte processes decreases the levels of transporters that take up the neurotransmitter glutamate released during synaptic transmission. This effect is critical for activity-dependent synaptic plasticity, which underlies learning and memory formation.

Eph receptors and ephrins continue to be expressed in the adult nervous system, although typically at lower levels than during development. Besides its role in synapse formation, in the adult nervous system, the EphB/ephrin-B system also regulates synaptic plasticity through effects on ion channels and the actin cytoskeleton (Pasquale 2008; Kania and Klein 2016; Henderson and Dalva 2018). Thus, both EPHA and EPHB receptors are crucial for synaptic plasticity. In fact, many studies implicate EphB/ephrin-B upregulation in the abnormal synaptic plasticity that occurs as a consequence of nerve damage and causes neuropathic pain (Barquilla and Pasquale 2015; Henderson and Dalva 2018). Furthermore, Eph receptors and ephrins are upregulated in multiple cell types in the injured nervous system, where their repulsive effects can negatively affect neural repair and nerve regeneration. Indeed, lowering EPHA4 expression or activity in rodent models can promote functional recovery after stroke and muscle reinnervation after nerve injury (Boyd et al. 2014; Barquilla and Pasquale 2015; Ling et al. 2018). Thus, Eph

receptors and ephrins are considered promising therapeutic targets to treat chronic pain and promote neural repair.

Epithelia

A number of Eph receptors and ephrins are expressed in epithelial cells, for example, in the skin, the lens, and the intestine. In the skin, the Eph/ephrin system plays a role in organizing the stratified layers of proliferating and differentiating keratinocytes, with EPHA2 and ephrin-A1 being particularly important for epidermal homeostasis (Lin et al. 2012). Multiple Eph receptors and ephrins are also expressed in hair follicles, where they affect stem cell position, self-renewal, and differentiation (Lin et al. 2012; Boyd et al. 2014). In the eye, EPHA2 and ephrin-A5 have been implicated in maintaining the integrity of cell-cell junctions in the lens epithelium, which is critical for the transparency of the lens (Lisabeth et al. 2013). Indeed, mutations impairing EPHA2 function can lead to cataracts. The EphB/ephrin-B system plays a critical role in the intestine, where epithelial cells are generated from EPHB2-expressing stem cells at the bottom of the crypts and migrate over several days toward the tips of the villi, before being shed (Pasquale 2008). As the cells migrate and differentiate, they lose EphB expression and acquire ephrin-B expression. The resulting opposite gradients of EphB receptors (highest in newly generated cells in the crypts) and ephrin-Bs (highest in the differentiated cells near the tip of the villi) control the position of the epithelial cells as they migrate along the crypt-villus axis.

Cardiovascular and Lymphatic Systems

Eph receptors and ephrins play critical roles in the development and homeostasis of the cardiovascular and lymphatic systems (Barquilla and Pasquale 2015; Boyd et al. 2014). This is exemplified by the early embryonic lethal phenotype due to severe defects in heart and blood vessel development, caused by inactivation of the EPHB4 or ephrin-B2 genes in the mouse (Boyd et al. 2014; Zeng et al. 2019). Inactivation of other Eph receptor and ephrin genes in the mouse also causes various abnormalities in heart development and function, suggesting roles for multiple family members in different aspects of cardiac development (Barquilla and Pasquale 2015; Boyd et al. 2014).

In most of the vasculature, EPHB4 and ephrin-B2 define venous and arterial endothelial cells, respectively (Boyd et al. 2014; Finney and Orr 2018). Consistent with the importance of these genes, EPHB4 and ephrin-B2 mutations in humans and gene inactivation in animal models have been linked to severe cerebrovascular malformations (Zeng et al. 2019). The EphB/ephrin-B system, particularly ephrin-B2, is also critical for the development of the lymphatic system (Boyd et al. 2014). In the adult, ephrin-A1/EPHA2 and ephrin-B2/EPHB4 are the most important family members that promote pathological forms of angiogenesis, including tumor angiogenesis (Barquilla and Pasquale 2015; Boyd et al. 2014). Interestingly, ephrin-B2 promotes endocytosis of the VEGFR2 and VEGFR3 vascular endothelial growth factor receptors, which surprisingly enhances the signaling ability of these receptors in the vasculature (Barquilla and Pasquale 2015).

Members of the Eph receptor and ephrin families are also expressed in perivascular support cells, such as pericytes and smooth muscle cells, and, in the brain, astrocytes (Barquilla and Pasquale 2015; Finney and Orr 2018). In these cells, they mediate interactions with endothelial cells, which is critical for vascular development and mature vessel integrity. In fact, disruption of Eph receptor/ephrin function in vascular support cells can destabilize blood vessels, leading to hemorrhage or increasing permeability, for example, in cancer, inflammatory processes, and possibly neurodegenerative diseases. In the adult, the EphB/ephrin-B system can also affect blood pressure through regulation of vascular smooth muscle contractility (Finney and Orr 2018; Zeng et al. 2019). In addition, EPHA2 is expressed in multiple cell types involved in the development of atherosclerotic plaques and contributes to disease pathology, thus representing a potentially important target against cardiovascular disease (Funk and Orr 2013; Finney and Orr 2018).

Immune System

Multiple Eph receptors and ephrins have been implicated in immune cell development, trafficking throughout the body, and activation (Pasquale 2008; Darling and Lamb 2019). Eph receptor/ephrin bidirectional signaling is particularly well-suited to play a role in the cell-cell communication processes that are critical for immune cell development and function. For example, the interaction of ephrin-B2 in hematopoietic progenitor cells with EPHB4 in bone marrow stromal cells promotes the release of progenitor cells from the bone marrow into the circulation. Furthermore, Eph/ephrin system-mediated communication between the hematopoietic progenitors that colonize the thymus (thymocytes) and thymic stromal cells controls thymocyte trafficking within the thymus, survival, maturation into T cells, and T cell selection (Pasquale 2008). In lymphoid organs, the interplay between ephrin-B1 expressed by germinal center B lymphocytes and EPHB4/EPHB6 expressed by follicular T helper cells regulates activated T cell trafficking and cytokine secretion and thus B lymphocyte selection and efficient plasma cell formation/affinity maturation during immune responses (Darling and Lamb 2019). EPHB receptors and ephrin-Bs are widely expressed in subsets of T cells and can enhance T cell receptor signaling (Pasquale 2008; Darling and Lamb 2019). High ephrin-B1 and ephrin-B2 levels, however, have also been linked to negative feedback mechanisms that dampen T cell receptor signaling. The EphA/ephrin-A system regulates T cell differentiation and trafficking. In addition, Eph receptor-/ephrin-mediated leukocyte interactions with endothelial cells enable extravasation across the blood vessel wall during routine immune surveillance and in response to damage or infection (Funk and Orr 2013). Furthermore, EPHA2 promotes blood vessel permeability during inflammation, and various other Eph receptors and ephrins play a role in diseases involving inflammatory processes, such as atherosclerosis (see above), inflammatory bowel disease, and arthritis (Coulthard et al. 2012; Funk and Orr 2013; Darling and Lamb 2019).

Other Functions

Given the large number of Eph receptors and ephrins and their widespread expression in most cell types, the Eph/ephrin system has a multitude of diverse physiological and pathological functions besides those outlined above (Pasquale 2008; Boyd et al. 2014). For example, EphA forward signaling and ephrin-A reverse signaling in pancreatic cells play opposite roles in the regulation of glucose homeostasis (Pasquale 2008). In addition, both A and B Eph receptors and ephrins play a role in bone remodeling through the regulation of both osteoblasts and osteoclasts (Pasquale 2008; Boyd et al. 2014). In some cases, the effects of human mutations have revealed unexpected functions. For example, EPHB2 mutations have been linked to functional defects in platelets causing severe bleeding (Darling and Lamb 2019), and ephrin-B1-inactivating mutations cause craniofrontonasal syndrome, a disease involving defects in the fusion of bones in the developing skull (Boyd et al. 2014). In addition, as mentioned above, EPHA2 mutations cause cataracts (Lisabeth et al. 2013), and EPHB4 mutations cause venous malformations (Zeng et al. 2019).

Diseases

Cancer

Many Eph receptors and ephrins, and most notably EPHA2 and EPHB4, are aberrantly expressed in cancer cells, cancer stem cells, and the tumor microenvironment (Pasquale 2010; Boyd et al. 2014; Barquilla and Pasquale 2015). Their activities affect cancer progression through a multitude of complex mechanisms and signaling pathways that can promote but also inhibit cancer malignancy. The effects of Eph receptor/ephrin signaling in tumors depend on the cellular context and interplay with other signaling systems, such as the epidermal growth factor and fibroblast growth factor receptor families, integrins, oncogenic pathways, and metabolic networks.

An opposite regulation of Eph receptor versus ephrin expression in cancer cells often results in low activation of bidirectional signaling (Pasquale 2010). This is consistent with the tumor-

suppressive activities that can be elicited by Eph receptor forward signaling, such as inhibition of the AKT and ERK oncogenic pathways or integrin-mediated adhesion. In addition, forward signaling induced in cancer cells by ephrins present in normal cells surrounding a tumor can limit tumor expansion through repulsive effects. For example, EphB/ephrin-B signaling plays a tumor-suppressive role in the intestine, where precancerous lesions (adenomas) typically express EphB receptors but form in ephrin-B-expressing regions (Pasquale 2010; Barquilla and Pasquale 2015; Kania and Klein 2016). As a consequence, loss of EphB receptor expression can drive colorectal cancer progression. Furthermore, cell repulsion triggered by an imbalance of EPHA2 forward signaling in an epithelial monolayer can lead to the extrusion of a cell in which EPHA2 is upregulated by HRAS (Porazinski et al. 2016). This may represent a safeguard against development of tumors originating from cells that acquire an oncogenic RAS mutation. On the other hand, repulsion between tumor cells expressing Eph receptors and ephrins can lead to their dispersion, increasing the metastatic potential of these more resilient cells (Barquilla and Pasquale 2015).

Eph receptors can promote tumorigenesis through non-canonical signaling mechanisms that do not involve kinase activity (Pasquale 2010; Boyd et al. 2014; Barquilla and Pasquale 2015). This is exemplified by EPHA2 signaling through S897 phosphorylation, which has been shown to promote invasiveness, metastasis, cancer stem cell properties, and drug resistance. Non-canonical oncogenic signaling activities of other Eph receptors have also been documented, but are not as widely studied. Reverse signaling can also promote cancer malignancy. For example, ephrin-Bs have been linked to increased invasiveness in glioblastoma.

Typically multiple Eph receptors are co-expressed in cancer cells and likely assemble together into oligomers whose signaling effects may depend on the Eph receptor composition and mutational status (Boyd et al. 2014). The Eph receptors are highly mutated in cancer, although mutational hotspots are rare and most mutations are heterozygous. Some of the mutations have been shown to disrupt receptor functions such as ligand binding and kinase activity, supporting a tumor-suppressing role for forward signaling (Lisabeth et al. 2013; Barquilla and Pasquale 2015). However, the functional role of most Eph receptor cancer mutations and the extent of their contribution to cancer progression remain to be demonstrated. It will be interesting to determine whether heterozygous mutations in different Eph receptors have synergistic effects in signaling clusters comprising multiple receptors.

Tumors can hijack the physiological activities of the Eph/ephrin system. A well-known example involves tumor angiogenesis, which supports tumor progression, particularly through the activities of ephrin-A1/EPHA2 and ephrin-B2/EPHB4 (Pasquale 2010; Boyd et al. 2014; Barquilla and Pasquale 2015). Another role that is increasingly attracting attention is the modulation of anti-tumor immune responses through interactions of immune cells with various tumor cellular components.

Neurodegeneration

An increasing number of studies suggest that deregulation of Eph receptor/ephrin signaling plays a role in neurodegenerative diseases (Boyd et al. 2014; Barquilla and Pasquale 2015; Henderson and Dalva 2018). For instance, EPHA4 was identified as a modifier gene in amyotrophic lateral sclerosis (ALS), a fatal disease involving a progressive motor neuron degeneration. Studies in ALS animal models suggest that decreasing EPHA4 expression or pharmacological inhibition of EPHA4-ephrin interaction could be of therapeutic benefit. Consistent with this, low EPHA4 mRNA levels or EPHA4 loss-of-function mutations in ALS patients correlate with late disease onset and prolonged survival.

Excessive EPHA4 signaling has also been implicated in Alzheimer's disease, which is characterized by cognitive decline due to a progressive loss of synapses and neurons (Boyd et al. 2014; Barquilla and Pasquale 2015; Henderson and Dalva 2018). Elevated EPHA4 tyrosine phosphorylation has been detected in the brain of Alzheimer's patients and mouse models.

Interestingly, β-amyloid oligomers, which are characteristic of Alzheimer's brain pathology, promote EPHA4 activation leading to neurotoxicity in culture. Furthermore, inhibiting EPHA4 expression or function ameliorates disease pathology in cell culture and mouse Alzheimer's models. In contrast, β-amyloid oligomers cause EPHB2 loss, which contributes to disease pathology since restoration of EPHB2 levels or pharmacological inhibition of EPHB2 interaction with β-amyloid can reverse cognitive and behavioral defects in mouse Alzheimer's models. Thus, it could be therapeutically beneficial to inhibit EPHA4 and increase EPHB2 activity. Several single nucleotide polymorphisms in non-coding regions of the EPHA1 gene and missense mutations in the coding region have been linked to late-onset Alzheimer's disease. However, EPHA1 has limited expression in neural cells, and the pathological role of the polymorphisms and mutations remains to be determined.

Single nucleotide polymorphisms in several Eph receptors have also been associated with Parkinson's disease, a neurodegenerative disorder characterized by motor and cognitive symptoms caused by a progressive loss of dopaminergic neurons (Boyd et al. 2014; Barquilla and Pasquale 2015). In addition, a study has shown that a soluble form of ephrin-A1 (ephrin-A1 Fc) promotes the regeneration of the brain dopaminergic neurons that are lost in a rat Parkinson's model, suggesting a possible strategy for therapeutic intervention. Overall, additional studies are needed to better understand the role of the Eph/ephrin system in neurodegenerative diseases and its therapeutic potential.

Parasitic Infections

EPHA2 can enable or facilitate infections by a wide variety of parasites, including (1) viruses such as Kaposi's sarcoma-associated herpesvirus (KSHV; an oncogenic virus that causes Kaposi's sarcoma and B lymphocyte malignancies), the hepatitis C virus, and the Epstein-Barr virus (which causes mononucleosis and several cancers); (2) the malaria parasite *Plasmodium*; (3) bacterial pathogens such as *Chlamydia* and *Staphylococcus*; and (4) fungal pathogens such as *Candida albicans* and *Cryptococcus neoformans* (Pasquale 2008; Barquilla and Pasquale 2015; Darling and Lamb 2019). EPHA2 may be particularly suitable to promote the uptake of parasites into host cells because of its propensity to move inside the cell by endocytosis or macropinocytosis when bound to a ligand (or a pathogen that mimics a ligand). Non-canonical EPHA2 signaling through S897 phosphorylation has also been implicated in some infections, although the precise mechanism remains to be determined. In some cases EPHA2 has additional roles besides serving as an entry receptor or co-receptor for a pathogen. For example, EPHA2 non-canonical signaling through S897 phosphorylation supports the growth and survival of *Chlamydia*-infected cells as well as promotes proinflammatory responses in *Candida*-infected epithelial cells and antifungal responses in neutrophils. EPHA2 also helps *Cryptococcus* cross the blood-brain barrier. Additional studies are also beginning to implicate other EphA receptors in infectious diseases (Darling and Lamb 2019).

Among the ephrins, ephrin-B2 and ephrin-B3 serve as entry receptors for several henipaviruses, a zoonotic family of viruses that in humans cause highly lethal respiratory and encephalitic pathologies (Pasquale 2008; Barquilla and Pasquale 2015). The preferential expression of the two ephrins in the brain and blood vessels and their conservation in mammals explain the tropism of these viruses and their ability to cross from bats and farm animals to humans.

Drugs

The aberrant expression and activities of Eph receptors and ephrins in many disease processes suggest that they could be exploited as therapeutic targets for a variety of applications. Accordingly, significant progress has been made in the development and preclinical/clinical evaluation of agents that target them.

One of the first strategies used to modulate the function of the Eph/ephrin system is with the recombinant Eph receptor or ephrin extracellular portions (Pasquale 2010; Boyd et al. 2014; Barquilla and Pasquale 2015). In monomeric form, these engineered proteins inhibit both forward and reverse signaling by interfering with endogenous Eph receptors-ephrin interactions (except for the monomeric ephrin-As, which can activate EphA receptors). Furthermore, they target multiple family members due to the promiscuity of Eph receptor-ephrin interactions, which may in some cases increase their effectiveness. To prolong their lifetime in the blood circulation, Eph receptor/ephrin extracellular regions have been coupled to the Fc portion of an antibody or to serum albumin. Fusion to Fc results in dimerization, which increases potency due to higher binding avidity and can confer agonistic properties. For example, ephrin-A1 Fc or ephrin-B2 Fc administered in vivo can inhibit tumor growth and malignancy in glioblastoma and breast cancer mouse xenograft models, respectively, by promoting the tumor-suppressing effects of Eph receptor forward signaling. As another example, EphA Fc proteins have demonstrated anti-cancer activity in mouse tumor models by inhibiting the angiogenic effects of EPHA2 forward signaling in the tumor vasculature (by blocking EPHA2 activation by endogenous ephrin-As). In addition, a portion of the EPHA7 extracellular region can block the oncogenic effects of EPHA2 forward signaling in B cell lymphoma, and fusion of the EPHA7 fragment with the B lymphocyte-targeting anti-CD20 antibody rituximab increases the therapeutic potential of both agents. On the other hand, EPHB4 fused to human serum albumin (sEPHB4-HSA) is under evaluation in multiple cancer clinical trials, alone or in combination with other treatments, as an inhibitor of the EphB/ephrin-B system (clinicaltrials.gov) (Lodola et al. 2017).

Monoclonal antibodies represent another targeting strategy (Pasquale 2010; Boyd et al. 2014; Barquilla and Pasquale 2015; Lodola et al. 2017). Antibodies can exhibit high binding affinity, specificity, and long in vivo half-life. Activating and inhibitory antibodies that recognize Eph receptor/ephrin extracellular regions have been developed, mainly for applications against cancer and pathological angiogenesis. In several preclinical mouse cancer models, activating antibodies targeting EPHA2 can inhibit tumor growth by promoting anti-oncogenic EPHA2 forward signaling and/or EPHA2 degradation. In addition, a humanized EPHA3-activating antibody lacking fucosylation to increase antibody-dependent cellular cytotoxicity (KB004/ifabotuzumab) is cytotoxic for different types of leukemia cells. This antibody is also effective against solid tumors by disrupting tumor stroma and vasculature, which express EPHA3 (Boyd et al. 2014), and is under early clinical testing in cancer patients with promising results (clinicaltrials.gov). Antibodies targeting Eph receptors have also been conjugated to chemotherapeutic agents to enable selective tumor targeting and receptor-mediated drug internalization. For example, EPHA2 antibodies coupled to derivatives of the cytotoxic drug auristatin can inhibit tumor growth and reduce metastasis in several mouse cancer xenografts models. However, toxicity due to severe bleeding was observed with the fully human MEDI-547 EPHA2 antibody-auristatin conjugate in a phase I clinical trial (clinicaltrials.gov). In addition, a humanized antibody to ephrin-A4 (PF-06647263), coupled to the DNA-damaging agent calicheamicin to target tumor-initiating cells in triple negative breast cancer and ovarian cancer, achieved sustained tumor regression in mice carrying patient-derived tumor xenografts (Lodola et al. 2017). A phase 1 clinical trial demonstrated manageable safety of this antibody-drug conjugate but limited anti-tumor activity (clinicaltrials.gov). Antibodies coupled to radioisotopes have also been successfully used in mouse cancer models for in vivo imaging of tumor cells and for targeted delivery of the radioisotope for anti-cancer therapy (Lodola et al. 2017).

Additional therapeutic efforts have aimed at redirecting immune cells to target Eph receptors, and particularly EPHA2, in tumors (Boyd et al.

2014; Barquilla and Pasquale 2015). For example, a bispecific antibody that simultaneously targets EPHA2 and the T cell receptor/CD3 complex can cause T cell-mediated destruction of EPHA2-positive cancer cells in vitro and decreased tumor growth in vivo. Chimeric antigen receptor (CAR) T cells targeting EPHA2-expressing cancer cells have also been successfully developed (Darling and Lamb 2019).

Peptides are also useful for targeting Eph receptors with high selectivity and for modulating their activities (Noberini et al. 2012; Riedl and Pasquale 2015). Several peptides (approximately 10–15 amino acids long) bind specifically to the ephrin-binding pocket of different Eph receptors and antagonize ephrin binding. Information from X-ray crystal structures of peptide-Eph receptor complexes has guided optimization efforts that have yielded peptides specifically targeting the EPHA2, EPHA4, and EPHB4 receptors with low nanomolar affinity. Some of these peptides, or their modified derivatives, are highly resistant to proteases and have substantial in vivo half-life. Most of the peptides are antagonists that inhibit forward signaling (and reverse signaling). Peptide antagonists targeting EPHA4 have applications for the treatment of neurodegenerative diseases and to promote nerve regeneration after injury (Boyd et al. 2014; Riedl and Pasquale 2015). Peptide antagonists targeting EPHA2 and EPHB4 have applications for inhibition of tumor angiogenesis. Interestingly, some of the EPHA2-targeting peptides are agonists that mimic the ephrin ligands in promoting receptor activation and internalization/degradation even in their monomeric form (Riedl and Pasquale 2015). Thus, these peptides could be useful to promote EPHA2 tumor suppressing forward signals and EPHA2 downregulation as well as to facilitate the uptake of chemotherapeutic drugs by tumor cells. Indeed, peptides that bind to EPHA2 or EPHB4 have been successfully used in mouse cancer models to enhance the targeted delivery of drugs, such as paclitaxel or doxorubicin, as well as of nanoparticles loaded with drugs and/or imaging agents (Pasquale 2010; Barquilla and Pasquale 2015; Riedl and Pasquale 2015).

Small molecules that bind to the ephrin-binding pocket or the ATP-binding pocket of Eph receptors (Fig. 1) represent yet another strategy to modulate the Eph/ephrin system. Some small molecules targeting the ephrin-binding pocket have been identified, but typically they have low potency and selectivity for a specific Eph receptor. These characteristics can be improved, but at the cost of an increase in size beyond that of a small molecule (consistent with the large size and flexibility of the targeted pocket) (Noberini et al. 2012; Lodola et al. 2017). Many small molecules that target the ATP-binding pocket in the Eph receptor kinase domain are also known (Noberini et al. 2012; Lodola et al. 2017). These compounds are kinase inhibitors that can bind with nanomolar affinity, but generally exhibit poor selectivity and target multiple kinases (in fact most of these compounds were identified in screens using kinase targets other than Eph receptors). However, high selectivity may not always be desirable, since multi-kinase inhibitors can be more effective, if their toxicity is manageable. Several kinase inhibitors that include Eph receptors among their targets are under evaluation in cancer clinical trials, including dasatinib, tesevatinib/XL647, nilotinib, and bosutinib (Boyd et al. 2014) (clinicaltrials.gov). The inhibitory effects of these compounds on Eph receptor forward signaling should inhibit tumor angiogenesis, but may in some cases promote cancer cell malignancy. Nilotinib and bosutinib are also under evaluation in clinical trials for Alzheimer's disease and other forms of dementia (clinicaltrials.gov), although they do not efficiently cross the blood-brain barrier. The ability of these compounds to inhibit EPHA4 and the downstream ABL and SRC family kinases could provide therapeutic benefits in neurodegeneration, although the contribution of Eph receptor targeting to their therapeutic effects remains to be determined. More selective Eph receptor inhibitors could be obtained in dedicated screens in which an Eph receptor is the primary target, in conjunction with structure-guided and

computational optimization approaches (Noberini et al. 2012).

Small interfering RNAs (siRNAs) and antisense oligonucleotides targeting Eph receptors or ephrins also show promise for the treatment of cancers and neurodegenerative disorders, respectively. Although the efficient in vivo delivery of nucleic acids can present challenges, these agents are highly selective and eliminate all activities of the targeted molecule. In promising examples, delivery of EPHA2 siRNA using liposomes or nanoparticles inhibits tumor growth and metastasis in mouse ovarian cancer xenograft models, particularly in combination with other treatments (Pasquale 2010; Boyd et al. 2014). The EPHA2 siRNA liposomes are now under evaluation in a phase I clinical trial (clinicaltrials.gov). For the central nervous system, a single intracerebroventricular administration of EphA4-antisense oligonucleotides in mice can substantially reduce EphA4 levels in the brain and spinal cord for prolonged periods, promoting reinnervation and functional recovery after sciatic nerve injury (Ling et al. 2018). However, this antisense treatment has not shown beneficial effects in mouse ALS models.

Ongoing efforts to translate Eph receptor/ephrin-targeting pharmacological agents into clinical candidates focus on improving potency, selectivity, and in vivo stability. The complex and incompletely understood biology of the Eph/ephrin system represents a major challenge in predicting the therapeutic or toxic effects of modulatory agents. However, new research findings combined with the results from preclinical and clinical studies increasingly inform the development and refinement of Eph receptor-/ephrin-based therapeutic strategies.

Cross-References

- Adaptor Proteins
- Alzheimer's Disease
- Blood-Brain Barrier
- Bone Remodeling
- Fibroblast Growth Factors
- Inflammation
- MAP Kinase Cascades
- Neurodegeneration
- Parkinson's Disease
- Small GTPases
- Tyrosine Kinases

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EPHA1

▶ Eph Receptors and Ephrins

EPHA10

▶ Eph Receptors and Ephrins

EPHA2

▶ Eph Receptors and Ephrins

EPHA3

▶ Eph Receptors and Ephrins

EPHA4

▶ Eph Receptors and Ephrins

EPHA5

▶ Eph Receptors and Ephrins

EPHA6

▶ Eph Receptors and Ephrins

EPHA7

▶ Eph Receptors and Ephrins

EPHA8

▶ Eph Receptors and Ephrins

EPHB1

▶ Eph Receptors and Ephrins

EPHB2

▶ Eph Receptors and Ephrins

EPHB3

▶ Eph Receptors and Ephrins

EPHB4

▶ Eph Receptors and Ephrins

EPHB6

▶ Eph Receptors and Ephrins

EPHX2

▶ Soluble Epoxide Hydrolase

Epigenetic Therapy

▶ Epigenetics

Epigenetics

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Synonyms

Epigenetic therapy; DNA methylation; Histone methylation; Histone acetylation; Bromodomain inhibitors

Definitions

2-OG – 2-oxogluterate
AML – Acute myelogenous leukemia.
BET – Bromodomain and extra-terminal domain.
BRD – Bromodomain.
CTA – Cancer testis antigen.
DLBCL – Diffuse large B-cell lymphoma.
DNMT – DNA methyltransferase.
DOT1L – Disruptor of telomeric silencing 1-like.
EVR – Endogenous viral RNA.
EZH2 – Enhancer of zeste homolog 2.
FAD – Flavin adenine dinucleotide.
H3 – Histone H3.
HDAC – Histone deacetylase.
HDM – Histone demethylase.
HMT – Histone methyltransferase.
HOX – Homeobox.
IDH – Isocitrate dehydrogenase.
MDS – Myelodysplastic syndrome.
MLL – Mixed lineage leukemia.
NSCLC – Non-small cell lung cancer.
PRC2 – Polycomb repressive complex 2.
PRMT – Protein arginine methyltransferases.
SAM – S-adenosyl methionine.
SET – Su(var)3–9, enhancer of zeste and trithorax.
TET – Ten-eleven translocation methylcytosine dioxygenase.

The fate and function of every cell in an organism can vary significantly despite having identical genetic material. Differentiated cells are characterized by distinct patterns of gene expression due to variations in transcription, translation, or protein modification. Patterns of gene expression that are stably maintained after cell division but not attributed to DNA sequence variation are regulated by epigenetic mechanisms. Epigenetic information is encoded by cytosine methylation, posttranslational modification of histone proteins, and nucleosome positioning (Fig. 1). Writers of epigenetic code catalyze DNA methylation or posttranslational modifications of histones, such as methylation or acetylation of N-terminal histone "tails" extending from the nucleosome

structure. Epigenetic modifications may be recognized and used as points of contact by "reader" proteins that either alter the chromatin architecture further or regulate enzymatic processes such as transcription factor binding and RNA polymerase processivity. Epigenetic modifications may be removed by "eraser" enzymes such as histone deacetylases or demethylases. In addition, chromatin remodelers can mobilize or exchange histones. Together these mechanisms maintain chromatin accessibility patterns that govern interaction of transcription machinery with genes and cis-regulatory regions (Lorch et al. 1987). Mutations in genes that regulate the cellular epigenetic state are among the commonest class of mutations found in cancer (Lawrence et al. 2014). These mutations result in reprogrammed gene expression that can directly contribute to cancer and cooperate with other genetic events such as mutations of oncogenes or tumor suppressor genes affecting cellular proliferation pathways. Because epigenetic marks are reversible, recent work to develop more effective anticancer therapeutics has emphasized identifying and targeting epigenetic mechanisms (Bennett and Licht 2018).

Basic Mechanisms

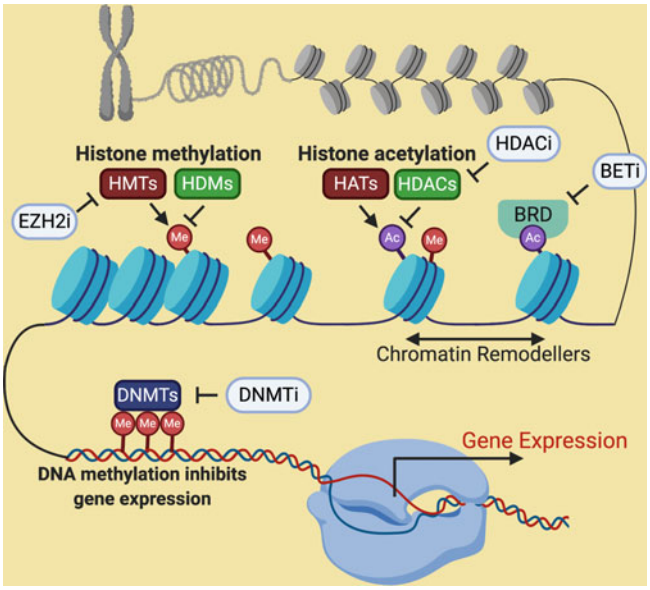
DNA Methylation

DNA methylation almost exclusively occurs on cytosines that precede a guanine (CpG) and plays a key role in regulation of gene expression. Endogenous palindromic methylation patterns are maintained in the genome and transmitted through the germline. CpG dinucleotides are often concentrated within "islands" of CpG-rich DNA regions located near transcription start sites (TSSs) (Saxonov et al. 2006). Hypermethylation of CpG islands may repress expression of the corresponding gene by preventing binding of transcription factors and recruiting methyl-CpG binding proteins that interact with repressive histone modifying enzymes (Herman and Baylin 2003). Changes in both global and individual gene methylation patterns are often found in cancer and aberrant methylation patterns have been used to differentiate tumor subtypes. DNA hypomethylation is often found in tumors due to loss of repeat region methylation and hypomethylation of regions that regulate the expression of proto-oncogenes such as ERBB2 and RAS (Nishigaki et al. 2005). In addition, hypermethylation of specific CpG rich regions has been observed to silence expression of tumor suppressors such as Rb and p16 (Herman et al. 1995; Greger et al. 1989).

The enzymes responsible for establishing and maintaining these patterns of DNA methylation are DNA methyltransferases (DNMTs). DNMT3A and DNMT3B establish de novo DNA methylation patterns that are maintained by DNMT1, which can recognize methylation in DNA and assures methylation of daughter strands after replication (Okano et al. 1999). Demethylation of DNA is facilitated by the TET enzymes which convert methyl cytosine into hydroxymethylcytosine which is not recognized by DNMT1 (Rasmussen and Helin 2016). Loss of function mutations of DNMT3A or TET enzymes are often observed in cancer, upsetting patterns of DNA methylation.

Histone Acetylation

Lysine acetylation of histones is important for regulation of chromatin structure, transcription, and DNA repair. Two competing enzyme families regulate this highly dynamic modification, histone lysine acetyltransferases (HATs), and histone deacetylases (HDACs). HATs transfer the acetyl group from acetyl-coenzyme A to the amino group of a histone lysine thereby neutralizing the positive charge of lysine and diminishing the interaction of histone with DNA. In general, this causes a more relaxed and accessible chromatin structure that favors binding of proteins, such as transcription factors, and thus acetylation of chromatin is associated with transcriptional activation while deacetylation is associated with gene repression (Haberland et al. 2009). HDACs play a key role in gene expression by removal of the activating histone acetylation and may also have other roles in the cell by controlling acetylation of nonhistone and nonnuclear proteins. HDACs are often overexpressed in cancer to silence tumor suppressor genes or they may be aberrantly recruited to target genes by overexpressed transcription factors or chimeric transcription factors



Epigenetics, Fig. 1 Epigenetic control mechanisms govern chromatin accessibility and gene expression DNA is wrapped around histone proteins to form nucleosomes (blue cylinders) which are then condensed into chromatin and ultimately chromosomes. Epigenetic mechanisms regulate nucleosome occupancy and positioning especially at regulatory elements of gene expression. Chromatin remodelers mobilize and reposition nucleosomes. Post-translational modification of N- and C-terminal "tails" of histones that extend beyond the nucleosome regulates nucleosome stability and chromatin compaction and may serve as docking sites for additional proteins such as bromodomain proteins (BRD). Histone methyltransferases (HMTs) catalyze transfer of a methyl group (Me) from S-adenyl methionine onto histone tails while histone demethylases (HDM) remove this mark. Similarly, histone acetyltransferases (HATs) catalyze the transfer of an acetyl group (Ac) from acetyl coenzyme A to histone lysine residues that function to weaken the interaction with DNA and increase chromatin accessibility. Histone deacetylases (HDACs) remove the acetyl mark from histones, decreasing chromatin accessibility and suppressing gene expression. Methylation of DNA at cytosines that precede guanine, CpG, by DNA methyltransferases (DNMT) near transcription start sites precludes the binding of transcription factors to inhibit gene expression. Several classes of inhibitors have been developed to reprogram the cellular epigenetic state in tumor cells (white ovals). EZH2i, such as tazemetostat, specifically inhibits methylation of histone H3 by the catalytic subunit of the polycomb repressive 2 complex that inhibits gene expression. HDACi such as entinostat or vorinostat inhibits histone deacetylation to reactivate gene expression. Inhibitors of bromodomain and extra-terminal domain proteins (BETi) such as JQ1 or BMS-986158 suppress aberrant gene expression driven by increased BRD activity in cancer cells. DNMT inhibitors (DNMTi) such as azacytidine promote DNA hypomethylation and reactivate gene expression

created by chromosomal fusions (Haberland et al. 2009).

Bromodomain-Containing Proteins Recognize Acetylated Histone

One of the best characterized proteins families that recognize epigenetic modifications is the bromodomain and extra-terminal (BET) family that includes BRD2, BRD3, and BRD4 (Kim et al. 2006; Xu and Vakoc 2017). These proteins share a conserved structural element consisting of two bromodomains that recognize acetylated lysine on the N-terminal tails of histones H3 and H4. Importantly, BRD4 associates with P-TEFb protein to promote transcription elongation by RNA polymerase II at paused sites (Yan et al. 2011). BRD4 is enriched at transcription start sites, enhancer and elongated or "super enhancer" regions, and BRD4 promotes expression of many transcription factors that are known to have roles in cancer development and progression such as Myc. BRD2 is overexpressed in B-cell lymphoma and increased BRD4 expression was reported in melanoma tissues and hepatocellular carcinoma

where it is associated with poor prognosis. In addition, the majority of patients that develop midline carcinoma have chromosomal rearrangements that create a fusion protein between BRD3 or BRD4 and the NUT protein (Yan et al. 2011). The BRD-Nut fusion maintains cancer cells in an undifferentiated state of self-renewal by recruiting HATs and sequestering cofactors normally associated with activated, acetylated chromatin away from normal gene targets, causing reduced expression of differentiation associated genes.

Histone Methylation

Posttranslational modification of histone N-terminal and C-terminal tails that extend beyond the nucleosome core influence downstream biological processes such as transcription, replication, and chromosomal stability. Reversible methylation of histones is orchestrated by transfer of a methyl group to the basic amino acids lysine, arginine, or histidine present in histone tails by histone methyltransferases (HMTs), while histone demethylases (HDMs) remove these marks (Fig. 1). Histone lysine residues can be modified to mono-, di-, or tri-methylated forms (me1, me2, or me3) and histidine can be mono-methylated but this modification is rare. Arginine may be mono- or di-methylated by protein arginine methyltransferases (PRMTs). Arginine dimethylation may be either symmetrical, meaning that methyl groups are added to both nitrogen atoms in the side chain or asymmetrical in which two methyl groups are added to only one of the side chain nitrogen atoms. Demethylation of histones is accomplished by two main classes of HDMs: the FAD-dependent amine oxidases and the Fe(II)/2-OG-dependent Jumonji C domain family (Klose and Zhang 2007). Dynamic changes in histone methylation state can occur based on cell type, tissue type, or cell cycle phase and are important for the response to factors such as DNA damage, mitogen signaling, and environmental stress. The balance between the methylated and demethylated states of histones at specific lysine residues can regulate transcriptional activity. For instance, lysine methylation of histone H3 at amino acid residue 4 (H3K4), 36 (H3K36), and 79 (H3K79) are associated with activation of gene expression while methylation at lysine 9 (H3K9), 20 (H3K20), and 27 (H3K27) are associated with repression of gene expression (Barski et al. 2007). Loss or gain of HMT or HDM activity can result from missense mutation, deletion, amplification, or chromosomal rearrangement affecting the genes encoding these enzymes. HMT or HDM dysfunction and the resultant aberrant gene expression have been linked with tumor grade, chromosomal instability, mutation burden, and clinical outcome.

Except for DOT1L1, which has a unique enzymatic domain, the enzymatic activity of all lysine methyltransferases resides in their SET catalytic domain. Type I and type II arginine methyl transferases share a well conserved SAM-binding catalytic core of about 350 amino acids (Klose and Zhang 2007). These enzymatic domains have pockets that bind SAM to be used as a donor cofactor for the transfer of methyl groups to substrates.

EZH2 is the catalytic component of the PRC2 complex that is responsible for trimethylation of H3K27 associated with chromatin compaction and transcriptional repression (Kim and Roberts 2016). EZH2 is an important regulator in several cellular pathways including cell cycle regulation, X-chromosome inactivation, and metastasis and is generally overexpressed in metastatic tumors relative to normal tissues or primary tumor specimens. Gain of function mutations of EZH2 are found in germinal center type diffuse large B-cell lymphoma of and more rarely in thyroid cancer and malignant melanoma (Morin et al. 2010). These heterozygous mutations enhance the ability of the enzyme to trimethylate H3K27. In lymphoma, these gain of function mutations cause aberrant repression of tumor suppressor genes and late B-cell genes, thus locking the B cell in a state of continuous proliferation at the germinal center stage of differentiation. Loss of function and deletion of EZH2 is found in MDS and AML and is associated with global decreases in the repressive H3K27me3 mark which activates oncogene expression (Ernst et al. 2010). Thus, depending on the cellular context or function of dysregulated gene expression, EZH2 can have either an oncogenic or tumor suppressor function.

Pharmacological Intervention

Targeting DNA Methylation

DNA methyltransferases inhibitors (DNMTi) have been developed to target aberrant methylation patterns observed in cancer cells. DNMTi such as 5-azacytidine, decitabine, and guadecitabine cause global hypomethylation, and their use as anticancer agents has been approved for use in patients with myelodysplastic syndrome or certain leukemias to reactivate tumor suppressor genes. These azanucleosides substitute nitrogen for carbon at the C-5 position of the pyrimidine ring and when incorporated into DNA irreversibly bind DNMT1 resulting in DNMT1 degradation and decreased DNA methylation (Ghoshal et al. 2005). The resulting loss of DNA methylation favors the re-expression of aberrantly silenced proteins, including tumor suppressor genes, cancer-associated antigens, and components of the antigen presentation machinery. While hypomethylating agents can reverse promoter methylation and reactivate silenced tumor suppressor gene expression in some instances, many reports attribute their anticancer activity to other mechanisms. For instance, treatment with decitabine causes the formation of DNA-DNMT adducts, subsequent double-stranded DNA breaks, and G2 arrest. In addition, reports suggest that DNMTs may associate with histone-modifying enzymes and a global increase of histone H3 and H4 acetylation has been observed after treatment with Aza. Furthermore, decitabine has been reported to stimulate expression of normally silent endogenous retroviral sequences in colon cancer cell lines to cause a host antiviral response, including activation of interferon response genes, independent of promoter demethylation. Taken together these results indicate that DNA methyltransferase inhibitors can have cooperative functions that contribute to their cytotoxic effect.

Defects in demethylation of DNA have also been reported to drive neoplasia. Mutations in the enzymes IDH1 and IDH2 that normally catalyze decarboxylation of isocitrate to α-ketoglutarate occur in gliomas, AML, chondrosarcomas, and cholangiocarcinoma. Mutant IDH1/2 reduces α-ketoglutarate to 2-hydroxygluterate, a competitive inhibitor of the TET family of DNA hydroxylases (Ward et al. 2010). In addition, mutations and translocations of *TET2* have been observed in numerous hematologic malignancies and associated with poor prognosis in AML (Delhommeau et al. 2009). TET enzymes normally convert 5′-methylcytosine to 5′-hydroxymethylcytosine, an important step in cytosine demethylation and inhibition of this mechanism results in DNA hypermethylation (Figueroa et al. 2010). Pharmacological agents (ivosidenib and enasidenib) that target mutant IDH1 and IDH2, respectively, have been approved for the treatment of AML. These agents block the production of 2-hydroxygluterate by the mutant enzymes, thus restoring TET2 function and reverting DNA methylation and gene expression patterns towards the normal state. In addition, high-dose vitamin C treatment activates TET to induce expression of EVRs, reverse cell lineage commitment, and restore tumor suppressive gene expression programs. High-dose vitamin C administration has been demonstrated to act synergistically with a variety of other anticancer agents such as BET inhibitors or decitabine, and active clinical trials that include vitamin C either as a single agent or in combination therapies are ongoing for patients with almost all types of cancer.

Targeting Histone Acetylation

Numerous HDAC inhibitors (HDACi) have been developed for cancers that display increased HDAC activity or HAT mutations. Because HDACs are Zn^{2+}-dependent, many nonselective broad-spectrum enzymatic inhibitors target the Zn^{2+} ion in the active site. Pan-HDACi such as vorinostat, belinostat, and panobinostat have been shown to reactivate tumor suppressor genes such as p21 and have either been approved or are in clinical trials to for many cancers. In addition, selective HDACi have been developed that target specific HDACs. These include romidepsin that targets HDAC1 and HDAC2 and has been approved for lymphoma patients and ricolinostat an HDAC6 specific inhibitor in clinical trials for patients with multiple myeloma or lymphoma that blocks the clearance of misfolded proteins (Santo et al. 2012).

Targeting Histone Methylation

While many HMTs have been implicated in diseases such as cancer, relatively few effective methyltransferase inhibitors have been identified that are being used clinically. One promising HMT target is DOT1L, a H3K79 methyltransferase that has an important role in *MLL* (KMT2A) leukemias. The DOT1L inhibitor pinometostat inhibits proliferation of cell lines with MLL rearrangements in association with decreases in H3K79me levels at *HOX* genes and is currently in clinical trials for patients with *MLL* rearranged leukemias (Waters et al. 2015). Another HMT target being pursued for anticancer therapies is EZH2 which functions to repress gene expression. EZH2 inhibitors may be useful to restore a balance between forces mediating gene repression and activation for cancers with overexpression or mutation of EZH2, as well as tumors having loss of function mutations of gene activator proteins such as the H3K27 demethylase KDM6A or inactivating mutations of the SWI/SNF proteins (van Haaften et al. 2009; Kim et al. 2015). Tazemetostat is one of the most promising EZH2 inhibitors that was recently approved for use with metastatic sarcoma patients and is in clinical trials as a single agent or in combination with other agents for hematologic malignancies (Knutson et al. 2012; Kurmasheva et al. 2017). In addition, increased histone arginine methylation by PRMT5 has been observed in leukemias and several inhibitors of PRMT5 have been developed such as JNJ-64619178, PF-06939999, and GSK3326595 that are in clinical trials for hematologic malignancies. Other small molecule HMT inhibitors have shown promise as potential anticancer therapeutics but have not yet advanced to the clinic. These include compounds targeting aberrant histone H3K9 methylation such as BIX01294 that targets the methyltransferase G9a which displays increased expression in lung cancer cell lines and inhibitors of SETDB1 which is frequently amplified in melanoma and lung cancers. The frequent disruption of histone methylation patterns in cancer highlights the need for continued development of this class of inhibitors.

Targeting Epigenetic Readers

Treatment of BRD4-NUT expressing cell lines with a small molecular inhibitor of BET domain acetyl-lysine binding led to a reactivation of gene expression and induction of differentiation (Yan et al. 2011). This finding motivated the ongoing development of pharmacological approaches to target BET domain proteins by inhibiting their tandem bromodomains. One of the first BET inhibitors developed, JQ1, induced terminal differentiation of leukemic stem cells in primary AML samples and prevented ovarian carcinoma growth in xenografts. Recently, new classes of improved BET inhibitors have been developed. Three of these, I-BET762, CPI-0610, and OTX015, are in clinical trials for hematologic malignancies and TEN-010 is in clinical trials for NUT midline carcinomas and advanced solid tumors.

In summary, as our understanding of the defective epigenetic mechanisms in cancer has improved so has the motivation for developing strategies to reprogram these dynamic mechanisms back to a normal cell state. Hypomethylating agents, HDAC inhibitors, and agents that reverse cancer associated histone modifications have proven to be clinically useful, particularly for treatment of hematologic malignancies. In addition, emerging therapeutic strategies take advantage of crosstalk between different epigenetic mechanisms. For instance, cooperation between DNA methylation and histone deacetylation in gene expression has led to clinical trials that test combinations of HDAC inhibitors and DNA hypomethylating agents. Furthermore, the widespread adoption of targeted immunotherapies for cancer has led to the need for a deeper understanding of how epigenetic mechanisms may govern immune surveillance escape by tumor cells. A key focus of future epigenetic research will be to leverage mechanistic insights into more effective anticancer therapies.

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Epithelial Na^+ Channel

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Definition

The epithelial Na^+ channel (ENaC) is a member of the ENaC/degenerin family of non-voltage-gated ion channels. This channel is localized in the apical membranes of Na^+-absorbing epithelia such as the aldosterone-sensitive distal nephron, respiratory epithelia, and distal colon. ENaC forms a heteromeric channel made of homologous subunits – α, β, and γENaC. The human genes encoding these three subunits are named *SCNN1A*, *SCNN1B*, and *SCNN1G* and are located in chromosome 12 (α) and 16 (β γ), respectively. In humans, but not in mice or rat, a fourth subunit, δENaC, exists. So far, little is known about the physiological role and the functional properties of this additional subunit; δENaC may replace the αENaC subunit to form functional heterotrimers.

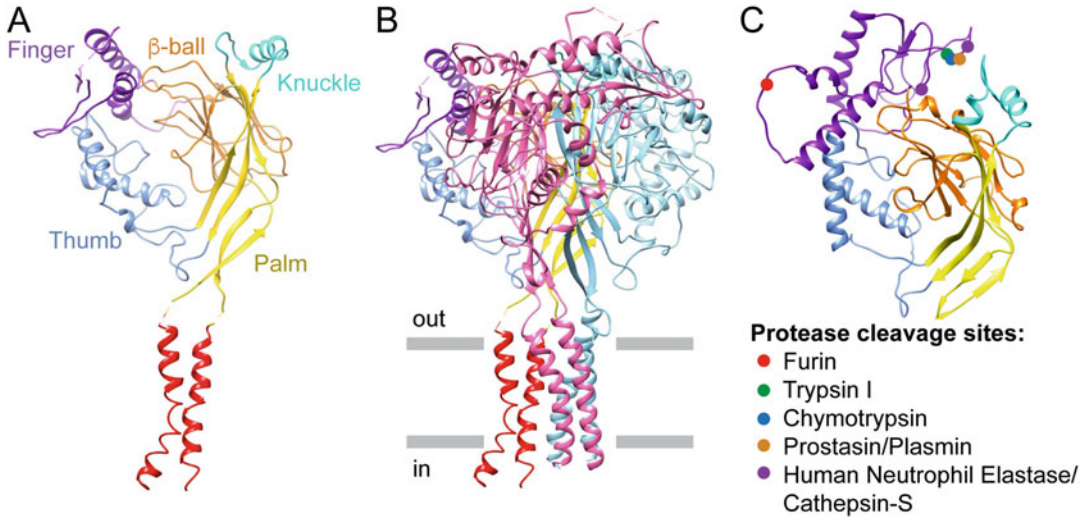
Basic Characteristics

Channel Family and Structural Organization

ENaC belongs to the ENaC/degenerin family of ion channels that include in mammals the neuronal acid-sensing ion channels (ASICs) and the bile acid-sensing ion channels (BASICs), in *C. elegans* the degenerin channel family involved in mechanosensation, and in mollusks the FMRF peptide-gated Na^+ channel FaNaC (Kellenberger and Schild 2002; Hanukoglu and Hanukoglu 2016; Kellenberger and Schild 2015). Soon after the cloning of ENaC subunits, it was concluded from primary sequence analysis and biochemical experiments that each ENaC subunit consists of a large extracellular loop, two transmembrane domains, and short intracellular N- and C-termini

and that the subunits are arranged pseudo-symmetrically around the channel pore. In 2007, the first ASIC structure, obtained from chicken ASIC1a, was published, which shares ~20% homology with human ENaC subunits (Jasti et al. 2007). This, and subsequently published ASIC structures, confirmed the predicted subunit topology and also showed that the assembled channel is a trimer. These structures provide detailed information on the organization of the extracellular and transmembrane domains of ASIC. The shape of the extracellular domain of each subunit was compared to that of a hand holding a small ball, and, consequently, subdomains were labeled as wrist, palm, thumb, finger, knuckle, and β-ball (Fig. 1) (Kashlan and Kleyman 2011; Stockand et al. 2008). Structural models of ENaC, based on the sequence homology to ASICs, allowed then the localization of functionally important ENaC residues and domains. Cleavage sites of proteases (see below) are localized in the finger domains. It was also shown that the selectivity filter is localized in the lower half of the transmembrane segments, whereas the binding site of the pore blocker amiloride is positioned one helical turn above the selectivity filter. The "degenerin site" is localized at a conserved position at the extracellular entry of the pore of ENaC/degenerin channels and is critical for channel activity. It is a Ser in α, β and γENaC. Replacing the degenerin site residue by a larger residue strongly increases the activity of degenerins, ENaC, and ASICs (Eastwood and Goodman 2012).

Very recently, the first cryo-electron microscopy structure of human ENaC was published (Noreng et al. 2018). The ENaC subunits used for the structural analysis were heavily truncated and mutated, and some regions – as the transmembrane regions and parts of the finger domains – could not be resolved. The structural analysis indicated basically the same subunit organization as in ASICs, and a diameter of the extracellular part of the ENaC trimer of 81 Å, and a height of the channel without the cytoplasmic ends (of which no structural information is available) of 120 Å. On the top of the trimer, facing outward, three regions rich in aromatic residues at subunit interfaces were identified, which are known from functional studies to be the target of ENaC



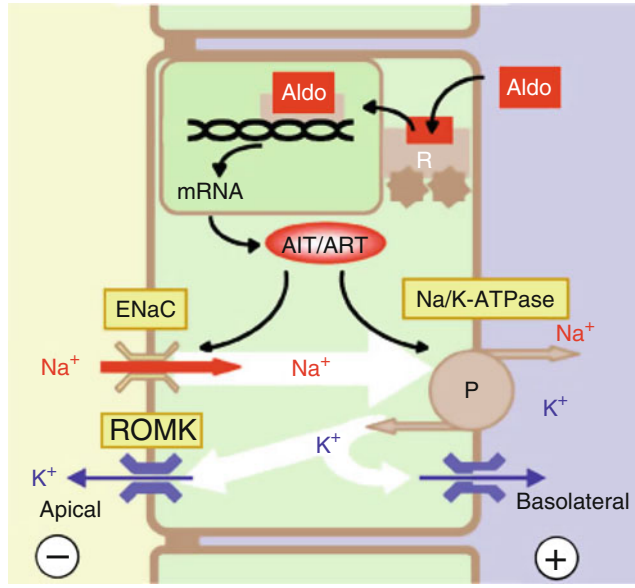
Epithelial Na^+ Channel, Fig. 1 ***ENaC structure.*** A, structural image of αENaC, with indication of the different domains. The interrupted lines indicate portions whose structure could not be determined (Figure based on (Noreng et al. 2018)). B, ENaC trimer, with the α subunit shown in the same colors as in A, β in pink, and γ in light blue. The approximate level of the plasma membrane is indicated. C, indication of the approximate location of cleavage sites by several proteases on γENaC, shown on a structural model of αENaC (Kashlan et al. 2011), since this part is not resolved in the ENaC structure

regulation by proteases. However, the parts containing the cleavage sites were not resolved in this structure.

Physiological Roles

ENaC is located in the apical membrane of polarized epithelial cells where it mediates Na^+ transport across tight epithelia (Marunaka et al. 2011). The most important tight epithelia expressing ENaC include the distal nephron of the kidney, the respiratory epithelium, and the distal colon. The basic function of ENaC in polarized epithelial cells is to allow vectorial transcellular transport of Na^+ ions. This transepithelial Na^+ transport through a cell involves basically two steps, as illustrated in Fig. 2 for a principal cell in the collecting duct of the distal nephron. The large electrochemical gradient for Na^+ ions existing across the apical membrane provides the driving force for the ENaC-mediated entry of Na^+ into the cell. Active Na^+ extrusion across the basolateral membrane is accomplished by the Na^+/K^+-ATPase. The Na^+ absorption in the distal nephron is coupled to K^+ secretion via K^+ channels (ROMK2) located at the apical membrane. ENaC mediates Na^+ absorption in the *aldosterone-sensitive distal nephron* (ASDN), which contains the late portion of the distal convoluted tubule, the connecting tubule, and the collecting duct (Loffing and Korbmacher 2009). This ENaC-mediated Na^+ absorption is critical to balance urinary Na^+ excretion with the daily intake and, thus, for the maintenance of the extracellular fluid volume and blood pressure. ENaC activity is under the control of the hormones aldosterone and vasopressin that are secreted in response to stimuli such as extracellular volume contraction, dehydration, or hyperkalemia. Aldosterone binds to intracellular mineralocorticoid receptors; the ligand-receptor complex is translocated to the nucleus and induces the expression of ENaC and the Na^+/K^+-ATPase proteins via aldosterone-induced transcripts (AITs) and/or aldosterone-repressed transcripts (ARTs). Vasopressin binds to the G-protein-coupled V2 receptor and activates ENaC via the cAMP-dependent pathway. The identification of mutations in genes encoding the ENaC, the mineralocorticoid receptor, and the 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD-2) that cause monogenic forms of hypertension strongly supports this notion.

Gain-of-function mutations of ENaC associated with an increased Na^+ absorption in the distal nephron lead to low plasma aldosterone levels and low plasma renin activity and cause Liddle's syndrome (pseudoaldosteronism), an autosomal



Epithelial Na^+ Channel, Fig. 2 ***Transepithelial ion transport in a principal cell of the cortical collecting duct.*** The figure illustrates the channels and transporters involved in the transepithelial Na^+ transport and the regulation of this transport by aldosterone. Aldo, aldosterone; AIT, aldosterone-induced transcript; ART, aldosterone-repressed transcript

dominant inherited form of salt-sensitive arterial hypertension. Conversely, loss-of-function mutations cause Na^+ salt losing nephropathy (pseudohypoaldosteronism type 1 (PHA-1)) and are characterized by urinary sodium loss, dehydration, hyperkalemia, elevated plasma renin, and aldosterone levels.

Mutations identified in patients with Liddle's syndrome are almost exclusively localized in the β- or γ-subunit of ENaC and mainly affect a conserved proline-rich motif (important for channel endocytosis and degradation) at the intracellular C-terminus of ENaC (Tetti et al. 2018). Recently, a missense mutation in the extracellular domain of αENaC has been reported to also cause Liddle's syndrome, probably by increasing intrinsic channel activity rather than by increasing channel expression at the cell surface (Salih et al. 2017).

ENaC activity in the lung is developmentally regulated and plays a critical role at birth for a rapid and normal fluid reabsorption from the lung which turns from a secretory (fetal) to a reabsorptive (adult) mode. Later in life, ENaC remains important for the ionic composition and the clearance of the airway surface liquid (ASL). The depth of the ASL is mostly determined by the equilibrium between chloride secretion by CFTR (cystic fibrosis transmembrane regulator) having a tendency of increasing this level and Na^+ absorption via ENaC, which will decrease the ASL depth. Dehydration of the airway surface leads to impaired mucociliary clearance and higher risk of infections. In mice, airway-specific overexpression of ENaC results in an increased Na^+ absorption and in a severe spontaneous lung disease sharing features with cystic fibrosis. Conversely, loss-of-function mutations of ENaC, as observed in recessive PHA-1, cause an increase of mucociliary clearance and a decrease in mucous viscosity. Several studies showed an increase of ENaC activity in the absence of CFTR, suggesting a negative control of ENaC activity by CFTR. The relevance of this possible regulation is currently still under debate. Cystic fibrosis (CF) is caused by mutations in CFTR which decrease its expression at the cell surface and/or its activity (Moore and Tarran 2018). In CF, there is a disequilibrium between CFTR and ENaC activity, leading to an increased fluid absorption and a reduction of the ASL depth and thereby to airway obstruction, inflammation, and chronic bacterial infections.

In *the tongue*, ENaC is expressed in taste bud epithelial cells. $\alpha ENaC^{(-/-)}$ mice show strongly reduced salt attraction and Na^+ taste response, indicating a role of ENaC in salt tasting (Chandrashekar et al. 2010).

In *the skin*, ENaC is expressed in keratinocytes of the epidermis and in hair follicles. It may contribute to the barrier function of the skin, since $\alpha ENaC^{(-/-)}$ mice show increased transepidermal water loss and disturbed stratum corneum lipid composition.

E

Mechanisms of ENaC Regulation

(a) Regulation of ENaC by proteases

Mechanisms of ENaC regulation are highly complex, vary from tissue to tissue, and include different aspects like transcription, regulatory proteins, kinases/phosphatases, the lipid environment, proteases, and amphiphilic substances like bile acids. Local factors affecting ENaC function are likely to be important for tissue-specific ENaC regulation. One mechanism, which plays a role in all tissues, is the regulation of ENaC by proteases. Cleavage results in the release of inhibitory peptides and probably activates the channel by changing its conformation. ENaC is cleaved at three furin sites (two in αENaC and one in γENaC) along the biosynthetic pathway before the channels reach the plasma membrane. A second cleavage event in proteolytic ENaC activation takes place at the plasma membrane where γENaC is cleaved by membrane-bound proteases (e.g., channel-activating proteases CAP1-3) and/or soluble proteases in a region distal to the furin site (see Fig. 1C).

The proteases activating ENaC under physiological and pathophysiological conditions have not been identified and may differ between tissues. Putative cleavage sites in γENaC have been described for the cysteine protease cathepsin-S, for the serine proteases prostasin, plasmin, elastase, kallikrein, trypsin, and chymotrypsin (see Fig. 1C) (Haerteis

et al. 2014, 2012a, b; Ray and Kleyman 2015; Kleyman et al. 2009; Rossier and Stutts 2009).

There is evidence that proteolytic ENaC activation by urinary proteases may contribute to Na^+ retention in nephrotic syndrome ("proteasuria") (Artunc et al. 2019). This is supported by data using aprotinin as pharmacological inhibitor of urinary serine protease activity in vivo in nephrotic mice showing protection of these mice from proteolytic ENaC activation and Na^+ retention (Bohnert et al. 2018). Renal diseases may not only affect the activity of proteases but may also shift the balance between endogenous proteases and protease inhibitors (Kitamura and Tomita 2010). Thus, the pathophysiological aspects of proteolytic ENaC regulation await further investigation and the identification of relevant proteases and protease inhibitors along the nephron and in urine samples in various disease states.

(b) Regulation of ENaC by amphiphilic substances (bile acids)

Recently it has emerged that amphiphilic substances, such as bile acids, also regulate ENaC (Ilyaskin et al. 2016; Wang et al. 2019). Bile acid sensitivity is not a unique feature of ENaC but rather a common phenomenon of channel activation that is shared by other members of the ENaC/degenerin ion channel family including BASIC and ASIC1.

Physiologically relevant tauro-conjugated bile acids (e.g., taurochenodeoxycholic, taurocholic, and taurodeoxycholic acid) stimulate ENaC in the human αβγ- and in the δβγ-configuration, whereas nonconjugated bile acids show a robust stimulatory effect only on δβγENaC. The degenerin site region in the channel's β-subunit seems to be critical for the functional interaction of bile acids with the channel. The physiological role of ENaC/BASIC/ASIC activation by bile acids remains still to be elucidated. Bile acids may be physiologically relevant modulators of ENaC function, e.g., in bile ducts where αβγENaC is expressed in cholangiocytes. Next to bile acids, other endogenous amphiphilic substances, capable of binding to the degenerin region of ENaC, may act as local modulators of channel function in a tissue-specific manner.

Drugs

ENaC is blocked from the tubular lumen of the distal nephron by the diuretic amiloride at submicromolar concentrations. By blocking ENaC activity at the apical membrane of the target cell of the distal nephron, the electrochemical gradient favoring the passive secretion of K^+ through ROMK is dissipated and K^+ secretion blocked. Unlike loop diuretics (furosemide and analogs) or distal convoluted tubule diuretics (i.e., *thiazide* diuretics), which cause K^+ secretion and hypokalemia, amiloride and analogs cause K^+ retention (and ultimately hyperkalemia) and are currently used as *K^+-sparing diuretics*. Since the distal nephron reabsorbs under physiological conditions less than 10% of the filtered load of sodium, blockade of ENaC results only in a slight increase in urinary excretion of sodium.

The biophysical characteristics of the block of ENaC by amiloride including voltage dependence and competitive interaction with permeant cations, such as Na^+ or Li^+ ions, strongly suggest that amiloride is a pore blocker which, upon binding to its receptor on the channel, physically occludes the ion permeation pathway (Garty and Palmer 1997). The binding site for amiloride is located in the channel pore, just above the ion selectivity filter, and involves specific amino acid residues on the α, β, and γENaC subunits. Amiloride shares its binding site on ENaC with triamterene which blocks the channel with a lower affinity (Kellenberger and Schild 2015).

Spironolactone, a competitive antagonist of aldosterone for the mineralocorticoid receptor, inhibits epithelial sodium transport and K^+ secretion and belongs to the same class of diuretics.

The development of amiloride analogs targeted to block selectively ENaC in the lung could be useful in the treatment of cystic fibrosis (CF) patients or more generally of patients suffering from chronic bronchitis, a condition in which an increased mucociliary clearance is highly desirable. Inhalation of amiloride has a moderate beneficial effect on lung function in CF patients, which is however limited by its rapid metabolism. Several companies have developed amiloride derivatives with higher potency and longer duration of action. Parion Sciences compounds 552-02 and P-680 showed increased efficacy and selectivity over amiloride. The studies of these compounds were however halted when hyperkalemia was observed in clinical trials. Many other ENaC antagonists have been developed by several companies, and several of them have entered clinical trials. While the development of some of them was stopped, due to lack of effect or the induction of hyperkalemia, several promising amiloride derivatives are currently in clinical trials, including AZD5634 of AstraZeneca, NVP-QBE170 and QBW 276 of Novartis, and BI 443651 of Boehringer Ingelheim (Moore and Tarran 2018). An alternative pharmacological approach is based on the peptide SPLUNC1 (short palate lung and nasal epithelial clone 1), a secreted protein that is abundant in the airways and contains an N-terminal region that binds to ENaC and inhibits ENaC activity. In experiments with a mouse model of reduced mucociliary clearance, the SPLUNC1-derived peptide SPX-101 reduced airway inflammation and increased survival. SPX-101 promotes ENaC internalization, leading to a prolonged reduction of Na^+/ASL absorption. The peptide nature of SPX-101 limits systemic exposure. SPX-101 showed no toxicity or systemic effects in phase I clinical trials, and phase II clinical trials with CF patients are underway (Moore and Tarran 2018; Couroux et al. 2019).

Channel activators are presently not available and will be useful for the treatment of *respiratory distress syndrome* in newborn, *high-altitude pulmonary edema* in adults, or, possibly, lung edema in congestive heart failure. So far, cellular studies have shown a stimulation of human ENaC by the small molecule S3969 and by the cyclic peptide solnatide (Kellenberger and Schild 2015).

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EPO, Recombinant Erythropoietin, Epoetin and Darbepoetin Alfa

▶ Hematopoietic Growth Factors

Epoxide Hydrolase 2

▶ Soluble Epoxide Hydrolase

Erythrocyte ChE

▶ Cholinesterases

Extracellular Matrix (ECM)

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Definition

The extracellular matrix (ECM) is a dynamic complex of interwoven fibrils and globular macromolecules, coming together to form the foundation of all biological tissue. Comprising of collagens, glycoproteins, and proteoglycans, as well as associated secreted factors and enzymatic regulators, this supramolecular assembly provides structural support for organs and surrounding cellular compartments and also exerts a great degree of extrinsic regulation over cellular phenotype and function. The ECM establishes the extracellular milieu in which cells exist, providing cells with context-specific cues that vary from tissue to tissue, spatially guiding cellular fate, fine-tuning developmental programs, and maintaining tissue order. Such cues are responsible for switching on and off intracellular signaling programs and establishing spatially distinct cellular phenotypes. In disease, genetic aberrations, or the loss of regulation of ECM homeostasis, are observed, leading to deviations in ECM composition and organization. As a consequence, the ECM's role as a cellular regulator is disrupted, resulting in a microenvironment that imparts a disordered phenotype on the surrounding cells, as well as organ dysfunction on a much larger scale. Our increasing knowledge of the physiology of ECM regulation and the mechanisms that precede and drive a pathological ECM in disease will continue to deliver a deeper understanding of the microenvironmental regulation of cellular phenotype and tissue function, and increase the potential for therapeutically modifying this network in disease.

Basic Characteristics

Characteristics of the Extracellular Matrix (ECM)

The biological function of the ECM can be generally classified as either biochemical or biomechanical in nature. These characteristics are determined by early developmental programs, which create tissue-specific networks necessary for site-specific function at both the cellular and organ level. For example, while tendons require dense and highly organized, fibrillar collagen matrices in order to mediate large tensile forces, the extracellular environment of the brain requires a loosely cross-linked, proteoglycan and glycosaminoglycan-rich network in order to sustain neuronal function (Mouw et al. 2014).

ECM composition is diverse and is largely synthesized by epithelial and distinct stromal cells (such as fibroblasts). Tissue-specific expression profiles determine the abundance of individual extracellular ligands and growth factors that guide resident cell fate and phenotype. ECM composition (i.e., fibrillar vs. globular constituents), density, and post-translational modification (such as cross-linking) result in differential tissue stiffness, porosity, and the overall topological landscape. This in turn influences the osmotic flow of nutrients (i.e., the tight-knit collagen IV–VII basement membrane (BM) lattice) (Pozzi et al. 2017) as well as controls the morphology and migration of resident cell populations. These features also determine overall tissue structure for organ function, for instance, elastin-rich pulmonary ECM for respiration and collagen-bioapatite mineral complexes for bone strength.

At the cellular level, the ECM exerts extrinsic control over phenotype through spatially patterned signaling gradients and hubs in the microenvironment, thus in turn regulating gene expression. These interactions may manifest in the form of cell receptors (i.e., integrins, discoidin domain receptors [DDRs], or syndecans), binding to growth factors (i.e., transforming growth factor beta (TGF-β)), and ECM motifs (e.g., RGD motifs), or biomechanical sensors of extracellular rigidity (i.e., mechanosensitive ion channels, focal adhesion complexes), leading to the activation of

various downstream signaling pathways (Hastings et al. 2019). The activation of these "mechanotransduction" pathways has various biological consequences, including the regulation of cell fate and differentiation. Importantly, it was demonstrated that ECM-mediated signal transduction can also regulate downstream expression of ECM molecules (Nelson and Bissell 2005), as well as their regulatory enzymes. The reciprocal interactions between cells and the ECM are termed "dynamic reciprocity," and this process leads to both positive and negative feedback loops between cells and the microenvironment, which are crucial to both development and disease (Hastings et al. 2019).

In a developmental context, the microenvironment has demonstrated great importance in guiding collective cell interaction during morphogenesis. Cell-cell and cell-ECM interactions in a developing embryo create a dynamically evolving ECM substrate with differential mechanical properties and geometries, which guide the formation of epithelial structures (i.e., intestinal villi) as well as organ structures (i.e., the undulations of the brain). The establishment of these "mechanochemical feedback loops" (Hannezo and Heisenberg 2019) is also observed between neural crest cells and the underlying tissue stiffness during embryogenesis. These dynamic regulatory loops enhance and direct cellular migration during zebra fish and *Xenopus* development (Hannezo and Heisenberg 2019). The specific expression of ECM components also regulates morphogenesis. For example, during the formation of the mammary ductal tree, stromally derived laminin-1 guides epithelial polarity and branching morphogenesis at the frontier of ductal formation (Nelson and Bissell 2006).

The ECM is considered a dynamic entity in that it is in constant flux. Remodeling of the ECM is crucial in producing essential stage-dependant networks during both homeostasis and development. The expressions of secreted factors such as proteases (e.g., matrix metalloproteases, (MMPs)) and their inhibitors (e.g., tissue inhibitor of MMPs, (TIMPs)), as well as protein cross-linkers (e.g., lysyl oxidase (LOX), transglutaminases, and peroxidasin), are central to the fine balance between ECM breakdown, construction, and stability (Filipe et al. 2018).

In situations such as trauma, or injury, stromal cells are recruited to the wound site secreting factors into the extracellular milieu. These include growth factors and structural ECM components necessary to facilitate the wound healing process. The deposition of fibrillar collagens is essential for providing structural support during wound closure, and glycoproteins such as fibronectin provide a transient substrate conducive to cellular proliferation for de novo synthesis of new tissue and wound resolution (Midwood et al. 2004). These transient matrices are then removed by MMPs in order to restore the ECM to its prior state. Similarly, during gestation and mammary involution, the expression of stage-specific ECM components and the subsequent activation of numerous proteolytic enzymes are observed. Together they are critical in the controlled synthesis of lactation-associated ECM and the subsequent degradation to restore the mammary gland ECM to its former state.

The ECM in Disease

In disease, homeostatic and developmental processes either run askew or are co-opted as disease-driving mechanisms, nurturing the progression of ECM pathologies and disrupting cell and organ function. Tissue fibrosis is one of the most prevalent ECM pathologies. Fibrosis results from an asymmetry between ECM synthesis and breakdown in response to chronic injury or exposure to noxious agents, as seen in liver cirrhosis, interstitial lung disease, or renal fibrosis (Wynn 2008). Fibroblast recruitment and activation typically lead to the excessive deposition of fibrillar collagens, which are post-translationally cross-linked by enzymatic mediators (i.e., LOX) into large collagen fibrils and fibers that accumulate, interfering with both cellular and organ functions through the reprogramming of resident cells.

On the contrary, mechanisms associated with aging tissue often result in ECM attrition. Decreased ECM synthesis (due in part to fibroblast senescence) and elevated proteolysis can result in BM thinning. Post-translational modifications that accumulate as a consequence of aging

and disease, such as advanced glycation end-products (AGEs) and extracellular oxidative stress-induced oxidative modifications, alter ligands present throughout the ECM as well as result in a stiffer and less elastic interstitial ECM. This "aged" ECM possesses altered mechanics and arrangement, modulating cell function and contributing to age-related pathologies (Frantz et al. 2010). Similarly, osteogenic diseases which also result in ECM molecule attrition are typically underpinned by increased ECM resorption, decreased deposition, and altered collagen mineralization, leading to bone brittleness and fracture. Analogous to this process is the continual decay and impaired repair of articular cartilage in osteoarthritis, mediated in part by deregulated proteoglycans in the microenvironment. In other situations, aging can lead to aberrant accumulation of ECM complexes, such as heparan sulfate proteoglycan-amyloid fibril complexes in Alzheimer's disease.

The ECM is also dysregulated in almost all solid tumors. The up- or downregulation of components over time may in part be understood in terms of gain of tumor-promoting and loss of tumor-suppressing components in the ECM. In cancer, tumor cell-secreted factors recruit fibroblasts, which in turn produce and remodel the ECM leading to the generation of a microenvironment that is typically conducive to tumor development. These cancer-associated fibroblasts (CAFs) continuously and progressively remodel the tumor ECM, thereby providing pro-tumorigenic signals to tumor cells. Increased collagen deposition and arrangement typically produce a stiffer tumor ECM. In synergy with the numerous differentially regulated glycoproteins, proteoglycans, and growth factors, these cues create a positive feedback loop driving tumor progression. The ECM also presents a barrier to chemotherapy efficacy. For example, increased hyaluronan in the tumor microenvironment is thought to increase solid stress and interstitial pressures, which can in turn decrease vascular patency and thus hinder drug delivery. The stiffness of the tumor ECM also imparts chemoresistance to tumors through mechanisms such as increased YAP/TAZ signaling. As we understand more about how these cues synergize and drive the progression of tumors, we are uncovering new targetable mechanisms to block tumor cell response to the ECM that will likely yield new treatment regimens to improve patient outcomes.

Aside from altered expression levels, variants of ECM genes may also be pathological. Mutations present in ECM genes typically result in either embryonic lethality or severe developmental disorders. For example, while *COL1A1* mutations can be lethal, *COL6A1*, *COL6A2*, and *COL6A3* mutations manifest as myopathies or tendinopathies. Similarly, *LAMB3* and *LAMC2* mutations hinder hemidesmosome formation and thus give rise to significant cutaneous blistering. Other monogenic ECM disorders include Ullrich congenital muscular dystrophy 2 (*COL12A1*), osteogenesis imperfecta (*COL1A1* or *COL1A2*), Ehlers-Danlos syndrome (*COL1A1 COL1A2*, *COL3A1*, *COL5A1*, *COL12A1*, or *TNXB*), and Marfan syndrome (*FBN1*). It is important to note here that the phenotypes of these monogenic mutations typically manifest through the disruption of the interconnected supramolecular assemblies of the ECM and explain why, in the case of diseases such as Ehlers-Danlos syndrome, mutations in several genes appear to underpin the same disease.

Drugs

Targeting the ECM

Historically the ECM was largely neglected as a therapeutic target due a perceived lack of bioactivity. Once theorized as solely the structural matter "outside of cells," the ECM is now understood to be a master regulator of cellular phenotype and is known to play key roles in numerous biological processes within the tissue microenvironment. As such, there are many underexplored therapeutic targets in the extracellular environment.

There are various strategies for therapeutically manipulating the ECM in disease. Approaches span from directly (or indirectly) targeting ECM genes, targeting their expression in specific cell types, or targeting the ECM-synthesizing and ECM-remodeling cells themselves. ECM

molecules and the enzyme families that regulate their assembly and turnover may also be directly targeted in the extracellular milieu, for example, blocking collagen I fibril formation through targeting the LOX family. More recent work has also focused on targeting the downstream effects (i.e., mechanoactivation) induced by a pathological ECM.

Gene Therapy

Due to the genetic or regulatory basis of various ECM pathologies, gene therapy may present a possible avenue for normalizing ECM networks and hence tissue function. Methods such as gene editing (e.g., CRISPR/Cas9, transcription activator-like effector, or zinc-finger nucleases), or the delivery of antisense oligonucleotides, ribozymes, small interfering RNA, short hairpin RNA, microRNA, or recombinant technologies (such as cDNA) to affected sites or organs, may be exploited either to correct genetic aberrations in monogenic ECM disease or to normalize the pathological expression of ECM components (Jarvelainen et al. 2009).

For instance, in junctional epidermolysis bullosa (JEB), which is underpinned by mutations in the *LAMB3* gene and results in severe cutaneous blistering, a combinatorial gene and cell therapy approach has been adopted to alleviate disease symptoms. The autologous transplantation of keratinocyte-derived skin grafts with gene-corrected *LAMB3* produces a fully functional epidermis in JEB patients for up to 21 months, mediated by self-regenerating holoclones (Hirsch et al. 2017). On the other hand, in instances where ECM components are differentially expressed, the synthesis of constituents may be fine-tuned using gene-editing systems to normalize expression levels. A proof-of-concept study has elucidated this potential through the upregulation of *LAMA1* using a skeletal muscle-specific CRISPR-Cas9 system in a preclinical murine model, further demonstrating the validity of ECM gene therapy as a novel therapeutic strategy in Duchenne muscular dystrophy (Perrin et al. 2017).

Genetic material can also be therapeutically administered to buttress the synthesis or suppression of ECM components and associated factors. In the rodent setting, the direct delivery of decorin cDNA alleviated renal fibrosis through the negative regulation of TGF-β-induced glomerulonephritis and fibrosis (Isaka et al. 1996).

MicroRNAs (miRNAs) are known to be a key intracellular regulatory element in gene expression and also ECM synthesis. As a result, promising therapeutic potential in ECM suppression has been observed (Piperigkou and Karamanos 2019). In particular, the miR-29 family has been observed to repress elastin, collagen (I and III), fibrillin-1, and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) 9 and 12 expression but is downregulated in hepatic fibrosis (Noetel et al. 2012; He et al. 2013). Exploiting this inhibitory function may be fruitful in targeting fibrotic pathologies. Similarly, collagen I synthesis can be selectively knocked down using small interfering RNA as a means to inhibit scarring and fibrosis long term (Schuppan et al. 2018). However, many ECM molecules exhibit long half-lives in the extracellular environment, spanning from 15 to 100 years for structural collagens in human skin and articular cartilage, respectively. Thus, targeting the expression of specific ECM components in later stages of disease may be ineffective, and hence alternative therapeutic approaches may be required once ECM molecules have been stably incorporated into the extracellular environment.

Direct Targeting of the ECM and Downstream Cellular Effectors

Depending on the rate of accumulation and longevity of ECM components, and the stage of clinical presentation for a given pathology, targeting the ECM at the protein level may present a more effective strategy as opposed to upstream processes involved in synthesis and deposition. Directly targeting ECM components can be achieved through enzymatic degradation of ECM components or through the blockade of cell- or enzyme-mediated ECM-remodeling processes. Otherwise the targeted blocking of specific ECM domains with antibodies or binding peptides, which target ECM-ECM or cell-ECM

interactions, may be employed to inhibit downstream biological consequences.

Targeting specific ECM-degrading enzymes may serve as a treatment in protease-driven disease (Lu et al. 2011). MMPs, ADAMTS, and cathepsins are dysregulated in a range of tissue disorders, including attrition-driven pathologies such as osteoarthritis and chronic obstructive pulmonary disease (COPD), malignant progression, and in cardiovascular disease (CVD). Hence, a range of pharmacological inhibitors have been produced to repress ECM degradation by these enzymes for the treatment of a range of diseases; however they have had limited success in the clinic. MMPs were seen as a promising target in cancer as their expression correlated with tumor stage and were functionally shown to play a role in tumor progression (Gialeli et al. 2011). Inhibitory approaches for MMPs include competitive inhibition using peptidomimetics, zinc chelators, tetracyclines, or TIMPs and have been extensively investigated at the preclinical level (Gialeli et al. 2011). Unfortunately, the preclinical findings did not translate into effective therapy in the clinic, likely due to stage-dependant roles of MMPs and on-target toxicity in healthy tissue (Gialeli et al. 2011).

On the contrary, the delivery of ECM-degrading enzymes has been exploited for therapeutic benefit due to their specificity for ECM constituents. Targeted enzymatic breakdown of hyaluronan (HA) using recombinant human hyaluronidase ($PEGPH_{20}$) is currently being trialed in HA-high breast cancer patients (NCT02753595). Importantly, studies of this nature are trialing the combined administration of hyaluronidase therapy with standard-of-care chemotherapy – in this case eribulin – since $PEGPH_{20}$ is not cytotoxic. $PEGPH_{20}$ has also been shown to successfully degrade HA and decrease pancreatic ductal adenocarcinoma (PDAC) tumor interstitial fluid pressure, normalize vasculature, and, as a result, increase perfusion of standard-of-care chemotherapies, increasing patient outcomes in a subset of HA-high PDAC patients (Hingorani et al. 2018).

The biogenesis of ECM components, such as fibrillar collagen, relies on a number of enzymatic steps that represent viable targets. The LOX family, which include LOX and four LOX-like isoforms (LOXL1-4), are copper-dependant amine oxidases and are essential in collagen and elastin fibril formation. LOXs form intra- and intermolecular cross-links in fibrillar collagen chains and elastin, increasing stability of fibrils by rendering them resistant to proteolytic degradation and fortifying tissues with a stiffened collagen scaffold. Thus, LOXs are a central node in fibrosis and stromal-mediated tumor progression and have been the focus of anti-fibrotic drug development. Some mechanisms of inhibition include copper-chelation via tetrathiomolybdate, inhibition by therapeutic antibodies, and small molecular LOX inhibitors (Leung et al. 2019; Trackman 2016; Chang et al. 2017), some of which are beginning to enter phase 1 clinical trials.

The TGF-β family is established as a key mediator of pro-fibrotic signaling. The canonical TGF-β signaling cascade begins with TGF-β binding to transmembrane type I and II serine/threonine kinase receptors. These heteromeric complexes then transduce signals through the SMAD family of transcription factors, which in TGF-β-activated fibroblasts results in increased ECM synthesis and deposition. Therefore, the efforts in inhibiting TGF-β to block fibrosis are a great example of both direct targeting and downstream inhibition. For instance, neutralizing antibodies generated against TGF-β (e.g., preclinical monoclonal antibodies 1D11 or 2G7) or synthetic or natural binding proteins can be administered therapeutically and are able to sequester the ligand. Blockade as this level inhibits cell-TGF-β interactions and the subsequent transduction of pro-fibrotic signals in effector cells (Katz et al. 2013). The signal transduction process may also be inhibited at the receptor level through the administration of receptor kinase inhibitors, such as SB-431542, inhibiting phosphorylation of SMAD-2 and SMAD-3 (Katz et al. 2013). Finally, peptide aptamers or small molecular inhibitors of intracellular signaling complexes, such as inhibitors of SMAD-2/SMAD-3 complexes formed with SMAD-4 (e.g., Trx-SARA (Katz et al. 2013)), can prevent signal transduction downstream of the TGF-β receptor.

Similarly, if ECM molecules cannot be targeted directly within the microenvironment, then downstream cell-ECM interactions offer a viable therapeutic approach. Cellular sensing of the ECM and cell-mediated adhesions to the ECM are largely mediated by cell surface receptors known as integrins. Integrins bind specific motifs in ECM components within the extracellular microenvironment. Consequently, these interactions initiate the transduction of signals that mediate various physiological functions such as cellular migration during morphogenesis and ECM architecture remodeling, as well as mediating disease progression through the activation of TGF-β-mediated fibrosis and cancer cell invasion (Hamidi and Ivaska 2018). As such, integrins pose an important avenue for the indirect targeting of the ECM.

Of the 24 known integrins, there are only 3 that are currently targeted by FDA-approved drugs (Ley et al. 2016). The first clinical monoclonal antibody targeting an integrin, abciximab, was developed against the platelet integrin αIIbβ3, which is known to bind fibrinogen, fibronectin, and vitronectin for platelet aggregation during haemostasis (Ley et al. 2016). Platelets and megakaryocytes exclusively express αIIbβ3, and thus drug development was suitably aimed at antiplatelet and antithrombotic outcomes. Eptifibatide, a cyclic heptapeptide, is also highly selective for αIIbβ3 and is administered in patients with non-ST segment elevation myocardial infarction and percutaneous coronary intervention (Ley et al. 2016). In cancer, integrins have demonstrated a role at each step of the tumor progression and spread and are largely involved in the development of resistance to chemotherapy (Hamidi and Ivaska 2018). Despite promising preclinical evidence, two clinical trials, CENTRIC EORTC (phase III 26071–22072) and POSEIDON (phase II NCT01008475), failed to improve patient survival when used in combination with standard-of-care therapy in glioblastoma and KRAS metastatic colorectal cancer, respectively (Ley et al. 2016). This may in part be explained by the capacity for cancer cell integrins to signal independently of ECM-ligand interactions (Ley et al. 2016), and thus efforts may benefit from shifting focus to downstream signal transduction.

In cancer, at the stage of clinical presentation, tumor-associated ECM-remodeling processes are typically already well underway and may have been for many weeks and months. These tumor-ECM feedforward processes can potentially be blocked by targeting the downstream cell-mediated ECM-remodeling processes. This has been demonstrated by the inhibition of cytoskeletal-mediated ECM contractility via Rho kinase inhibition, in the preclinical setting (Rath et al. 2017; Vennin et al. 2017). Researchers demonstrated that combination therapies targeting pathways involved in fibroblast activation and subsequent ECM deposition and remodeling in the tumor microenvironment have the potential to normalize the tumor ECM, decrease cancer spread, and improve response to chemotherapy in pancreatic cancer.

Future Considerations in Drug Development

As our understanding of the ECM continues to evolve on a fundamental basis, there are various clinical factors to consider for the optimal design of therapeutics targeting the ECM. Firstly, not all ECM profiles are equal. Not only does ECM composition vary between organs or spatially within a given tissue; stage-dependant ECM networks and patient-to-patient variation within a disease must be accounted for in order to understand patient-specific pathology and therefore accurately predict response to a particular ECM-targeting therapy. Also, in multifactorial diseases such as COPD, CVD, and cancer, the ECM will synergize with intrinsic cellular effectors to drive disease progression. In such instances a combined therapeutic approach, which includes ECM targeting, will likely yield the most promising increase in patient outcomes in the clinic.

Determining the balance between drivers and suppressors of disease within the ECM is another key consideration when designing ECM-targeting drugs. This is highlighted by the observation that complete CAF ablation, and therefore ablation of tumor fibrosis, accelerates tumor progression and decreases survival, suggesting both pro- and anti-tumorigenic roles of the ECM. Similarly, while high ECM stiffness is known to impart tumor cells

with chemoresistance and a pro-invasive phenotype, low stiffness is observed to instate stemlike characteristics, which may be equally problematic in cancer. Thus, rather than ablating the ECM or ECM producing cells, ECM normalization approaches may offer an optimal therapeutic strategy in halting cancer spread.

Finally, the ECM is a complex network and derives its bioactivity from its biochemical and biomechanical composition as well as the numerous ECM-ECM and cell-ECM interactions in the microenvironment. Advances in our understanding of the ECM on a holistic level, such as those established by "The Matrisome Project" (Naba et al. 2012), are providing tools to interrogate systems levels information. Such investigations lay the foundation to uncover deeper biological insights into disease mechanisms that can be applied to the development of new approaches to target the ECM in disease.

Final Thoughts

The ECM is a key regulatory agent of cellular programs and is crucial to overall organ function. Our increasing appreciation of the diverse biological function of the ECM in health and disease has led to a solid fundamental understanding of matrix biology, as well as numerous preclinical and clinical investigations aiming to modify this supramolecular assembly for the attenuation of disease. In principal, drug design may focus at any point along an ECM-mediated pathogenic process, from the synthesis of ECM components to the downstream biological consequences of disease drivers within the ECM. As the ECM is a complex network or system, investigators should aim to understand such a complex interconnected entity at the global level, in order to understand how numerous elements in the ECM act in concert in order to progress disease.

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Ferroptosis

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AA	Arachidonoyl
ACC	Acetyl coenzyme A carboxylase
ACSL3	Acyl coenzyme A synthetase long-chain family member 3
ACSL4	Acyl CoA synthetase long chain family 4
ACSL4	Acyl coenzyme A synthetase long-chain family member 4
AMPK	AMP-activated protein kinase
BH2	Dihydrobiopterin
BH4	Tetrahydrobiopterin
CoA	Coenzyme A
DFO	Iron chelator deferoxamine
Fe	Iron
Fer1	Ferrostatin1
FIN56	Ferroptosis inducing 56
FSP1	Ferroptosis suppressor protein 1
FSP1	Ferroptosis suppressor protein 1
GCH1	GTP cyclohydrolase 1
GCS	Glutamylcysteine synthetase
GLS	Glutaminase
GPX4	Glutathione peroxidase 4
GPX4	Glutathione peroxidase 4
GSH	Glutathione (gama-glu-cys-gly)
GSH/GSSG	Glutathione
GSR	Glutathione S-reductase
GSS	Glutathione synthetase
HMG CoA	3-hydroxy-3-methylglutaryl CoA
HNE	Hydroxynonenal
HSPB1	Heat shock protein family B member 1
INFɣ	Interferon gamma
IRI	Ischemia reperfusion injury
LOX	Lipoxygenase
LOXs	Lipoxygenases
LPCAT3	Lysophosphatidylcholine acyltransferase 3
LPCAT3	Lysophosphatidylcholine acyltransferase 3
MDA	Malondialdehyde
MDM2	Mouse double minute 2
MDMX	Mouse double minute 4
MUFA	Monounsaturated fatty acid
NCOA4	Nuclear receptor coactivator 4
NRF2	Nuclear factor E2-related factor 2
OXPHOS	Oxidative phosphorylation
PE	Phosphatidylethanolamine
PL	Phospholipid
POR	Cytochrome P450 oxidoreductase
PPARa	Peroxisome proliferator-activated receptor alpha
PUFA	Polyunsaturated fatty acid
RCD	Regulated cell death
RIPK1	Receptor-interacting protein kinase 1
ROS	Reactive oxygen species

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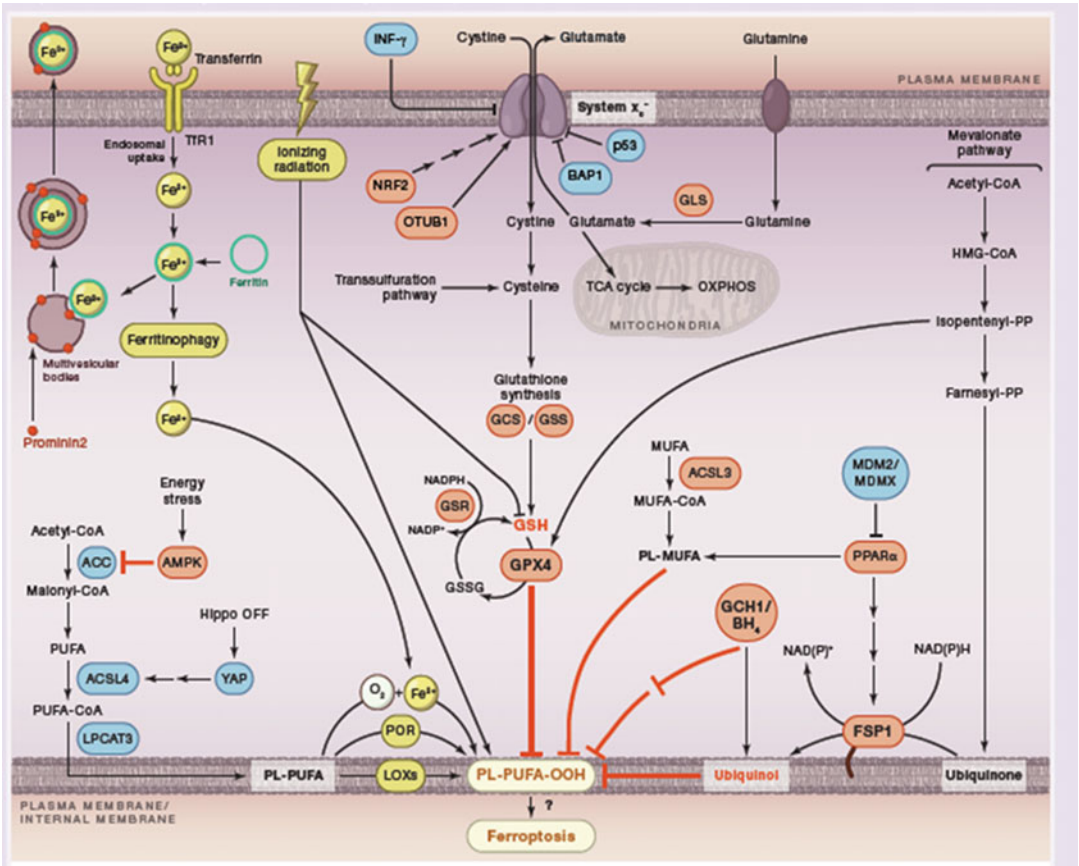
RSL3	RAS-selective lethal 3-selective lethal 3
TBARS	Thiobarbituric acid reactive substances
TCA	Tricarboxylic acid
TfR1	Transferrin receptor 1
Xc	The cystine/glutamate antiporter
YAP	Yes-associated protein

Regulated cell death is vital for cell division, differentiation, and the development of most multicellular organisms. Ferroptosis is a form of regulated cell death that is caused by iron-dependent lipid peroxidation that is lethal to cellular membranes. It is so designated according to the Nomenclature Committee on Cell Death (NCCD) because it is regulated by genetic and pharmacological modulators. Ferroptosis is different from apoptosis, the classical regulated and programmed cell death and the other forms of regulated cell death such as necroptosis, autophagy, or pyroptosis as its initiation is not characterized by defined canonical effector protein machinery. It is therefore independent of effectors of apoptosis such as the caspases, BAX, and BAK and the receptor-interacting protein kinase 3 (RIPK1/RIPK3) of necrosis. Instead, the induction and the execution ferroptosis are modulated by intracellular metabolic pathways that culminate in lipid peroxidation as a selective distinguishing biomarker.

Dixon et al. (2012) described ferroptosis as a distinct form of cell death that is induced by erastin, a small molecule that inhibits the import of cystine which causes glutathione (GSH) depletion and the subsequent inactivation of the phospholipid peroxidase glutathione peroxidase 4 (GPX4). The substrates during ferroptosis are phospholipids with polyunsaturated acyl tails (PL-PUFAs) which are particularly sensitive to peroxidation reactions (Yang and Stockwell 2016). Hence the regulation of ferroptosis is dependent on the factors that modulate the aberrant accumulation of lipid hydroperoxides (L-OOH) and the processes that inhibit GPX4 and hinder the elimination of toxic peroxides (L-OOH) as non-toxic lipid alcohols (L-OH). The state-of-the-art snapshot of ferroptosis is displayed in Fig. 1 (Hadian and Stockwell 2020). Cell death by ferroptosis is thus mediated by metabolic pathways encompassing biosynthesis and the metabolism of amino acids, GSH, iron, PUFA phospholipids, NADPH, and coenzyme Q10. However, the manifestation of ferroptosis is subject to variability due to different cell types, genetic mutations, type of target organelles, and the pleiotropy and redundancy of metabolites. Moreover, heterogeneity of in vitro cell culture studies of ferroptosis could be confounded by factors that are nonexistent in systemic metabolism (Conrad and Pratt 2019). Nevertheless, ferroptosis as a distinct form of cell death is initiated or inhibited by specific pharmacological and genetic perturbations, and these have been exploited as tools such as metabolomics, lipidomics, small molecule inhibitors, in vitro and in vivo molecular biology techniques to understand the functions of the key metabolites, regulators, and molecular biomarkers of the cell death process (Table 1).

Lipid Peroxidation of Membranes in Ferroptosis

Polyunsaturated fatty acids (PUFAs) are precursors of signaling molecules in the regulation of cellular proliferation, differentiation, and development. They are also vital structural constituents that confer fluidity and dynamic architecture on lipid bilayers of cellular membranes. The substrates during ferroptosis are phospholipids with polyunsaturated acyl tails (PL-PUFAs) which are particularly sensitive to peroxidation reactions. Oxidative disruption or damage to PL-PUFAs in the membrane is pivotal to the manifestation of ferroptosis. Inhibition of acetyl coenzyme A carboxylase (ACC) by AMP-activated protein kinase (AMPK) and energy stress can decrease endogenous biosynthesis of PUFAs needed for PL-PUFA biosynthesis. Free PUFAs are esterified into membrane phospholipids (PL-PUFAs) in reactions that are catalyzed by acyl-CoA synthetase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3) (Fig. 1). Specifically, phosphatidylethanolamines (PEs) containing arachidonic acid (C20:4) or its elongation product,



Ferroptosis, Fig. 1 Metabolic pathways and key molecular processes of ferroptosis. (Hadian K, Stockwell BR. Cell. (2020). SnapShot: Ferroptosis. 181(5):1188–1188. Copyright Permission Cell Elsevier)

adrenic acid (C22:4), are key PL-PUFAs that undergo oxidation by lipoxygenases to execute ferroptotic cell death (Doll et al. 2017, Kagan et al. 2017). Several lines of evidence have shown that the expression of ACSL4 is vital in the regulation and the sensitivity to ferroptosis, and it has been proposed as a marker of ferroptosis (Yuan et al. 2016). Spermidine/spermine N1-acetyltransferase 1 (SAT1) was also shown to promote lipid peroxidation, possibly through lipoxygenases. However, the insertion of monounsaturated fatty acids (MUFAs) into phospholipids by ACSL3 suppresses ferroptosis by a process that is not yet clear.

Although the function of lipid peroxidation in ferroptosis has been deciphered by lipidomic analysis, the ultimate toxic process of peroxides that damage membranes remains unclear. However, an aldehyde ketone reductase, encoded by AKR1C1–3 gene, was shown to inhibit ferroptosis by reducing lipid peroxides to nontoxic alcohol products. It has been proposed that excess peroxides may decrease and destabilize membrane structure, fluidity, and integrity (Kajarabille and Latunde-Dada 2019). Moreover, alteration of membrane components may result in loss of function and the degradation of lipid peroxides that decompose into reactive aldehydes and electrophiles which damage proteins and nucleic acids (Gaschler and Stockwell 2017).

Critically, iron is essential to the two main mechanisms of lipid peroxidation pathways, namely, (a) an autoxidation non-enzymatic and iron-catalyzed spontaneous peroxyl radical-mediated chain reaction and (b) the enzymatic oxidation of lipids primarily mediated by the non-heme iron-dependent lipoxygenase

Ferroptosis, Table 1 Features of ferroptosis

Definition	Ferroptosis is an iron-dependent form of oxidative, non-apoptotic cell death that is tightly linked to metabolism and disease
Morphological	
Cell membrane	Normal spherical cells
Cytoplasm	Diminutive mitochondria with decreased crista and collapsed and ruptured membrane
Nucleus	Normal
Biochemical features	Inhibition of system Xc^- Reduced GSH, inhibition of GPX4. Increased Fe, ROS accumulation, and lipid peroxidation, mitochondrial membrane potential dissipation
Key genes	GPX4, FSP1, TFR1, SLC7A11, NRF2, NCOA4, P53, HSPB1, ACSL4, LOX5, HO-1, GCH1
Pathways	GSH synthesis, trans-sulfuration, mevalonate, lipid oxidation/peroxidation, iron metabolism
Immune features	Pro-inflammatory

(15-LOX) family. Recent evidence has however revealed that LOXs possibly generate hydroperoxides to promote lipid autoxidation as rather being directly involved in the execution of ferroptosis (Shah et al. 2018). Cytochrome P450 oxidoreductase (Zou et al. 2020) has also recently been implicated as a driver of ferroptosis. MDM2/MDMX complex regulates PPARα activity by remodeling lipid profiles to enhance ferroptosis (Fig. 1).

Iron and Ferroptosis

Excess free redox-active Fe(II) has the propensity to cause ROS and oxidative stress that has been associated with various pathologies. Consequently, the regulation of the metabolic pathways of iron absorption, utilization, recycling, and ubiquitination of iron-containing proteins maintains homeostasis during normal physiology in the body. Moreover, iron could act as an intracellular second messenger that is released in response to cytosolic stress to induce cell death. Iron availability is thus an important hallmark of ferroptosis that render cells highly sensitive to erastin and RSL3-induced cell-death process. Hence iron transport and metabolism proteins are important for the regulation and sensitivity to ferroptosis. Inducers of ferroptosis have been shown to enhance the expression of iron import protein, transferrin receptor 1 (TfR1), and heme oxygenase 1 (HO-1) to promote iron availability for ferroptosis. Heat shock protein family B member 1 (HSPB1), also decreases intracellular iron level by inhibiting TfR1 expression to reduce sensitivity to ferroptosis.

Moreover, overexpression of ferroportin (FPN), the iron efflux protein, inhibited erastin-induced ferroptosis, while it's silencing in neuroblastoma cells had the opposite effect (Geng et al. 2018). Silencing of the iron-responsive element binding protein 2, IREB2, a transcriptional regulator of iron metabolism genes, also increases sensitivity to ferroptosis (Hadian and Stockwell 2020). IREB2 inhibits the degradation of TfR1 mRNA and represses the translation of ferritin translation. Inhibition of IREB2 increases the expression of FTL and FTH1, to decrease erastin-induced ferroptosis. Reduced ferritin (FTH) expression in fibroblasts by erastin, however, increased intracellular iron levels in cells. The classical ferroptosis inducer erastin also promotes the expression of CISD1(CDGSH iron-sulfur domain 1), an iron-containing outer mitochondrial membrane protein to reduce ferroptosis. The nuclear receptor coactivator 4 (NCOA4)-mediated autophagy of ferritin (ferritinophagy) plays a vital role in releasing iron to enhance ferroptosis. NCOA4 chaperones ferritin to autophagosomes for lysosomal degradation and the release of free iron (Mancias et al. 2014). This has been shown to enhance erastin-induced ferroptosis in cells. However, hypoxia was shown to inhibit ferritinophagy by repressing the transcription of NCOA4 and coupled with a micro RNA 6862-5p-dependent degradation of NCOA4 mRNA increases mitochondrial ferritin (FTMT) to prevent ferroptosis in macrophages. NCOA4 and FTMT were however not regulated in HT1080 ferroptosis-sensitive cells. Moreover, prominin2 suppresses ferroptosis by facilitating

the formation of multivesicular bodies containing ferritin-bound iron which exports iron out of cells (Fig. 1) (Brown et al. 2019).

Remarkably, nuclear factor-erythroid 2-related factor 2 (NRF2) plays a vital function in modulating cellular redox homeostasis by regulating several genes encoding proteins and enzymes engaged in the induction and inhibition of ferroptosis. These include genes for regulating glutathione activity, NADPH recycling, iron regulation, and lipid peroxidation (Kajarabille and Latunde-Dada 2019). In general, antioxidants, glutamate-cysteine ligase (GCLC), SLC7A11, HO-1, and ferritin are examples of genes that are transcriptionally regulated by Nrf2.

Scavenging and Detoxification of Lipid Peroxides

The deleterious consequences of PL-PUFAs peroxidation in ferroptosis are neutralized and nullified by three main metabolic pathways. These are as follows.

GPX4 employs cysteine-containing tripeptide glutathione as a cofactor to eliminate phospholipid peroxides. System Xc- is a central hub for regulation of ferroptosis, and it is a disulfide-linked heterodimer composed of SLC7A11 and SLC3A2, which imports cystine, the extracellular oxidized form of cysteine, in exchange for intracellular glutamate antiporter to generate GSH (Stockwell et al. 2017). Inhibition of the xc-system by erastin decreases cysteine levels and the depletion of GSH, and this suppresses the function of GPX4 in the reduction and detoxification of hydroperoxides (LOOH) to neutral alcohols (LOH). Another source of cysteine for GSH synthesis is methionine from the transsulfuration pathway, and this can render system Xc redundant in the elimination of peroxidized PL-PUFAs. Loss of cysteinyl-tRNA synthase (CARS) upregulated the genes in the transsulfuration, thereby suppressing erastin-induced ferroptosis. Moreover, a deubiquitylase, Otubain 1 (OTUB), stabilizes SLC7A11 to enhance its function in the GSH/GPX4 axis in preventing ferroptosis. On the contrary, however, the tumor suppressor BRCA1-associated protein 1 (BAP1) also in a deubiquitinating-dependent manner represses SLC7A11 expression. BAP1 reduces cysteine uptake by SLC7A11 and results in elevated lipid peroxidation and ferroptosis. However, tumor protein 53 (TP53) represses SLC7A11 to promote ferroptosis, by increasing sensitivity to ferroptosis via reduced cysteine level, thereby accentuating its role in the cell death process. SLC7A11 with its multiple regulatory effector mechanisms is, thus, a prime target for pharmacological applications of ferroptosis for therapy (discussed further below).

Erastin's function as an inducer of ferroptosis transverses proximal SLC7A11 inhibition to GPX4 degradation via the promotion of lysosomal-associated membrane protein 2a/autophagy. Erastin also binds the voltage-dependent anion channel (VDAC) to cause increasing levels of ROS mitochondrial dysfunction and ferroptosis.

RAS-selective lethal 3 (RSL3), on the other hand, alkylates the nucleophilic active site of selenocysteine of GPX4 and causes its inactivation and loss of function in the hydroperoxides repair process. This causes the accumulation of peroxides that exert a direct enhancement of ferroptosis. Ferroptosis inducing 56 (FIN56) promotes the degradation of GPX4, while $FINO_2$ leads to inhibition of its activity. Several activators and inhibitors of the different processes of ferroptosis in different model systems are shown in Table 2.

Second, Ferroptosis Suppressor Protein 1 (FSP1) (formerly known as apoptosis-inducing factor mitochondrial 2 (AIF-M2)) has been shown as independent of glutathione/GPX4 in the suppression of phospholipid peroxidation and ferroptosis (Doll et al. 2019). FSP1, an NAD(P)H oxidoreductase, reduces ubiquinol to ubiquinone-10 (CoQ10), a lipophilic radical-trapping antioxidant that suppresses the formation of PL-PUFA-OOHs. Remarkably, the mevalonate pathway (MVP) is a nexus that transverses GPX4 and FSP 1 via isopentenyl pyrophosphate GPX4 and ubiquinol (Bersuker et al. 2019). Furthermore, inhibition of MDM2/MDMX expression causes elevation of FSP1 protein and resultant higher levels of coenzyme Q10.

Ferroptosis, Table 2 Some inducers and inhibitors of ferroptosis

	Function	Mode of action
Erastin, piperazine erastin (PE), imidazole ketone erastin (IKE), erastin analogs, sulfasalazine, glutamate, sorafenib	Inhibit system Xc−	Prevents cystine import, causes GSH depletion
Cystine/cysteine deprivation, buthionine sulfoximine (BSO), cisplatin	GSH	GSH depletion
(1S,3R)-RSL3, ML162, diphenyleneiodonium (DPI) compounds	GPX4	Inhibitor of GPX4 induce accumulation of lipid hydroperoxides
FINO2	Lipid peroxidation	Induces lipid peroxidation; indirectly inhibits GPX4
FIN56	Squalene synthase (SQS) and GPX4	Depletes CoQ10; decreases GPX4 levels
Statins	HMGCR	Block CoQ10 synthesis by mevalonate pathway inhibition
Phospholipids with two PUFA tails	Phospholipids	Induce lipid peroxidation
Trigonelline, brusatol	NRF2	Blocks NRF2 proteins
INF-γ	Downregulates expression of system Xc	GSH depletion
Ferroptosis inhibitors		
Ferrostatins, liproxstatins	ROS lipid peroxidation	Block lipid peroxidation
Cycloheximide	Protein synthesis	Suppresses ferroptosis induced by system Xc inhibitors
Vitamin E, alpha-tocopherol, CoQ10, idebenone	Lipid peroxidation	Block lipid peroxidation, inhibition of lipoxygenases
Deferoxamine (DFO), ciclopirox, deferiprone	Deferoxamine (DFO), ciclopirox, deferiprone	Deplete iron
Dihydrobiopterin (BH2), tetrahydrobiopterin (BH4)	Ubiquinol	Antioxidant effect; lipid remodeling
Deuterated PUFAs (D-PUFAs), MUFAs	Lipid peroxidation	Block lipid peroxidation
Lipoxygenase inhibitors (e.g., cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC), baicalein and zileuton)	Lipoxygenases	Block lipoxygenase-induced lipid peroxidation
CoQ10, idebenone	Lipid peroxidation	Block lipid peroxidation
Dopamine	Neurotransmitter	Blocks GPX4 degradation
Vildagliptin, alogliptin, and linagliptin	Dipeptidyl peptidase-4 (DPP4)	Block lipid peroxidation

Adapted from Hadian and Stockwell (2020) and Stockwell et al. (2017)

Thirdly, coenzyme Q10 is also a product of GTP cyclohydrolase-1 (GCH1). GCH1 and its metabolite tetrahydrobiopterin (BH4) have a dual function of generating reduced CoQ10 (ubiquinol) and of remodeling lipids to selectively favor the preservation of phospholipids with two polyunsaturated fatty acyl tails while causing the peroxidation of aberrant phospholipids (Kraft et al. 2020). The GCH1-BH4-phospholipid axis thus presents a unique mechanism of ferroptosis resistance through the promotion of the abundance of the antioxidant BH4 and CoQ10 that is independent of the GPX4/glutathione system (Fig. 1).

Ferroptosis and Disorders

Ferroptosis is an iron-dependent form of regulated cell death involving the dysregulation of glutathione/GPX4 activity, iron metabolism, and lipid peroxidation leading to degenerative processes and tumor suppression. A compelling body of evidence over several years independently has revealed the association of tissue iron accumulation, lipid peroxidation and/or decreased levels of GSH and GPX4 in various pathologies. This is particularly pertinent to neurological diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS). Other neurodegenerative diseases that exhibit features of ferroptosis include acute renal failure drug-induced hepatotoxicity, tissue ischemia/reperfusion injury, T cell immunity, and cancer cell death (Li et al. 2020). Furthermore, the generation of ROS in the presence of iron via Fenton chemistry has also been linked to oxidative stress and diseases. Consequently, regulated GPX4 and FSP1 antioxidant defense machinery against oxidative stress when compromised results in ferroptotic cell death in tissues and organs. For example, in dementia, decreased GPX4 cortical content and impairment, elevated iron levels, and lipid peroxidation biomarkers such as hydroxynonenal, malondialdehyde, and acrolein have been reported. Furthermore, iron chelation, inhibitors of ACSL4 and intervention of vitamin E have been clinically beneficial to AD patients. These hallmarks of ferroptosis are the underlying factors driving a range of degenerative diseases and cancer. Consequently, inhibitors and inducers of ferroptosis can be exploited as targets for therapeutic purposes. Animal models of GPX4 gene deletion have revealed features of ferroptosis in pathologies in the kidney; ischemia-reperfusion injury (IRI) was observed in the liver, heart, and the brain. Glutathione/GPX4 enzymatic activity is essential for the maintenance of membrane integrity by inhibiting and repairing membrane protein damage and the elimination of electrophiles arising from lipid peroxidation. This function can only be partially redundant in the presence of hydrophobic, membrane-restricted chain-breaking radical-trapping antioxidants (RTAs) such as α-tocopherol (vitamin E) or phenothiazine. While overwhelming evidence alludes to ferroptosis as an anticancer process, the molecular mechanisms are intricate. P53, a tumor suppressor protein apart from being a transcriptional regulator of SLC7A11 also modulates the expression of spermidine/spermine N1-acetyltransferase 1 (SAT1) and glutaminase 2 (GLS2) to promote ferroptosis. SAT1 elevates the expression of arachidonate 15 lipoxygenase, while GLS2 increases glutamine levels through glutaminolysis which depending on the context could be equivocal. Other independent inducers of ferroptosis are CD8+ T-driven IFN-ɣ that downregulates the expression of SLC7A11 as well as the effect of radiation which directly induces peroxidation of lipids (Hadian and Stockwell 2020). **A** Yes-associated protein (YAP) is a transcriptional coactivator in the Hippo tumor-suppressing signaling pathway that could also promote ferroptosis.

Chemical compounds that inhibit the activity of GPX4 and lipid peroxidation need to be tested and evaluated in clinical trials for the therapy of degenerative diseases (Stockwell et al. 2017). Conversely, inducers of players of ferroptosis from the proximate to the terminal process such as system xc and ACSL4 could be employed in a regulated manner to promote ferroptosis in cancer therapy. Pharmacological strategies could also be targeted at processes such as the Nrf2 antioxidant system, transsulfuration pathway, ferritinophagy and hypoxia-inducible factors (Fuhrmann et al. 2020), and other pathways that are associated with ferroptosis.

In summary, the discovery of ferroptosis has opened up a new vista that has high potentials for the treatment of a myriad of diseases (Li et al. 2020). Research in ferroptosis is ripe to decipher and clarify issues that are yet to be resolved. For example, there is a need for a clear understanding of ferroptosis regulatory genes and their potentials in different cancer cells or tumors. What are the vital effectors of cell death downstream of lipid peroxidation? Research is required to define the unequivocal role of ferroptosis in tumorigenesis, inflammation, and immunogenicity. Another

important area of interest is to define the nature of the association and interactions of ferroptosis and other forms of cell death. The availability of stable inhibitors of ferroptosis in in vivo situations will facilitate research in the field. The definition of its physiological functions in normal metabolism and the identification of specific and selective biomarkers will be breakthroughs in the field. The definition of physiological functions of ferroptosis in normal metabolism, and the identification of specific, selective and sensitive biomarkers (such as the measurement of real-time GSH/ROS dynamics, analysis of iron-independent expression of TfR1 and lipid peroxidation kinetics) particularly in vivo, will be vital advances in the field.

Summary

Ferroptosis is a form of regulated iron-dependent cell death that is characterized by a lethal accumulation of lipid peroxides and impaired functionality of the antioxidant defense system. Ferroptosis is thus induced by the loss of function of the glutathione/GPX4-dependent antioxidant defenses and lack of the FSP1/ubiquinol compensatory/overlapping mechanism that results in excessive lipid peroxidation and cell death. Lipophilic antioxidants, iron chelators, and chemical compounds that inhibit or induce ferroptosis have potentials for therapy of degeneration diseases and cancer.

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Fever

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Definition

Fever is defined as a state of elevated body temperature (T_b), which is often part of the defensive responses of the host to the invasion of live or inanimate matter recognized as pathogenic or alien. The febrile rise of T_b is due to a change in the thermoregulatory characteristics, which was formerly called elevation of a "set point," a reference temperature that is compared with the regulated value of T_b. Since a neuronal correlate for such a reference signal does not exist, the term set point should no longer be used or be replaced by the term "balance point." The elevation of T_b under febrile conditions is actively achieved by coordinated behavioral and physiological responses with the aim to reduce heat loss (warm-seeking behavior, peripheral vasoconstriction) and increase heat production (shivering or non-shivering thermogenesis). The threshold T_bs, at which these responses are initiated, are shifted to higher values in the initial phase of fever (Fig. 1).

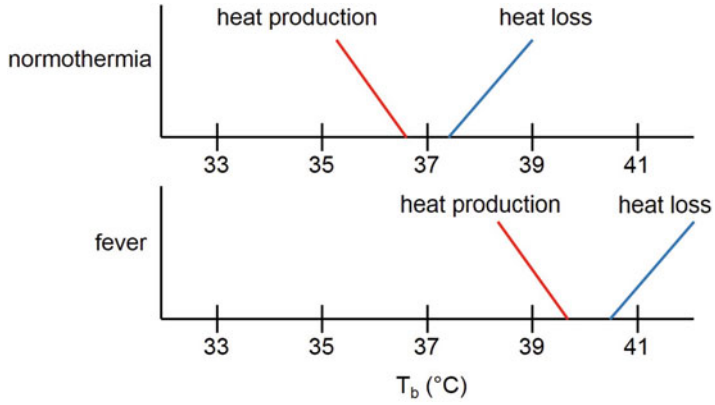
This shift represents the elevated thermoregulatory balance point during fever, which is above that during normothermia. The febrile upward shifts of thermoregulatory thresholds are caused by the action of pyrogenic substances, namely, prostaglandin E_2 (PGE_2), on neurons within the preoptic-anterior hypothalamic area (POA), which control efferent neuronal pathways to thermoregulatory relevant peripheral effector organs (Roth and Blatteis 2014).

Basic Mechanisms

Exogenous and Endogenous Pyrogens

Fever is a critical component within a series of defensive responses that occurs when live or inanimate agents enter the body through a break in one of its natural barriers. Here, they are quickly recognized as foreign or pathogenic by the innate immune system of the invaded host. Many different substances can provoke these responses (Table 1). Since they originate outside the body, they are called *exogenous* pyrogens. These exogenous pyrogens can be microbial or non-microbial or infectious or non-infectious in nature (Roth and Blatteis 2014).

The exogenous pyrogens listed in Table 1 are not the agents that directly cause fever and various other aspects of the acute-phase response (APR). Rather, upon their first contact with the invaded host's immune cells, they stimulate these cells to generate and release a number of factors that are the proximate mediators of the febrile response; these factors are termed *endogenous* pyrogens. The mechanisms whereby exogenous pyrogens are recognized, excite immune cells, and induce the generation of endogenous pyrogens can be briefly summarized as follows. The general assumption of this concept is that after the appearance of an exogenous pyrogen (e.g., lipopolysaccharide, LPS, the most frequently used exogenous pyrogen in experimental studies), the endogenous pyrogen would appear in the circulation of the LPS-treated individual to induce fever.

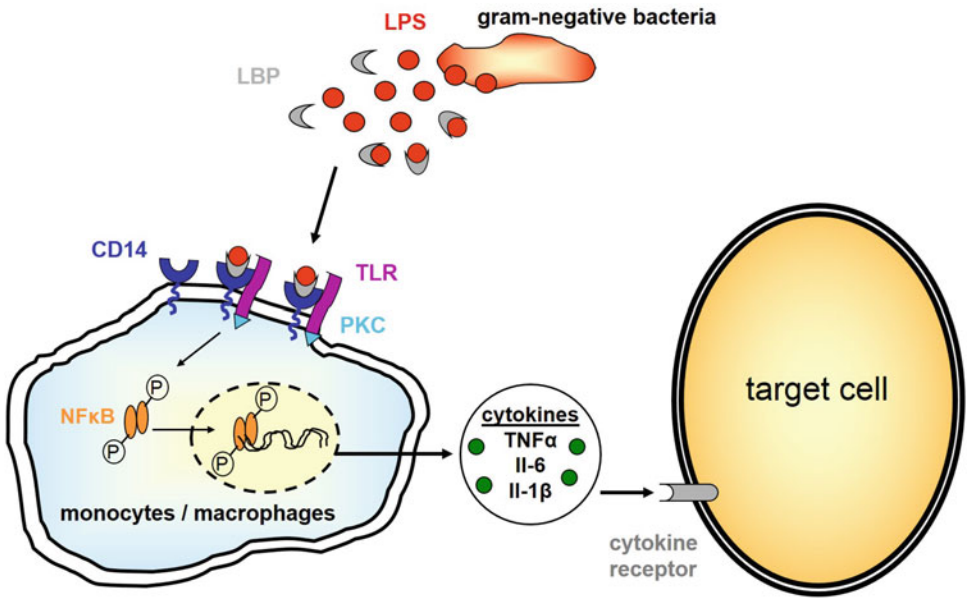


Fever, Fig. 1 Thermoregulatory thresholds in normothermic subjects (upper panel) and under febrile conditions (lower panel). The thresholds represent T_b, at which heat production or heat loss mechanisms are activated, when the subject is exposed to cold or heat. During the rising phase of fever, both thresholds are symmetrically shifted to higher values, indicating the elevated thermoregulatory balance zone. (Modified from Roth and Blatteis (2014))

Fever, Table 1 Pathogenic stimuli of microbial and non-microbial origin (exogenous pyrogens) that induce fever. (Modified from Roth and Blatteis (2014))

Examples for exogenous pyrogens	
1. Microbial	
Viruses	Whole organism, hemagglutinin, single or double stranded RNA
Bacteria	Gram-positive-whole organisms, cell walls, peptidoglycans, muramyl dipeptide, lipoteichoic acids, exotoxins, enterotoxins, erythrogenic toxins, group B polysaccharides
	Gram-negative-whole organisms, peptidoglycans, lipopolysaccharides (lipid A)
Mycobacteria	Whole organisms, lipopeptides (macrophage activating lipopeptide-2, fibroblast-stimulating lipopeptide-1) polysaccharides, lipoarabinomannan
Fungi	Whole yeast, zymosan, capsular polysaccharides, proteins
2. Non-microbial	
Antigens	Bovine serum albumin, bovine gamma globulin, ovalbumin, penicillin
Inflammatory agents	Turpentine, asbestos, silica, UV radiation
Plant lectins	Concanavalin A, phytohemagglutinin
Drugs	Synthetic polynucleotides (e.g., polyriboinosinic-polyribocytidylic acid, cytosine-phosphate-guanine-rich CpG-DNA), imidazoquinolines (imiquimod, gardiquimod), antitumor agents (bleomycin), plant alkaloids (colchicines), synthetic immunoadjuvants
Host-derived	Antigen-antibody complexes, activated complement fragments, inflammatory bile acids, urate crystals, androgenic steroid metabolites, lymphocyte products

Endogenous pyrogens are represented by a family of small proteins termed cytokines (Roth and Blatteis 2014; Garami et al. 2018). With a few exceptions, circulating cytokines are not detected in healthy individuals but appear in the blood during various states of disease to regulate and coordinate innate and adaptive immune responses. Pathogens share highly conserved microbial components called "pathogen-associated molecular patterns" (PAMPS). These activate the innate immune system via "Toll-like receptors" (TLRs) or other pattern recognition receptor families. Employing a number of intracellular adapter proteins, the interaction of a given PAMP with its TLR causes activation of the transcription factor nuclear factor-κB (NF-κB), which then is translocating into the nucleus of the PAMP-stimulated cell (Fig. 2).



Fever, Fig. 2 Schematic illustration of the coupling between exogenous and endogenous pyrogens at the cellular level with LPS from gram-negative bacteria as a representative example for an exogenous pyrogen. After binding of LPS to a LPS-binding protein, this complex is bound to CD14. LPS-loaded CD4 causes the activation of TLR4, the cognate receptor for LPS. In the LPS-stimulated cell, protein kinase C (PKC) and the transcription factor NF-κB are activated. Activated NF-κB migrates into the nucleus and causes the transcription of inflammatory target genes including those coding for the pyrogenic cytokines TNF-α, IL-1β, and IL-6. These cytokines are released, spill over into the bloodstream, and influence many types of target cells, which possess receptors for these cytokines. The target cells, which are relevant for the induction of fever, are located in the brain

Here, the transcription of a number of target genes is initiated, some of which are related to fever. These include the most important pyrogenic cytokines, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), which appear in the blood during fever in a characteristic sequence. The following further arguments support a role for peripherally released TNF-α, IL-1β, or IL-6 as endogenous pyrogens. Injections of all the three of these cytokines evoke fevers in man or experimental animals. Pharmacological tools, which aimed to neutralize, antagonize, or block the formation of single circulating cytokines after injection of a given exogenous pyrogen, attenuate the febrile response. Other cytokines, chemokines, adhesion molecules, receptors for inflammatory molecules, and the inducible forms (as opposed to constitutive forms) of the inflammatory enzymes cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1) are further products of target genes, which show enhanced expression due to NF-κB activation. COX-2 and mPGES-1 are strongly coupled in their induction after an inflammatory insult and considered to be the critical enzymes for the generation of PGE_2 during fever (Blomqvist and Engblom 2018). Hepatic and pulmonary macrophages belong to the first cells, in which LPS causes a coupled induction of COX-2 and mPGES-1 (Garami et al. 2018). LPS, the most potent exogenous pyrogen, employs a second, faster way for peripheral formation of PGE_2 by activation of the complement cascade via the alternative pathway, namely, in Kupffer cells of the liver (Roth and Blatteis 2014). The rapid peripheral generation of PGE_2 seems to contribute to the initiation of a febrile response. In addition to TNF-α, IL-1β, IL-6, and PGE_2, there are more peripherally produced molecules with intrinsic pyrogenic properties upon stimulation of TLRs by a given exogenous pyrogen.

Actions of Pyrogens (PGE_2) on the Thermoregulatory System

The mammalian thermoregulatory system consists of a sensory afferent part, a central integrative part, and an efferent part terminating at the distinct effector organs. Information about environmental temperature is generated by peripheral thermosensors (predominantly cold sensors) and transmitted to the spinal dorsal horns. Here, secondary neurons receive and transport the thermal signals via the pontine lateral parabrachial nucleus (LPB) to the thermoregulatory relevant neurons within the POA (Morrison and Nakamura 2019). These structures represent the so-called pyrogenic zone within the POA, where smallest amounts of PGE_2 cause a rise of T_b (Blomqvist and Engblom 2018). The critical neurons within the POA use, in part, gamma amino butyric acid (GABA) as neurotransmitter. These GABAergic neurons exert a tonic inhibitory influence on secondary neurons located in the dorsomedial hypothalamus and in the rostral medullary raphe pallidus nucleus, which directly control thermogenesis and vasomotor tone via the sympathetic nervous system. After disinhibition by external cooling or by the appearance of PGE_2 within the POA, appropriate thermoeffector responses are activated via the descending neuronal pathways to brown adipose tissue (BAT), blood vessels, or skeletal muscles (Morrison and Nakamura 2019). The inhibitory GABAergic neurons are, in part, warm-sensitive and equipped with PGE_2 receptors of the subtype EP3. An inhibition of central GABAergic warm-sensitive neurons by action of PGE_2 on the EP3 receptor is considered as the central drive for the generation of fever (Blomqvist and Engblom 2018).

Transfer of Fever-Generating Signals Across the Blood-Brain Barrier

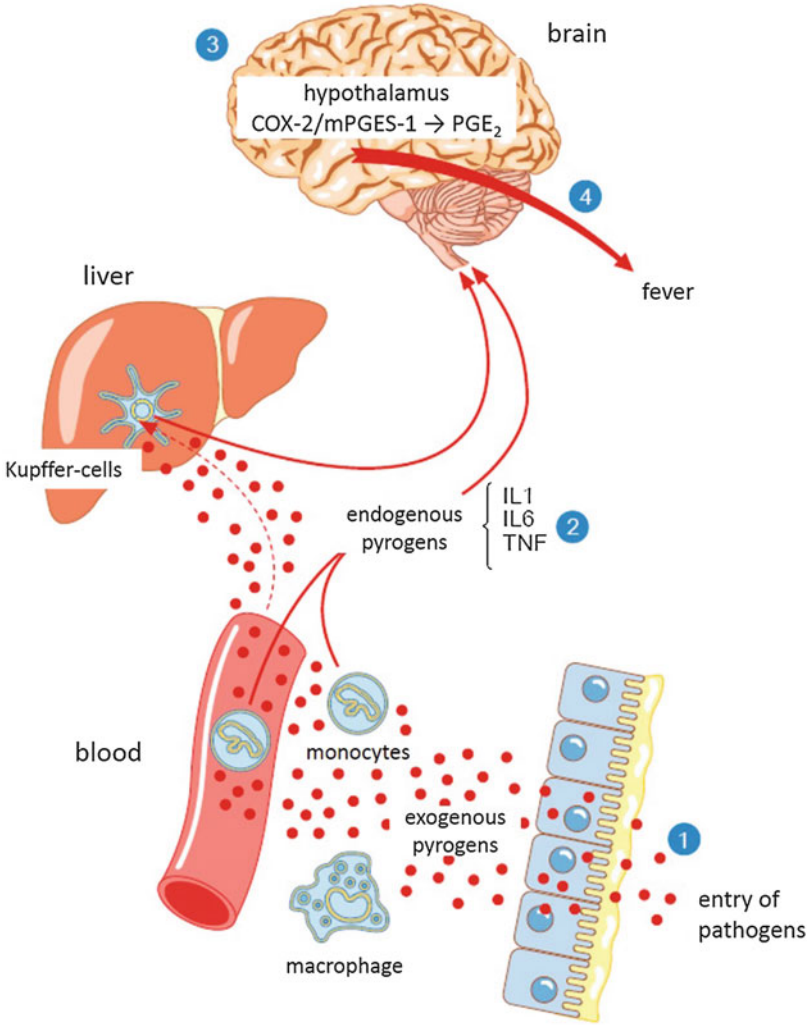
The structures of the POA, where PGE_2 (or any other pyrogenic substance) causes the febrile shift of the thermoregulatory balance point to a higher level, are located inside the blood-brain barrier (BBB). Four possible routes for the transfer of febrile signals to the brain are briefly introduced, (I) the circumventricular organs lacking a tight BBB, (II) cells forming the BBB and acting as relay stations for the transfer of febrigenic signals to the brain, (III) transport of endogenous pyrogens into the brain, and (IV) the activation of afferent nerves by peripherally generated pyrogens (Dantzer 2018). Mechanisms I–III can be summarized as the "humoral pathway for the induction of fever." This predominant pathway is illustrated in Fig. 3.

Sensory Circumventricular Organs (CVOs)

The parenchyma capillaries of the sensory CVOs possess a fenestrated endothelium, thus allowing circulating hydrophilic signal molecules, including endogenous or exogenous pyrogens, to enter the brain. With regard to fever, special attention has been directed to the *organum vasculosum laminae terminalis* (OVLT) due to its close proximity to the POA (Roth and Blatteis 2014). There is an extensive expression of receptors for cytokines and even of TLRs in the OVLT. The sensory CVOs including the OVLT belong to the most responsive sites within the CNS to systemic inflammatory stimulation. Their role as exclusive central sensors for pyrogenic cytokines or PAMPs is challenged by the fact that cells forming the BBB in the surrounding regions of the POA, namely, endothelial cells and perivascular macrophages, are also equipped with receptors for PAMPs and cytokines and are also highly responsive to inflammatory stimulation (Blomqvist and Engblom 2018).

Cells Forming the Blood-Brain Barrier (BBB)

Cells forming the BBB, vascular endothelial cells, and perivascular macrophages, within the entire brain, have the capacity to secrete cytokines (IL-6), PGE_2, and other mediators into the brain parenchyma after their respective inflammatory stimulation from the luminal blood side. Especially brain endothelial cells play an important role in the transduction of inflammatory signals from the blood to the brain (Blomqvist and Engblom 2018). The expression of TLRs and of receptors for pyrogenic cytokines on brain endothelial cells enable their direct responsiveness to many kinds of pyrogens. In brain endothelial



Fever, Fig. 3 Humoral pathway for the induction of fever. Fever is generated in a sequence of four steps. (1) A pathogen (red circles) enters the host through one of the natural barriers (gastrointestinal tract, respiratory tract, urogenital tract, or skin). PAMPs activate TLRs on monocytes or macrophages. A break within the gastrointestinal tract causes the transport of exogenous pyrogens predominantly to the liver, where Kupffer cells are activated by the same mechanisms. (2) Monocytes, macrophages, and Kupffer cells produce endogenous pyrogens/cytokines (TNF-α, IL-1β, IL-6). Cytokines and PAMPs reach the brain via the bloodstream. (3) In the brain (endothelium; OVLT), a coupled induction of COX-2/mPGES-1 occurs, and large amounts of PGE_2 are produced and released abluminally into the POA (hypothalamus). (4) In the POA PGE_2 inhibits warm-sensitive/GABAergic neurons via the EP3 receptor, with the consequence that heat production is activated at a higher T_b – fever develops. (Modified from Roth and Blatteis (2014))

cells, circulating pyrogens thus cause an activation of NF-κB (Fig. 1) and other inflammatory transcription factors (Rummel 2016). In the context of fever, the target genes of these transcription factors include COX-2 and mPGES-1. Like in peripheral macrophages, the coupled induction of COX-2 and mPGES-1 in brain endothelial cells is important for inflammatory formation of PGE_2 in the brain. The co-localized expression of both these inducible enzymes in brain endothelial cells is the prerequisite for sustained PGE_2 synthesis in the POA during fever and the subsequent activation of the EP3 receptor (Blomqvist and Engblom 2018).

Transport of Pyrogens Across the Blood-Brain Barrier

Small amounts of pyrogenic cytokines can pass the BBB through specific saturable trans-endothelial transport systems (Roth and Blatteis 2014). It is a matter of debate whether the amounts of transported cytokines are sufficient to trigger a febrile response. An entry of peripherally derived PGE_2 through the BBB by specialized transporters is also possible and has been suggested to contribute to the early onset of fever prior to the formation of PGE_2 by the cells forming the BBB (Garami et al. 2018).

Inflammatory Signal Transfer from the Periphery to the Brain Via Sensory Nerves

Afferent fibers of the vagus nerve and of somatic nerves have been suggested to contribute especially to the early stage of fever. In the ganglia containing the cell bodies of these fibers, receptors for cytokines and the EP3 receptor for PGE_2 are expressed. This means that afferent nerve fibers can be readily activated by PGE_2, which is released by Kupffer cells in the liver (vagus nerve) or within other inflamed tissues (somatic nerves). Evidence for a role of sensory afferent parts of peripheral nerves in the generation of fever has mainly been obtained in experimental models, which mimic localized peripheral inflammation (Roth and Blatteis 2014; Blomqvist and Engblom 2018; Dantzer 2018). This mechanism might reflect situations, when fever is observed in absence of circulating pyrogens.

Pharmacological Intervention

During infection and inflammation, the prominent febrile increase of body temperature (T_b) is not an isolated event. Fever is rather an integrated component of a complex array of brain-mediated, nonspecific host defense responses to the invasion of the body by pathogens. It is thus a physiological, not a pathological, reaction. These brain-mediated responses include a range of behavioral changes collectively termed "sickness behavior" (Dantzer 2018; Harden et al. 2015). Presumptively the sense of these behavioral changes is to restrict physical activity of the infected host in order to conserve energy for the restoration of health (Dantzer 2018). Antipyretic (self-)administered drugs are routinely used to antagonize other components of the sickness syndrome rather than fever, for example, pain or general malaise. The question of whether fever is of maladaptive value and should thus be treated or rather an adaptive response to improve the fight against infection is still discussed controversially. Experimental studies have shown that nearly all components of innate and adaptive immunity are enhanced and accelerated by febrile temperatures. Meta-analyses of clinical studies could, however, not provide an unequivocal response to the question, whether treatment of fever in populations of patients in intensive care units is beneficial of detrimental (Harden et al. 2015). Two principle strategies are available for interventions with aim to reduce fever: pharmacological therapy with nonsteroidal anti-inflammatory drugs (NSAIDs like ibuprofen) or acetaminophen (paracetamol), on the one hand, or physical therapy by use of cooling mechanisms on the other hand. Pharmacological strategies most frequently interfere with the mechanisms leading to the formation of PGE_2, the final mediator of fever. In this case, active heat production by the infected host is suppressed. Physical cooling reduces T_b without depressing endogenous heat production. The inhibition of PGE_2 formation can be achieved at distinct steps with the signal pathways for fever induction shown in Figs. 2 and 3. Traditionally, COX-inhibitors are used for antipyretic treatment. Non-selective COX-inhibitors block the constitutive (COX-1) as well as the inducible isoform (COX-2). The side effects of these drugs include gastrointestinal bleeding or disturbance of renal blood flow. Selective COX-2 inhibitors, on the other hand, which were approved for clinical use, exert adverse cardiovascular effects when used at higher doses. Especially in experimental studies, other drugs are frequently employed, which antagonize formation and action of pyrogenic cytokines or inhibit inflammatory transcription factors. A summary of antipyretic and anti-hyperthermic strategies is provided in Table 2.

Fever, Table 2 Some antipyretic or antihyperhermic therapies currently in use or under test in experimental studies. (Modified from Roth and Blatteis (2014))

Routine
Acetaminophen (paracetamol)
Glucocorticoids (cortisone, prednisolone)
COX-inhibitors, nonselective (NSAIDs, aspirin, ibuprofen, and others)
COX-2 inhibitors, selective or preferential (celecoxib, meloxicam, and others)
Emergency
Sponging
Ice packs
Cooling blankets
Inhaled or ambient helium-oxygen
Others (mostly experimental)
Antioxidants (catechins, vitamin E, platonin)
LPS sequestrants, anti-lipid A
Anti-inflammatory cytokines (IL-4, IL-10)
Cytokine antagonists (IL-1-receptor antagonist, soluble TNF-receptors, antibodies)
mPGES-1 inhibitors
Phospholipase (PL) A2 inhibitors
Antibacterials, antivirals, antifungals
Complement fragment 5a (C5a) antibodies, synthetic C5aRas
Anti-NF-κB (parthenolide)
Anti-JAK-STAT agents (AG 490)
μ-opioid receptor antagonists (naloxone)

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Fibrates

► Lipid-Lowering Drugs

Fibroblast Growth Factors

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Definition

Fibroblast Growth Factors (FGFs) are a family of proteins that regulate a variety of physiological and pathological processes, including embryogenesis, tissue homeostasis, wound repair, and cancer. They exert these functions by binding to Fibroblast Growth Factor Receptors (FGFRs), triggering the activation of multiple signal transduction pathways that modulate the proliferation, differentiation, survival, migration, and metabolism of target cells.

Basic Characteristics

FGFs

The FGF family contains 22 members that act as paracrine, autocrine, or endocrine factors for different cell types. They are divided into seven subfamilies in accordance with their sequence similarity, biochemical function, and evolutionary relationship.

Canonical FGFs encompass subfamilies FGF1/2/5, FGF3/4/6, FGF7/10/22, FGF8/17/18, and FGF9/16/20. To induce their paracrine/autocrine responses, canonical FGFs bind to and activate tyrosine kinase (TK) FGFRs. FGF/FGFR interaction is stabilized by heparan sulfate (HS) proteoglycans (HSPGs), resulting in the formation of biologically active FGF/FGFR/HSPG ternary complexes, fundamental for signal transduction. During development, canonical FGFs are involved in several regulatory functions,

including the patterning of germ cell layers, formation of body axes, morphogenesis, and induction of organogenesis. Moreover, FGFs play a prominent role in the adult, where they contribute to tissue homeostasis, remodeling, and repair.

Intracellular FGF11/12/13/14, also known as FGF homologous factors (FHFs), act at the intracellular level in a FGFR-independent manner. They play a pivotal role in neuronal functions at postnatal stages and in regulating the cardiac rhythm by interacting with the intracellular domains of voltage-gate sodium and calcium channels and with the neuronal mitogen-activated protein kinase (MAPK) scaffold protein islet-brain-2.

The members of the FGF19/21/23 subfamily are hormone-like FGFs that exhibit poor affinity for HSPGs, a characteristic that results in more diffusive properties through blood circulation. They play a crucial role in regulating various metabolic processes, including phosphate, glucose, and lipid metabolism. Besides FGFRs, they interact in a specific manner with Klotho co-receptors to activate intracellular signaling responses (Presta et al. 2017; Giacomini et al. 2016; Ornitz and Itoh 2015).

FGFRs and HSPGs

Four distinct genes encode for FGFRs in mammals (*FGFR1–4*). FGFRs consist of three extracellular immunoglobulin-like (Ig) domains (D1–3), a single transmembrane domain and an intracellular TK domain. D2 and D3 domains are responsible for FGF binding, whereas the acid box between the first and second Ig loop is involved in HSPG interaction. FGFRs exhibit diverse specificities for FGF ligands and the alternative splicing of the D3 domain generates IIIb and IIIc isoforms of FGFR1, 2, and 3 that are endowed with additional ligand-binding properties. Interestingly, FGFR1–3IIIb and FGFR1–3IIIc variants often display a distinct pattern of expression in epithelial and mesenchymal tissues, respectively. Under physiological conditions, a ligand produced by epithelial cells will usually activate a mesenchymal receptor and vice versa, with exceptions.

FGFs can interact with different cell surface co-receptors, thus facilitating their productive binding to FGFRs. Among them, the interaction with HSPGs plays a pivotal role in mediating the biological activity of FGFs. HSPGs are glycoproteins expressed by almost all the cell types as membrane-associated receptors, extracellular matrix (ECM) components, and/or free molecules. They are composed by HS chains covalently linked to a core protein. HSPGs of the ECM may represent a reservoir for FGFs by sequestering the growth factors near the site of action and may allow the formation of FGF gradients that are essential for paracrine signaling. HSPGs are needed for a productive FGF/FGFR interaction. Indeed, HSPGs, FGFs, and FGFRs form 2:2:2 ternary complexes that induce receptor dimerization, a change in the receptor conformation and *trans*-phosphorylation of tyrosine residues in the FGFR TK domain. Multiple signal transduction pathways are activated by receptor phosphorylation, thus generating distinct cellular responses. HS chains can undergo structural modifications that affect FGFR signaling and are responsible for its fine-tuning regulation. At variance with canonical FGFs, the activity of hormone-like FGFs depends on the presence of Klotho proteins as co-receptors. Cell surface β-Klotho and α-Klotho act as co-factors for FGF19/21 and FGF23, respectively. They limit nonspecific/off-target signaling by converting FGFRs into high affinity receptors for endocrine FGFs (Presta et al. 2017; Giacomini et al. 2016).

FGF/FGFR Signaling

FGFR engagement induces the activation of multiple signal transduction pathways. As mentioned above, the phosphorylation of specific tyrosine residues in the TK domain of FGFRs results in the formation of docking sites for adaptor proteins that regulate downstream signaling. The activation of the intracellular specific adaptor protein fibroblast growth factor receptor substrate 2 (FRS2) recruits the adaptor proteins guanine nucleotide exchange factor 2 (GRB2) and son of sevenless (SOS) and activates the RAS/mitogen activated protein kinase (MAPK) pathway, mainly implicated in cell proliferation and

differentiation. In turn, GRB2 recruits GRB2-associated binding protein 1 (GAB1), leading to the activation of the phosphoinositide 3 kinase (PI3K)/RAC-alpha serine/threonine-protein kinase (AKT) pathway associated with cellular survival and cell fate determination. Phospholipase C gamma (PLCγ) is a further target of activated FGFRs. PLCγ hydrolyzes phosphatidylinositol-diphosphate (PIP2) into inositol-triphosphate (IP3) and diacylglycerol (DAG). DAG and cytoplasmic calcium released from the endoplasmic reticulum in response to IP3 lead to the activation of protein kinase C (PKC). This pathway sustains MAPK and AKT signaling and plays a role in cell migration, adhesion, and cell morphology. Other pathways activated by FGFRs include the signal transducers and activator of transcription (STAT)-dependent signaling, p38 MAPK, and ribosomal S6 protein kinase (RSK).

FGFR-mediated signaling is modulated by various intracellular mechanisms. The RAS/MAPK pathway can act as a negative feedback modulator by inhibiting FGFR activation. Sprouty proteins (SPRY) interact with GRB2 in order to inhibit or modulate RAS/MAPK and PI3K/AKT signaling, respectively. SHP2 dephosphorylates and switches off FGFR signaling, whereas MAPK phosphatase-3 (MKP-3) directly dephosphorylates extracellular signal-regulated kinases$_{1/2}$ (ERK$_{1/2}$). Lastly, the transmembrane protein similar expression to FGF (SEF) prevents FGFR activation by binding the extracellular moiety of the receptor and suppresses FGF signaling by inhibiting the RAS/MAPK pathway (Fig. 1).

FGFs in Physiology

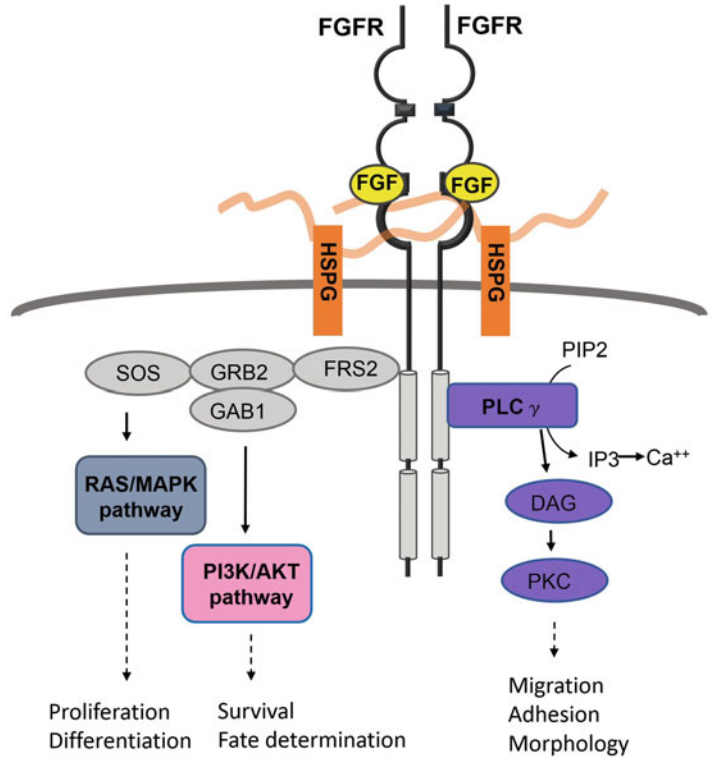
Embryonic Development

FGFs regulates several developmental events, including patterning of germ cell layers, formation of body axes, coordination of morphogenetic movements, induction of organogenesis, and morphogenesis.

Germ cell layer patterning. FGF signaling is highly involved in the specification of the mesoderm and neuroectoderm. Several studies indicate that FGFs act as diffusive signaling proteins implicated in the formation of the mesoderm (axial induction and paraxial maintenance) and of the neural tissue derived from the embryonic ectoderm. FGFs are involved in the induction of the posterior nervous system and are crucial for embryonic morphogenesis during gastrulation by regulating cell proliferation, differentiation, and senescence and by coordinating morphogenetic movements. The concentration gradient of the FGF morphogen across the embryo is regulated by HSPGs and induces cellular responses in a dose-dependent manner. FGF gradients are controlled also by ligand trafficking and secretion, ligand and receptor endocytosis, and by feedback mechanisms triggered by inhibitors of the FGF/FGFR axis.

Axes specification. FGFs are implicated in the patterning of the dorsoventral, anteroposterior, and left/right axes of the embryo. The maternal Wnt/β-catenin pathway and the zygotic bone morphogenetic protein (BMP) pathway contribute with FGFs in the definition of the dorsoventral axis. FGF signaling promotes dorsal fates and inhibits ventral fates by restricting the expression and activity of BMPs. As in dorsoventral patterning, FGF activity regulates anteroposterior patterning by a coordinated action with retinoic acid and Wnt. During anteroposterior extension, the formation of a FGF activity gradient from posterior to anterior that occurs in parallel with an inverse gradient (from anterior to posterior) of retinoic acid activity represents a conserved mechanism that regulates the patterning and timing of differentiation in the posterior region. Regarding the left/right axis determination, FGF signaling is involved in the formation of the node and nodal cilia and in the modulation of the expression of genes that determine the right and left side of the embryo body.

Organogenesis. At first, the FGF/FGFR system plays a crucial role in the specification of the trophectoderm and of the primitive endoderm. Later in development, FGFs exert nonredundant functions in organogenesis, including the formation of the anterior and secondary heart fields, induction of limb and lung buds, and in the development of liver, pancreas, kidney, inner ear, and



Fibroblast Growth Factors, Fig. 1 FGFR-mediated signaling. See text for details (Presta et al. 2017; Giacomini et al. 2016; Turner and Grose 2010)

brain. In this frame, FGF signaling plays a key role in regulating the interaction between epithelial cells and the mesenchyme. As described above, alternative mRNA splicing leads to the production of the IIIb or the IIIc FGFR isoforms. These receptor variants are regulated in a tissue-specific manner and determine ligand-receptor binding specificities. While the FGFR-IIIb isoforms are expressed in epithelial lineages, FGFR-IIIc variants are expressed mainly by mesenchymal lineages. Adjacent tissues produce FGFs specific for the distinct FGFR isoforms, thus generating a directional epithelial-mesenchymal signaling. Even though with some exceptions, branched organs (lung, salivary gland, and lacrimal gland), intestine, liver, and limb bud development are guided by the binding of mesenchymal FGFs (e.g., FGF7 and FGF10) to epithelial FGFR-IIIb variants. Conversely, FGFs expressed by epithelia (e.g., FGF8 and FGF9) can activate mesenchymal FGFR-IIIc isoforms (Dorey and Amaya 2010; Bottcher and Niehrs 2005).

Tissue Homeostasis

FGFs exert homeostatic functions in the adult, being involved in tissue maintenance, repair, remodeling processes, and metabolism. In adults, FGFs/FGFRs are expressed in a cell type-specific fashion to maintain tissue homeostasis and function. FGFR-IIIb variants and their ligands are essential for the homeostasis of different tissues. For instance, FGFR-IIIb receptors are important in the maintenance of skin annexes and epidermal barrier function following their engagement by FGF1, FGF7, FGF10, and FGF22 expressed in different compartments of the skin. Liver homeostasis and regeneration is regulated by the expression of the FGFR2-IIIb variant in hepatocytes. FGF10 signaling is known to play an essential role in cardiac homeostasis. Stromal FGF7/FGF10 and epithelial FGFR2 are important in the maintenance of prostate tissue homeostasis and androgen signaling. FGF2, as well as FGF4, FGF8, FGF9, FGF10, and FGF18, play important roles in bone homeostasis. FGF2/FGFR1 and FGF18/FGFR3 axes have been implicated in the

regulation of articular and intervertebral disc cartilage homeostasis. Finally, the FGFR1-IIIc isoform is involved in vascular permeability.

Hormone-like FGF19, FGF21, and FGF23 coordinate different metabolic pathways. They lack conventional heparin-binding domains, making them able to be readily present in the blood circulation, thus acting in an endocrine-like manner. These factors require Klotho co-receptors that define tissue selectivity and determine their distinct physiological roles. FGF19 (FGF15 in mice) regulates cholesterol/bile acid synthesis and homeostasis, stimulates glycogen and protein synthesis in the liver, represses gluconeogenesis, and governs nutrient metabolism. FGF21 is involved in the control of glucose and lipid metabolism by modulating gluconeogenesis, hepatic lipid oxidation, and ketogenesis. FGF23 plays a critical role in phosphate homeostasis and vitamin D metabolism in the kidney.

As described below, abnormal expression of FGFs and FGFRs and aberrant activation of FGF signaling are observed in various adult tissue-specific pathologies and may cause developmental disorders (Ornitz and Itoh 2015; Itoh et al. 2015; Yang et al. 2015; Steiling et al. 2003).

Wound Healing

The importance of FGFs and their receptors in tissue regeneration and repair is well documented. Wound repair requires the cooperation of various cell types, including keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets. They orchestrate a series of coordinate events, including cell proliferation and migration, ECM deposition and remodeling, wound contraction, and angiogenesis. FGF7, FGF10, and FGF22, all ligands of the FGFR-IIIb variant, exert a prominent role in wound repair. They target epithelial cells, including keratinocytes, stimulating cell proliferation and migration, and reduce reactive oxygen species production, thus preserving cells from apoptosis. In addition, inflammatory cells, vascular endothelial cells, fibroblasts, and keratinocytes produce FGF1 and FGF2 in the acute wound. They act predominantly on stromal cells that express the FGFR-IIIc variant. They play a role in granulation tissue formation, re-epithelialization, angiogenesis, and tissue remodeling, regulating the synthesis and deposition of various ECM components and promoting fibroblast migration. Deregulation of the FGF/FGFR system is associated with chronic wound. Angiogenesis (the formation of new blood vessels from pre-existing ones) represents a key step during tissue repair, delivering nutrients and oxygen to support the energy-consuming process of tissue remodeling. FGF1, FGF2, FGF4, FGF8b, and FGF10 trigger an angiogenic response by acting on FGFR1 and FGFR2 expressed by endothelial cells. They modulate different steps of the angiogenesis process, including endothelial cell proliferation and migration, ECM degradation, morphogenesis, and vessel maturation. FGFs have been implicated also in tumor angiogenesis (Muller et al. 2012; Werner and Grose 2003; Presta et al. 2005; Maddaluno et al. 2017; Beenken and Mohammadi 2009).

FGFs in Pathology

Genetic Diseases

The wide range of disorders observed when the FGF/FGFR axis is genetically mutated reflects the complex function exerted by this system during tissue development and homeostasis. *FGF/FGFR* genes show germline mutations in at least 24 distinct human congenital disorders (Table 1). Most of these syndromes are characterized by skeletal, cranial, and limb abnormalities. At the skeletal level, altered ossification may result in short-limbed dwarfism and craniosynostosis, characterized by the premature fusion of one or several cranial sutures. Gain-of-function mutations of the D3 domain of FGFRs, that cause ligand-independent FGFR dimerization and activation, are observed in this type of syndromes. In addition, genetic disorders characterized by deafness, endocrine or renal abnormalities, and neurological diseases have been described, frequently associated with FGF/FGFR loss-of-function mutations. As for canonical FGFs, FHFs have been implicated in genetic disorders, providing insights about their role in ion channel function (Helsten et al. 2015; Moosa and Wollnik 2016).

Fibroblast Growth Factors, Table 1 Genetic disorders and malignant conditions associated with aberrant FGF/FGFR signaling

	Heritable FGF/FGFR gene mutation	Acquired FGF/FGFR deregulation in malignancy
Gene	**Genetic disorder**	**Type of cancer**
FGF alterations		
FGF1		Ovarian cancer
FGF2		Bladder cancer, prostate cancer, small cell lung carcinoma, melanoma, hepatocellular carcinoma
FGF3	Michel aplasia, labyrinthine aplasia, microtia, and microdontia (LAMM) syndrome	Breast cancer
FGF4		Breast cancer
FGF5	Trichomegaly	Glioblastoma
FGF6		Prostate cancer
FGF7		Lung adenocarcinoma
FGF8	Hypogonadotropic hypogonadism 6 with/without anosmia (Kallmann syndrome)	Breast cancer, prostate cancer, hepatocellular carcinoma, colorectal cancer
FGF9	Multiple synostoses syndrome 3	Colorectal and endometrial carcinomas, non-small cell lung cancer
FGF10	Lacrimo-auricular-dento-digital (LADD) syndrome, aplasia of the lacrimal and salivary glands (ALSG)	Breast carcinoma, prostate cancer
FGF12	Brugada syndrome	
FGF14	Spinocerebellar ataxia type 27 (SCA27)	
FGF16	Metacarpal 4–5 fusion	Ovarian cancer
FGF17	Hypogonadotropic hypogonadism 20 with/without anosmia	Prostate cancer, hepatocellular carcinoma
FGF18		Hepatocellular carcinoma
FGF19		Prostate cancer, hepatocellular carcinoma
FGF20	Renal hypodysplasia/aplasia 2	
FGF23	Hypophosphatemic rickets, Familial hyperphosphatemic tumoral calcinosis	Increased risk of prostate cancer
FGFR alterations		
FGFR1	*Skeletal dysplasia:* Osteoglophonic dysplasia *Craniosynostosis:* Jackson–Weiss syndrome, Pfeiffer syndrome *Others:* Hartsfield syndrome, Hypogonadotropic hypogonadism 2 with or without anosmia, Trigonocephaly 1	Small cell lung cancer, squamous cell lung cancer, breast cancer, ovarian cancer, pancreatic ductal adenocarcinoma, tongue squamous cell carcinoma, melanoma, pilocytic astrocytoma, leukemia, lymphoma, alveolar rhabdomyosarcoma, glioblastoma, myeloproliferative syndrome
FGFR2	*Skeletal dysplasia:* Bent bone dysplasia, *Craniosynostosis:* Antley–Bixler syndrome without genital anomalies or disordered steroidogenesis, Apert syndrome, Beare–Stevenson cutis gyrata syndrome, craniofacial-skeletal-dermatological dysplasia, Crouzon syndrome, Jackson–Weiss syndrome, Pfeiffer syndrome, Saethre–Chotzen syndrome,	Gastric cancer, breast cancer, Cholangiocarcinoma, Endometrial carcinoma

(continued)

Fibroblast Growth Factors, Table 1 (continued)

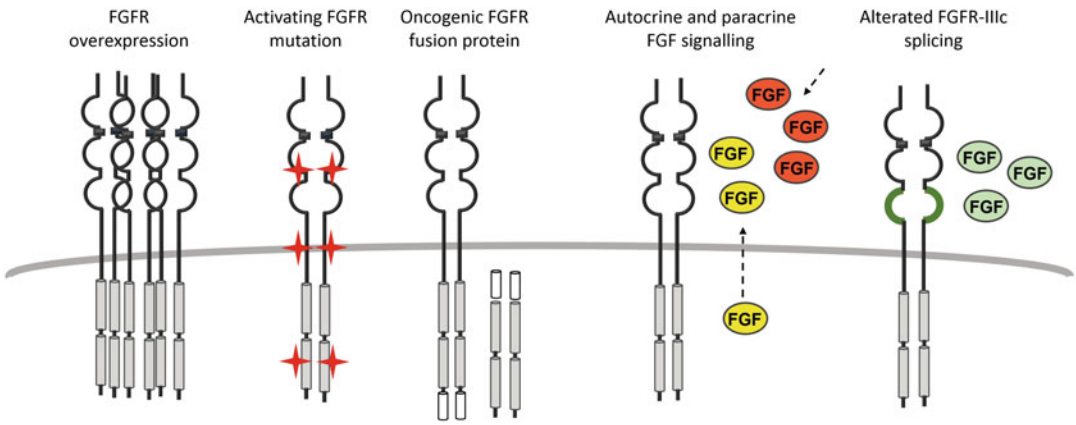
	Heritable FGF/FGFR gene mutation	Acquired FGF/FGFR deregulation in malignancy
	Scaphocephaly, maxillary retrusion, and mental retardation *Others:* LADD syndrome	
FGFR3	*Skeletal dysplasia:* Achondroplasia, Hypochondroplasia, Severe achondroplasia with developmental delay and acanthosis nigricans (SADDAN) syndrome, Thanatophoric dysplasia type 1 and 2, *Craniosynostosis:* Crouzon with acanthosis nigricans, Muenke syndrome *Others:* Camptodactyly-tall stature-scoliosis-hearing loss (CATSHL) syndrome, LADD syndrome	Gastric cancer, colorectal cancer, breast cancer, endometrial carcinoma, urothelial carcinoma, bladder tumor, skin tumor, myeloma, Squamous cell lung cancer, bladder cancer, glioblastoma, lymphoma, Colon cancer
FGFR4		Rhabdomyosarcoma, adenoid cystic carcinoma, breast Cancer, Ovarian cancer, hepatocellular carcinoma

Cancer

Tumors are characterized by a tight cross-talk between cancer and stromal cells, including inflammatory cells (e.g., lymphocytes, macrophages, and mast cells), fibroblasts, and vascular components. The various cellular components express different FGFRs and can release FGFs in the tumor microenvironment. This triggers a complex network of autocrine/paracrine loops of stimulation that acts on both the parenchymal and stromal compartments of the neoplastic tissue. Deregulation of the FGF/FGFR system occurs in human tumors, leading to the aberrant activation of ligand-dependent or ligand-independent FGFR signaling (Table 1). Different genetic alterations that occur in cancer cells, including activating *FGFR* gene mutations of the extracellular, transmembrane or TK domain of the receptor, chromosomal rearrangements, *FGFR* gene amplification, translocation, or transcriptional deregulation, may result in aberrant FGFR signaling. Unlike *FGFRs*, somatic *FGF* mutations are rarely observed in human tumors and appear to be limited to *FGF9* mutations as occurs in colorectal and endometrial cancers. Nevertheless, aberrant expression of different FGFs has been observed in a variety of solid and hematological human malignancies, contributing to cancer progression by acting on both parenchymal and stromal compartments. Autocrine and paracrine stimulation by overexpressed FGFs lead to cancer cell proliferation, resistance to cell death, increased motility and invasiveness, enhanced metastatic activity, stem cell maintenance, and resistance to chemotherapy. In addition, FGFs play a pivotal role in the recruitment, activation, and maturation of endothelial cells, fibroblasts, and infiltrating myeloid cells. Due to its impact on human tumors, the FGF/FGFR system may represent a promising target for anticancer therapies (Fig. 2).

Other FGF-Related Pathologies

Even though genetic diseases and cancer represent the predominant pathological conditions related to defects of the FGF/FGFR system, other disorders could be linked to its deregulation. For instance, the FGF/FGFR axis has received considerable attention in recent years for its role in various diseases of the nervous system, including epilepsy and Parkinson's disease, and in psychiatric disorders. Defects of FGF2, FGF21, and FGF23 signaling have been observed in heart diseases in which FGF15/19, FGF21, and FGF23 can be exploited as serum biomarkers. Regarding inflammatory conditions, patients suffering from inflammatory bowel disease show



Fibroblast Growth Factors, Fig. 2 Alterations of the FGF/FGFR system in cancer. See text for details

elevated levels of FGF7 while upregulation of FGF7 and FGF10 has been observed in the psoriatic skin. Finally, the blood levels of FGF23 are increased in patients with fibrous dysplasia, a disorder in which normal bone is replaced by fibro-osseous tissue (Presta et al. 2017; Ornitz and Itoh 2015; Moosa and Wollnik 2016).

Drugs

Several agents have been developed to target the FGF/FGFR system with different selectivity and specificity. Some of these compounds are commonly used in basic research as FGF/FGFR inhibitors, while others have been evaluated in preclinical settings and in clinical trials as therapeutic agents for the treatment of FGF/FGFR-dependent tumors (www.clinicaltrials.gov). Acting at the extracellular or intracellular level, these compounds exert their inhibitory action by preventing the interaction of FGFs with their receptors or by impairing the signal transduction pathways activated by a deregulated autocrine/paracrine FGFR activity. FGF/FGFR inhibitors can be classified as nonselective and selective TK inhibitors (TKIs), neutralizing monoclonal antibodies (mAbs), allosteric inhibitors, and FGF traps.

Nonselective TKIs bind to the ATP-binding domain of the catalytic site of FGFRs, but at the same time they can also suppress the activity of other TK receptors. The advantage of these inhibitors is their ability to concurrently targeting various signaling pathways in tumor epithelial and stromal cells. However, they show a multiplicity of toxic side effects, including proteinuria, cardiotoxicity, gastrointestinal disorders, and cutaneous reactions. Nonetheless, some of these compounds have been approved for their use in combination with chemotherapy or after first-line therapy failure in several cancer types. The nonselective TKI lucitanib (E3810) has shown antitumor/antiangiogenic effects in preclinical models of endometrial, gastric, breast, and lung cancers, leading to significant benefits in patients with solid tumors expressing aberrant FGFRs. By targeting FGFR1–3 and other TK receptors, nintedanib (BIBF1120) has shown an interesting antitumor activity on colorectal, prostate, liver, lung, and female tumors with controllable adverse effects. Encouraging results for the treatment of FGFR1-altered prostate cancers with bone metastases, advanced renal cell carcinoma, multiple myeloma, and squamous cell carcinoma of the lung have been obtained also with the nonselective TKI dovitinib (TKI158). Other nonselective FGFR TKIs, including brivanib, cediranib, lenvatinib, orantinib, pazopanib, ponatinib, regorafenib, and sorafenib, are under evaluation in preclinical and clinical models (Table 2).

Fibroblast Growth Factors, Table 2 FGF/FGFR inhibitors: main intracellular and extracellular drugs

Type of inhibitor		Compound	Target
Intracellular FGFR inhibitors	Nonselective TKIs	AXL1717	FGFRs, VEGFRs, IGF1R
		Brivanib	FGFRs, PDGFRs, VEGFRs
		Cediranib	FGFRs, VEGFRs, c-KIT
		Danusertib (PHA-739358)	FGFR1, BRC-Abl, c-RET, Aurora
		Dovitinib (TKI 258)	FGFRs, PDGFRs, VEGFRs, FLT3, c-KIT
		ENMD2076	FGFR1/2, PDGFRs, VEGFR2, FLT3, c-KIT, Aurora
		Lenvatinib (E7080)	FGFRs, PDGFRs, VEGFRs
		Lucitanib (E3810)	FGFR1–2, VEGFRs, PDGFRs
		MAX40279	FGFRs, FLT3
		MK2461	FGFR1–3, PDGFR, c-met, FLT1/3, Ron, Mer
		Nintedanib (BIBF1120)	FGFRs, PDGFRS, VEGFRs
		Orantinib ()TSU-68	FGFRs, PDGFRs, VEGFR2
		Pazopanib	FGFR1/3, PDGFRs, VEGFRs, c-KIT
		Regorafenib	FGFRs, PDGFRs, VEGFRs, c-KIT, c-RET
		Sorafenib	FGFR1, VEGFR2, FLT3, PDGFRs
	Selective TKIs	ARQ087	FGFR1–3
		ASP-5878	FGFR1–4
		AZD4547	FGFR1–3
		BAY-1163877	FGFR1–4
		BGJ398	FGFR1–3
		Debio1347	FGFR1–3
		Erdafitinib (JNJ-42756493)	FGFR1–4
		FGF401	FGFR4
		H3B-6527	FGFR4
		INCB062079	FGFR4
		LY2874455	FGFR1–4
		Pemigatinib	FGFR1–3
		TAS-120	FGFR1–4
Extracellular FGF/FGFR inhibitors	Antibodies	B-701	FGFR3
		BAY 1179470	FGFR2
		FPA144	FGFR2IIc
		MFGR1877S (RG7444)	FGFR3
		KRN23 (Burosumab)	FGF23
	Allosteric inhibitor	Alofanib (RPT835)	FGFR2
	FGF trap agents	FP-1039 (GSK3052230)	FGFs

VEGFR2: Vascular endothelial growth factor 2; PDGFR: Plated-derived growth factor receptor; FLT3: fms-like tyrosine kinase 3; RET: REarranged during Transfection tyrosine-protein kinase receptor; c-KIT: Mast/stem cell growth factor receptor; c-MET: Hepatocyte growth factor receptor; IGF1R: Insulin-like growth factor 1 receptor; Ron: Macrophage-stimulating protein receptor; Mer: Tyrosine-protein kinase Mer; BCR-Abl: Breakpoint cluster region protein-Tyrosine protein kinase Abl (Presta et al. 2017; Helsten et al. 2015; Carter et al. 2015) (https://clinicaltrials.gov)

Selective FGFR TKIs have been developed in order to limit the side effects of nonselective molecules. Most of them are able to target more than one FGFR. AZD4547 is specific for FGFR1–3 and is under evaluation in combination with conventional therapies for the treatment of different tumors, including breast, lung, and gastric cancers. BGJ398 (NVP-BGJ398) is a FGFR1–3 inhibitor that causes tumor shrinkage in FGFR-aberrant non-small cell lung cancer, bladder carcinoma, and cholangiocarcinoma with tolerable side effects. JNJ-42756493 (erdafitinib) acts onto FGFR1–4 and induces the stabilization of advanced tumors characterized by FGFR translocations or fusions, like glioblastoma, bladder carcinoma, lung and breast cancers. Other selective TKIs (including ARQ-087, BAY-1163877, Debio 1347, LY287445, and TAS-120) are under investigation in preclinical models and in clinical trials on different oncotypes (Table 2). The most significant side effects of these compounds are related to the modulation of the FGF23 pathway that can induce hyperphosphatemia and tissue calcification. In any case, selective TKIs show reduced adverse effects when compared to nonselective TKIs.

New strategies for the treatment of tumors with an aberrant activation of the FGF/FGFR system may consist in the use of neutralizing anti-FGFR mAbs, anti-FGF mAbs, or FGF traps. Neutralizing anti-FGFR mAbs are promising therapeutic agents characterized by a high specificity and possible absence of off-target effects. Among them, MGFR1877S is a specific anti-FGFR3 mAb under evaluation for the treatment of solid tumors and multiple myeloma, whereas the mAb FPA144 binds the FGFR2-IIb variant and inhibits tumor growth in experimental models of gastric cancers.

Compounds able to sequester one or more FGFs may represent a therapeutic option for those tumors characterized by the upregulated expression of these growth factors. In this frame, anti-FGF mAbs have shown encouraging results in different tumor models in vitro and in vivo. Moreover, the anti-FGF23 mAb KRN23 (burosumab) is on clinical evaluation for the treatment of hypophosphatemia in adults and children.

In parallel with mAbs, small molecules acting as pan-FGFs traps are under development. The activity of FGFs is modulated by their interaction with a plethora of molecules present in the extracellular microenvironment. The structural characterization of this “interactome” has been exploited for the development of FGF trapping agents. FP-1039 (GSK3052230) is a FGF ligand trap derived from the extracellular domain of FGFR1. FP-1039 exerts its inhibitory activity on angiogenesis and tumor cell growth in different tumor models and has shown limited side effects when tested in clinical trials. In addition, small molecules derived from the FGF ligands thrombospodin-1 (TSP-1), heparin/HSPGs, and the soluble pattern recognition receptor long pentraxin-3 (PTX3) have been developed and their antiangiogenic and antitumor activity has been demonstrated in different tumor models. Notably, FGF traps show a low toxicity profile when compared to TKIs (Presta et al. 2017; Ghedini et al. 2018).

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Fondaparinux

► Heparin and Related Drugs

Foot

► Ryanodine Receptor

Fragment-Based Drug Discovery

► SAR-by-NMR

Free Fatty Acid Receptors

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Definition

Free fatty acids (FFAs) consist of a carboxylic acid which is linked to an aliphatic tail, and they can be classified according to the chain length of this tail as short-chain fatty acids (SCFAs), which have 1–6 carbon atoms; medium-chain fatty acids (MCFAs), which have 7–12 carbon atoms; and long-chain fatty acids (LCFAs), which have more than 12 carbon atoms. FFAs play important roles as energy carriers, basic components of biological structures, and precursors of various mediators. In addition, FFAs can directly regulate biological processes, such as metabolic and immune functions. Some of these effects are mediated by a subgroup of G protein-coupled receptors (GPCRs). These include four receptors that have been classified as members of the FFA receptor family (FFA1-FFA4). FFA1 and FFA4 are activated by MCFAs and LCFAs, whereas FFA2 and FFA3 are activated by SCFAs. FFA1, FFA2, and FFA3 are structurally related, and their human genes are located in tandem on the long arm of chromosome 19. In contrast, FFA4 shows only minor structural similarity to FFA1-FFA3. In addition to FFA receptor family members, several additional GPCRs have been shown to be activated by FFAs. These include GPR84, a receptor

for MCFAs, and the olfactory receptor OR51E2 (Olfr78 in the mouse) and hydroxycarboxylic acid receptor 2 (HCA2), which are activated by particular SCFAs.

Basic Characteristics

FFA1

FFA1 (Table 1) is activated by MCFAs and LCFAs with chain lengths of ten or more carbon atoms, and this includes both saturated and unsaturated free fatty acids. 20-Hydroxyeicosatetraenoic acid (20-HETE) activates FFA1 with particularly high efficacy. Activated FFA1 couples predominantly to G_q/G_{11} family G proteins. FFA1 is expressed in insulin-producing β-cells of the pancreas. Expression has also been found in enteroendocrine cells, immune cells, taste buds, osteocytes, and cells of the central nervous system. The best-understood physiological function of FFA1 is its ability to mediate a stimulation of glucose-stimulated insulin secretion from β-cells by dietary free fatty acids and by 20-HETE. The latter is produced by β-cells themselves under the influence of glucose and thereby functions as a positive feed-forward mediator (Fig. 1).

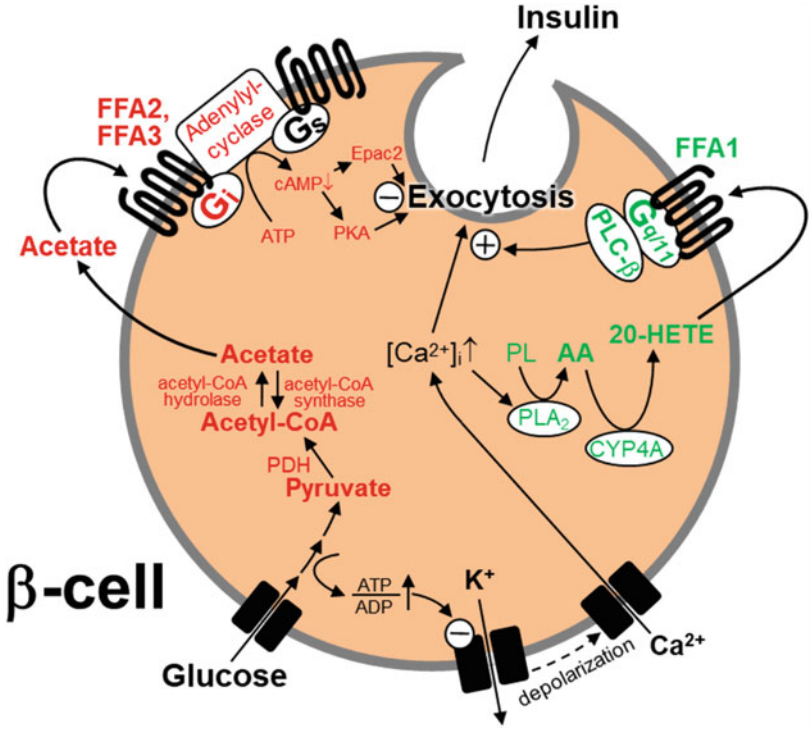
FFA2 and FFA3

Human FFA2 and FFA3 (Table 1) are activated by micromolar concentrations of propanoic acid (C3) and butyric acid (C4). Acetic acid (C2) activates FFA2 but is a relatively poor agonist of FFA3, and valeric acid (C5) and caproic acid (C6) prefer FFA3. Both FFA2 and FFA3 couple to G proteins of the G_i family. FFA2 can also activate G_q/G_{11} family members. FFA2 and FFA3 are expressed in the pancreatic islets, including α- and β-cells and in various enteroendocrine cells. FFA2 has also been described to be expressed in white adipocytes and various immune cells including neutrophils, monocytes, and intestinal Treg cells, whereas FFA3 is expressed in the peripheral nervous system including the sympathetic ganglia and enteric neurons.

Since SCFAs are primarily generated through the fermentation of fiber by the gut microbiota, the by far highest levels of SCFAs in the organism are

Free Fatty Acid Receptors, Table 1 Free fatty acid receptors

Receptor	G protein coupled to	Endogenous ligands	Synthetic ligands	Expression (function)
FFA1 (GPR40)	G_q/G_{11}	Saturated and unsaturated LCFAs	*Agonists:* Fasiglifam (TAK-875), GW-9508, AMG837, AMG1638, MG5262, TUG424 *Antagonist:* GW1100	Pancreatic β-cells (enhancement of glucose-stimulated insulin secretion). Enteroendocrine cells (stimulation of GLP-1 and GIP secretion)
FFA2 (GPR43)	G_q/G_{11}, G_i	SCFAs (acetate, propionate, butyrate)	*Agonists:* Phenylacetamide-1/AMG7703, Euroscreen compounds 1 and 2 *Antagonists:* GLPG0974, Euroscreen compound 3	Pancreatic β-cells (inhibition (in some cases also stimulation) of glucose-stimulated insulin secretion), enteroendocrine cells (stimulation of GLP-1 secretion), adipocytes (inhibition of lipolysis), immune cells (proinflammatory effects)
FFA3 (GPR41)	G_i	SCFAs (acetate, propionate, buryrate)	*Agonist:* AR420626 *Antagonist:* AR399519	Pancreatic β-cells (inhibition of glucose-stimulated insulin secretion), enteroendocrine cells (stimulation of GLP-1 secretion), sympathetic neurons (stimulation of activity)
FFA4 (GPR120)	G_q/G_{11}, G_i	Saturated and unsaturated LCFAs	*Agonists:* GW9508, TUG891, GSK137647A, NCG21 *Antagonist/inverse agonist:* AH7614	Immune cells (anti-inflammatory), adipocytes (increased insulin-mediated glucose uptake), enteroendocrine cells (inhibition of hormone secretion)



Free Fatty Acid Receptors, Fig. 1 Free fatty acid receptors. Regulation of β-cell function through FFA1, FFA2, and FFA3 receptors. Glucose-induced increases in free cytosolic Ca^{2+} concentrations activate phospholipase A_2 (PLA_2), which converts phospholipids (PL) into arachidonic acid (AA). AA is then further converted by CYP4A to 20-HETE, which, in an auto- and/or paracrine fashion, activates FFA1. FFA1 downstream signaling promotes glucose-stimulated insulin secretion. 20-HETE/FFA1 thereby mediates a positive feed-forward mechanism. Glucose is converted through glycolysis and pyruvate dehydrogenase (PDH) into acetate, which is released from β-cells and, in an auto- and/or paracrine fashion, can activate FFA2 and FFA3. FFA2/FFA3 activation via inhibition of cAMP formation inhibits glucose-stimulated insulin secretion. This mechanism functions in particular at high glucose concentrations and might help to avoid overshooting insulin secretion in response to high glucose concentrations. PKA, cAMP-regulated protein kinase

found within the gastrointestinal tract. Given that FFA2 and FFA3 are expressed in enteroendocrine cells as well as in immune cells and neurons of the intestinal wall, multiple studies have analyzed the role of FFA2 and FFA3 in the intestine in part with contradictory results. There is good evidence that FFA2 mediates SCFA-induced stimulation of GLP-1 release from enteroendocrine cells. It appears also to be involved in SCFA-induced inhibition of ghrelin release. The role of FFA2 and FFA3 in regulating intestinal immunity is less clear. Both, anti- and proinflammatory roles of FFA2 activation have been described. In adipocytes, FFA2 can mediate an inhibition of lipolysis as well as of insulin signaling and of glucose uptake. In pancreatic β-cells, FFA2 and FFA3 mediate an inhibition of glucose-stimulated insulin secretion by acetate, which is released from β-cells in response to high glucose concentrations. This mechanism might protect β-cells from overshooting insulin secretion in response to high glucose concentrations (Fig. 1). However, under certain conditions, FFA2 can also mediate a stimulation of glucose-stimulated insulin secretion by coupling to G_q/G_{11}. The physiological, pathophysiological, and pharmacological roles of these mechanisms are still not clear.

FFA4

FFA4 (Table 1) is activated by a similar spectrum of MCFAs and LCFAs as FFA1, but polyunsaturated fatty acids (PUFAs) appear to have higher

efficacy than saturated fatty acids. FFA4 is present in two splice variants. A more common short isoform couples to G_q/G_{11} as well as to G_i-type G proteins and recruits arrestin, while the long isoform recruits only arrestin. Major sites of FFA4 expression are white adipocytes, macrophages and some enteroendocrine cells.

Evidence has been provided that FFA4 on adipocytes can mediate free fatty acid-induced inhibition of triglyceride lipolysis. Controversial data are existing with regard to the role of FFA4 in enteroendocrine cells. In immune cells, FFA4 appears to mediate anti-inflammatory effects. Loss of FFA4 in mice and presence of an FFA4 version with reduced signaling function have been shown to promote obesity and type 2 diabetes.

Drugs and Pharmacological Relevance

Since FFA1 mediates an enhancement of glucose-stimulated insulin secretion, primarily agonists of FFA1 have been developed (Table 1) (Offermanns 2014; Milligan et al. 2017). Fasiglifam (TAK-875) has by far had the greatest impact on understanding the therapeutic potential of FFA1. Fasiglifam showed in phase II and III studies beneficial effects on hyperglycemia and HbA_{1C} levels in diabetic patients without a risk of producing hypoglycemia. However, because of liver toxicity, probably due to an off-target effect due to inhibition of hepatobiliary transporters, further development of fasiglifam was stopped.

Developing potent and selective ligands for FFA2 or FFA3 has been challenging. However, a growing number of synthetic ligands for FFA2 have been reported, in particular orthosteric agonists and antagonists (Table 1) (Bolognini et al. 2016). One compound, GLPG0974, an orthosteric antagonist of FFA2, entered clinical trials in patients with mild to moderate ulcerative colitis. Although GLPG0974 was well-tolerated and reduced neutrophil activation and influx, no measurable clinical effect was observed. While allosteric ligands for FFA3 have been developed (Table 1), no potent orthosteric ligands of FFA3 have been described.

Several lines of evidence suggested that agonism of FFA4 would be beneficial for the treatment of metabolic disorders, such as obesity and type 2 diabetes (Husted et al. 2017; Milligan et al. 2017), and, consequently, the majority of synthetic ligands of FFA4 are orthosteric agonists (Table 1). A few compounds have shown beneficial effects in animal models of metabolic disorders, but no studies in humans have been reported yet.

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GABAergic System

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Synonyms

Anterograde amnesia; Anticonvulsant; Anxiolysis; Barbiturates; Benzodiazepines; General anesthetics; Inhibitory neurotransmission; Myorelaxation; Neurosteroids; Sedation; Synapse

Definition

γ-Aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the central nervous system. Structures involved in releasing and binding GABA as a neurotransmitter constitute the GABAergic system. Of pharmacological interest, the GABAergic system is involved in the regulation of vigilance, anxiety, muscle tension, epileptogenic activity, and memory functions (Engin et al. 2018; Olsen 2018; Rudolph and Antkowiak 2004; Rudolph and Knoflach 2011; Sieghart and Savic 2018).

Basic Characteristics

Inhibitory GABAergic neurotransmission in the brain is crucial for temporally precise activity of neuronal circuits and the synchronized oscillatory activity of neuronal populations. By far the most frequent pharmacological interventions of GABAergic neurotransmission target GABA receptors. There are two types of GABA receptors which are both targets of clinically used drugs: ionotropic GABA receptors mediating fast responses, called $GABA_A$ receptors, on which this entry is focused and which are targeted by benzodiazepines, barbiturates, neurosteroids, and some general anesthetics, and metabotropic GABA receptors mediating slow responses, called $GABA_B$ receptors, which are targeted by the $GABA_B$ agonist baclofen, which reduces spasticity. Other pharmacological targets in the GABAergic system are GABA transaminase and GABA reuptake. Vigabatrin, which is effective in the treatment of focal seizures, is a GABA transaminase inhibitor, an enzyme involved in the breakdown of GABA. Tiagabine, which is also used in the treatment of focal seizures, is an inhibitor of the GAT-1 GABA transporter, a GABA reuptake mechanism which removes GABA from the synaptic cleft.

In many brain regions, e.g., cortical regions or hippocampus, interneurons which release GABA modulate the activity of excitatory principal neurons or of other GABAergic interneurons. Receptors for GABA are present on both excitatory and

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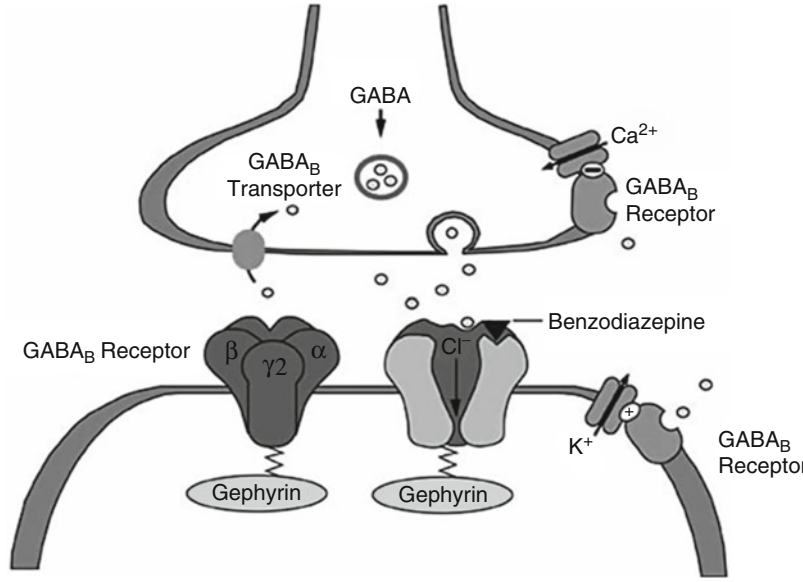
inhibitory interneurons, and some GABA receptors may thus also have disinhibiting functions. In fact, cognitive deficits caused by excess inhibition can be reversed by disinhibition, which thus plays an important role in learning and memory.

$GABA_A$ receptors are typically postsynaptic pentameric complexes that are assembled from a subunit repertoire of at least 19 subunits (α1–6, β1–3, γ1–3, δ, ε, θ, π, ρ1–3) which form a central channel with permeability for chloride ions. They may be linked to the postsynaptic protein gephyrin (Fig. 1), and α5-ontaining $GABA_A$ receptors have been shown to be linked to radixin. GABA-mediated chloride influx results in hyperpolarization of the postsynaptic neuron. $GABA_A$ receptors most frequently contain α-, β-, and γ-subunits. The GABA binding site is formed by the α- and β-subunits, whereas the benzodiazepine site is formed by the α- and γ-subunits. α1β2γ2 is the most abundant GABA receptor subtype, representing approximately 60% of all $GABA_A$ receptors in the brain. α2β3γ2 is the second most abundant $GABA_A$ receptor subtype, representing approximately 15–20% of all $GABA_A$ receptors in the brain. Pharmacologically, $GABA_A$ receptors are most frequently differentiated by their α-subunits, in part as they contribute to the benzodiazepine binding site. $GABA_A$ receptors containing the α1-, α2-, α3-, or α5-subunits are sensitive to modulation by classical, clinically used benzodiazepines, whereas $GABA_A$ receptors containing the α4- and α6-subunits are not. For practical purposes, $GABA_A$ receptors are frequently referred to as, for example, α1-containing $GABA_A$ receptors or α1-$GABA_A$ receptors, which simply indicates that this subunit is part of the pentameric complex, which also contains other subunits. The function of GABA receptor subtype can be assessed in a variety of different ways: studies using gene knockout mice; studies on mice carrying single amino acid point mutations (knock-in mice) changing the pharmacological properties of the respective receptor, e.g., mice with point mutations in α-subunits rendering the respective $GABA_A$ receptors insensitive to modulation by clinically used benzodiazepines, alcohol, and volatile general anesthetics; pharmacological studies using $GABA_A$ receptor subtype-selective compounds; knockdown studies using short hairpin RNA (shRNA) approaches; or knockdowns or other mutational studies using genome editing by CRISPR/Cas-9 or similar systems. Studies with gene knockout mice have to take into account that compensatory regulations of related genes, e.g., upregulation of other $GABA_A$ receptor subunits, may occur, which may make interpretations of results difficult. Knock-in



GABAergic System, Fig. 1 GABAergic synapse. Schematic of a GABAergic synapse, depicting the major elements of signal transduction. Typically postsynaptic $GABA_A$ receptors are pentameric ligand-gated ion channels assembled from various types of subunits. On the cytoplasmic side, they are linked to gephyrin. $GABA_B$ receptors are coupled to calcium or potassium channels

mice with single point mutations generally do not exhibit such compensatory regulations, or if so, to a more limited degree, and have thus been valuable for assigning specific functions to individual $GABA_A$ receptor subtypes. In such experiments, benzodiazepines such as diazepam are tested on mice with a histidine to arginine point mutation which renders the respective receptor insensitive to modulation by classical benzodiazepines. Any benzodiazepine action missing could then be ascribed to the receptor subtype containing the point-mutated α-subunit. Mice have been generated with histidine to arginine point mutations in three out of the four diazepam-sensitive α-subunits. In these mice, diazepam is a highly subtype-specific positive allosteric modulator. Mice with such point mutations in all four diazepam-sensitive α-subunits are insensitive to modulation by clinically relevant doses of diazepam.

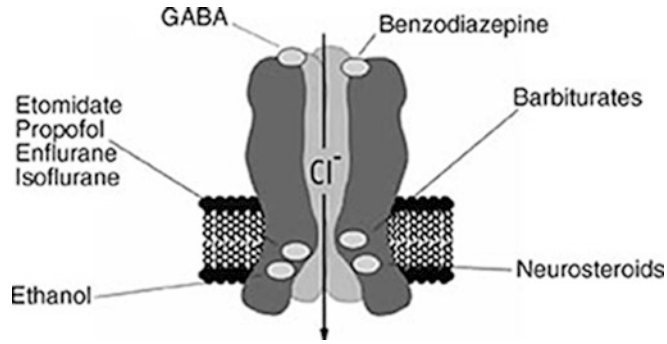
Whereas in other fields of research, e.g., adrenoceptors, the synthesis of subtype-selective compounds has been achieved and contributed significantly to the assignment of functions to specific receptor subtypes, the success of this strategy to $GABA_A$ receptors was more limited. As the similarity between $GABA_A$ receptor subtypes is high, it has been difficult to develop compounds that are truly subtype-specific, and assignment of functions to individual $GABA_A$ receptor subtypes based on the activity of "subtype-selective" compounds has to be done with caution. Specifically, if a compound displays some selectivity for a specific $GABA_A$ receptor subtype and has a certain action, it may be erroneous to conclude that this action is mediated by the subtype for which the compound displays some specificity; it might also be mediated by another $GABA_A$ receptor subtype which the compound also modulates.

In addition to functions of $GABA_A$ receptors that have long been known and are pharmacologically exploited, e.g., by benzodiazepines (see below), studies with knock-in mice and with subtype-selective compounds also revealed previously unrecognized functions of $GABA_A$ receptor subtypes. Such actions may become apparent if a specific subtype-selective compound (or diazepam in knock-in mice) does not positively modulate α1-containing $GABA_A$ receptors, which mediate reduction of motor activity, i.e., sedation. In other words, such actions are not visible with clinically used benzodiazepines simply since they are sedating the experimental animal (or the patient). This includes an antihyperalgesic action that is mediated by α2- and α3-containing $GABA_A$ receptors in the spinal cord in models if inflammatory and neuropathic pain in rodents. These receptors have also been shown to have an itching suppression effect, reducing acute histaminergic and non-histaminergic itch in mice. Positive modulation of these receptors alleviated chronic itch in a mouse model of atopic dermatitis and in dogs sensitized to house dust mites.

Recent studies were able to identify specific neuronal populations in which a defined $GABA_A$ receptor subtype mediates specific actions. The anxiolytic-like action of diazepam was shown to be dependent on α2-containing $GABA_A$ receptors in dentate gyrus granule cells and in hippocampal CA3 pyramidal neurons, whereas the fear-reducing action of diazepam is dependent on α2-containing $GABA_A$ receptors in hippocampal CA1 pyramidal neurons. Moreover, α5-containing $GABA_A$ receptors on PKCδ-positive neurons in the central amygdala mediating tonic inhibition have been shown to modulate anxiety-like behavior. Furthermore, α5-containing $GABA_A$ receptors in dentate gyrus granule cells were found to play a role in the management of interfering memories. In addition to synaptic inhibition, $GABA_A$ receptors are also involved in mediating extrasynaptic inhibition. Some $GABA_A$ receptors, e.g., α5-containing $GABA_A$ receptors in the hippocampus, are located in extrasynaptic locations, where they detect GABA that is spilled over from synapses. These receptors are sensitive to lower concentrations of GABA compared to synaptic $GABA_A$ receptors and have been shown to be involved in learning and memory.

Drugs

Several groups of drugs exert all or some of their action via the GABAergic system (Fig. 2).



GABAergic System, Fig. 2 Model of a $GABA_A$ receptor and its binding sites. In addition to binding sites for the neurotransmitter GABA, $GABA_A$ receptors have modulatory binding sites for a variety of ligands, including benzodiazepines, barbiturates, neurosteroids, and ethanol and general anesthetics such as isoflurane, enflurane, etomidate, and propofol. The positioning and the size of the binding sites are arbitrary. One subunit has been removed to visualize the pore

Benzodiazepines

Benzodiazepines act by shifting the GABA dose-response curve to the left and thus increase the affinity of the receptors for GABA. At a given concentration of GABA in a synapse, the chloride current will be increased. At the molecular level, benzodiazepines increase the opening frequency and thus the number of channels that are opened by a given concentration of GABA. Benzodiazepines have no action in the absence of GABA (use-dependence) and cannot increase maximal physiological stimulation by a high concentration of GABA, i.e., their action is self-limiting, which most likely contributes to the safety of these drugs with respect to overdoses. They are used as anxiolytics, sedatives, anticonvulsants, and muscle relaxants. Experiments with knock-in mice (see above) revealed that the anxiolytic-like action of benzodiazepines is mediated by α2-containing $GABA_A$ receptors, whereas the sedative action, the anterograde amnestic action, and in part the anticonvulsant action are mediated by α1-containing $GABA_A$ receptors. The myorelaxant actions of benzodiazepines have been shown to be mediated by α2- and α3-containing $GABA_A$ receptors.

Barbiturates

Barbiturates act by increasing the conductance level. In contrast to benzodiazepines, they also have direct agonistic actions at $GABA_A$ receptors. Also in contrast to benzodiazepines, their action is not self-limiting, i.e., they can activate the $GABA_A$ receptor to higher levels than high concentrations of GABA alone. These features may be responsible for the fact that overdoses of barbiturates are life-threatening. Studies in β3(N265 M) knock-in mice in which β3-containing $GABA_A$ receptors are largely insensitive to pentobarbital revealed that the immobilizing and in part the hypnotic actions of pentobarbital are mediated by β3-containing $GABA_A$ receptors, but not its respiratory depressant actions.

General Anesthetics

Both volatile and intravenous general anesthetics have been shown to modulate the activity of the $GABA_A$ receptor, and these actions likely contribute to their clinical effects. Amino acid residues in the α- and β-subunits contribute to the binding site for volatile anesthetics. In contrast, only mutations of amino acid residues in the β-subunit contribute to the binding site for intravenous anesthetics like etomidate and propofol. For the intravenous anesthetics etomidate and propofol, using mice with etomidate (and propofol-)-insensitive β3-containing $GABA_A$ receptors [β3 (N265 M)], it was shown that this receptor subtype is required for the immobilizing action and the respiratory depressant action of these drugs

and contributes to the hypnotic action of these drugs. Interestingly, these mice still experience motor sedation in response to etomidate. Studies in mice with etomidate-insensitive β2-containing $GABA_A$ receptors [β2(N265S) mice] have shown that this receptor subtype contributes to the sedative and hypothermic action of etomidate. In β3 (N265 M) mice, higher concentrations (EC_{50}) of the volatile anesthetics like isoflurane, halothane, and enflurane were required to achieve immobilization. In recombinant systems, the ε-subunit, which is found in the amygdala and thalamus, and which is particularly abundant in the subthalamic nucleus, confers insensitivity to general anesthetics, potentially protecting essential physiological functions.

Neurosteroids

Neurosteroids prolong the mean open time of recombinant $GABA_A$ receptor channels. They enhance phasic inhibition (through synaptic $GABA_A$ receptors) and tonic inhibition (through extrasynaptic $GABA_A$ receptors), and as δ-containing $GABA_A$ receptors are also found in extrasynaptic locations, such receptors are particularly sensitive to modulation by neurosteroids. The transmembrane domains of $GABA_A$ receptor α-subunits contribute to a binding site for potentiating neurosteroids. Levels of progesterone-derived neurosteroids fluctuate over the ovarian cycle, with a large increase during pregnancy and a precipitous decline at parturition. In pregnant mice, the $GABA_A$ receptor subunits γ2 and δ are downregulated, which goes along with a decrease in tonic and phasic inhibition in pregnant mice. Mice heterozygous or homozygous for a knockout of the δ-subunit exhibit depression-like behaviors and abnormal maternal behaviors, and in heterozygous δ-knockout mice, abnormal postpartum behaviors can be attenuated by the δ-subunit-selective agonist at the GABA site, THIP (gaboxadol). An intravenous formulation of the endogenous neurosteroid allopregnanolone, brexanolone, showed significant antidepressant activity in postpartum depression in humans and has been approved by the FDA for this indication.

$GABA_A$ Receptor Mutants as Models for Disease

Some $GABA_A$ receptor knockout mice display distinct phenotypes, e.g., α2-knockout mice and heterozygous γ2-knockout mice an anxiety-related and depression-related phenotype, α3-knockout mice have increased dopaminergic activity in the ventral tegmental area and deficits in sensorimotor gating, and α5-knockout mice actually perform better in the Morris water maze, which led to the development of α5-negative allosteric modulators as potential cognitive enhancers, and β3-knockout mice display cleft palate, neurological impairments including hyperresponsiveness to sensory stimuli, motor impairment, and epileptic seizures. β3-knockout mice also display impaired social and exploratory behaviors. Mice with a double point mutation in the β3-subunit which abolishes phosphorylation sites for serine/threonine phosphorylation [β3(S408A/S409A)] and in which modulation by phosphorylation/dephosphorylation is abolished, actually by mimicking a state corresponding to the phosphorylated state, displayed increased phasic and decreased tonic inhibition and exhibited alterations in dendritic spine structure, increased repetitive behavior and decreased social interactions, hallmarks of autism spectrum disorders, and an increase in sensitivity to seizures. Interestingly, phosphorylation of S408/S409 was significantly enhanced in Fmr1 knockout mice, a model of fragile X mental retardation, suggesting that alterations in phosphorylation and/or activity of β3-containing $GABA_A$ receptors may contribute to the pathophysiology of autism spectrum disorders and other neurodevelopmental disorders.

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Gap Junction Hemichannels

► Connexin Hemichannels

Gastric Motility

► Ghrelin, Physiological Roles and Clinical Relevance of

Gene Expression Analysis and Next-Generation Sequencing

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Synonyms

Gene expression profiling; NGS; RNA-sequencing; Transcriptome analysis

Definition

Next-Generation sequencing technologies enable sequencing of millions of DNA molecules simultaneously. This is facilitated by fragmentation of DNA, adaptor ligation, and immobilization on a solid matrix, from which the nucleotide sequence is read out. Through the prior conversion of RNA into cDNA, NGS also enables the quantification of RNA produced by its coding gene (Gene expression profiling).

Description

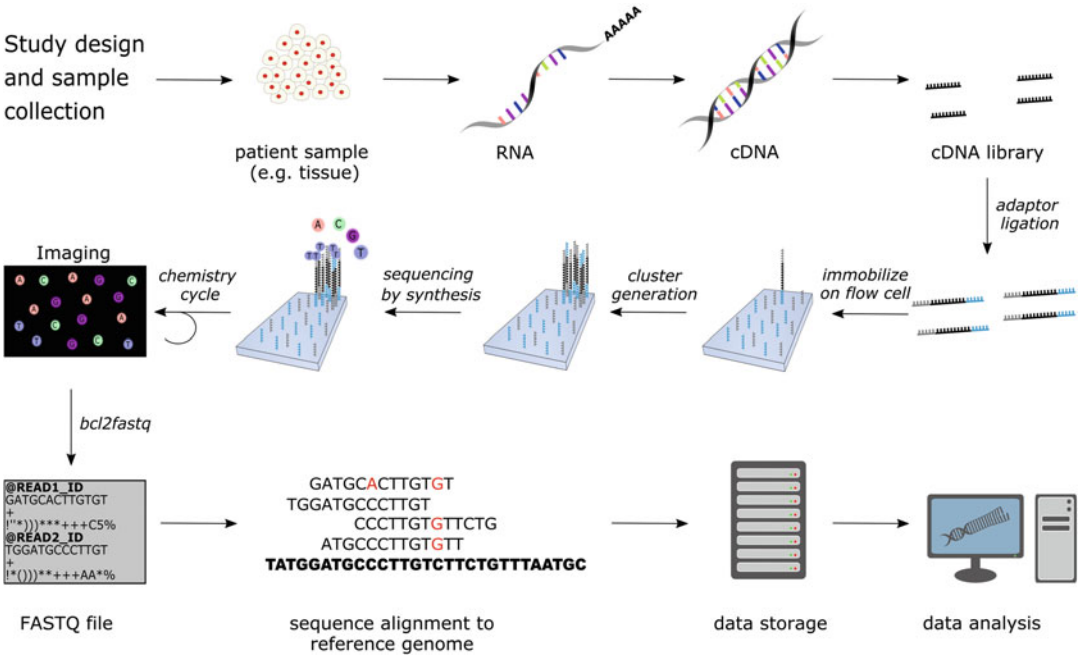
High throughput sequencing technologies such as Next-Generation sequencing (**NGS**) measure gene activity in a highly parallel manner. Current approaches allow sequencing of full **genomes**, epigenomes, exomes, **transcriptomes**, methylomes, and translatomes. Sequencing technologies help us to further our understanding and interpretation of the complexity of the genome, for example, by enabling the identification of single-nucleotide polymorphisms (SNPs), small insertions and deletions (Indels), or copy-number variants (CNVs) (Goodwin et al. 2016). Current NGS protocols for gene expression profiling, or RNA-Sequencing (RNA-Seq), require a **reverse transcription** step from RNA into cDNA, which is sequenced instead. This protocol enables a quantitative measure of gene expression, de-novo identification of splicing events and fusion genes (e.g., in cancer). Microarrays rely in contrast to sequencing technologies on *a priori* knowledge of a reference sequence annotation, which impedes identification of novel sequence alterations or transcript isoforms.

NGS technologies are derived from First-Generation Sequencing technologies, which are based on the chain termination reaction. First-Generation methods include Maxam-Gilbert-sequencing, which was firstly published in 1977 by Allan Maxam and Walter Gilbert and Sanger sequencing, published in the same year by Frederick Sanger. The first automated sequencer based on Sanger dideoxy sequencing was the Model 370A DNA Sequencing System, which was marketed by Applied Biosystems in 1986. This platform revolutionized the field of functional genomics, as it enabled reading of more bases in shorter time with higher accuracy. Despite the currently dominant position of NGS platforms, Sanger sequencing is still used for validation of NGS findings and the verification of DNA sequences such as plasmids, inserts, and genomic mutations. Due to its targeted approach, Sanger sequencing is still a cost-efficient option for low numbers of samples and targets.

NGS, sometimes also termed “deep” or “high-throughput” sequencing, terms a collection of methods which were developed between 1990

and 2000. NGS is characterized by a higher throughput, which is achieved by random fragmentation of DNA followed by simultaneous sequencing of a randomly selected subset. This concept has higher power to discover novel genomic or transcriptomic variations resulting from the higher read coverage of the sequenced product. Further cost efficiency is achieved by the possibility of pooling multiple samples in one sequencing run (multiplexing), allowing the user to achieve a desired, sample-specific sequence depth and hence a pragmatic means to increase sample throughput at limited expense. There are various protocols available, depending on the sequencing platform, e.g., Solexa/Illumina, Roche 454, Ion Torrent /Proton and ABI SOLiD, with Illumina currently being most often used in studies. The protocol of NGS data generation is a multi-step process (Fig. 1): (i) design and sample collection, (ii) isolation of RNA and transcribing into cDNA (this step is not necessary in case DNA is sequenced), (iii) fragmentation and adaptor ligation, (iv) adaptor hybridization to solid matrix (Illumina sequencing), (v) cluster generation and bridge amplification, (vi) sequencing by synthesis with fluorescence-tagged nucleotides, (vii) text file generation (demultiplexing), (viii) read alignment to reference genome, (ix) data storage, (x) statistical analysis and biological interpretation. Bioinformatical processing is often time consuming, as no standard pipelines are available and it requires multiple steps of data processing. NGS technologies so far dominate the market since their development.

Third-generation sequencing (TGS) was developed to achieve longer sequencing read lengths with less sample preprocessing. Despite these advantages, high error rates and cluster cross-talk currently make routine application challenging. At the moment there are three platforms commercially available, i.e., Pacific Bioscience (PacBio) Single Molecule Real Time (SMRT) Sequencing, Oxford Nanopore Technologies sequencing platform (MinION, Promethion, to name a few), and the Illumina Tru-Seq Synthetic Long-Read technology. Some of the currently released TGS devices are portable, e.g., the MinION commercialized by Oxford Nanopore Technology with a weight of approximately 100 g, which will allow



Gene Expression Analysis and Next-Generation Sequencing, Fig. 1 Schematic representation of the experimental process of global gene expression analysis using Illuminas Next-Generation Sequencing protocol

incorporation of sequencing technologies in the clinics and field (Heather and Chain 2016).

Sequencers return base call files, which need to be converted into a text-based format (FASTQ file) for downstream computational analysis. For Illumina platforms, this can be done by using Bcl2Fastq conversion software. FASTQ files contain a sequence identifier, raw sequence letters and ASCII encoded quality scores for each base called. Errors or low-quality base calls can originate during library preparation (e.g., **PCR** artifacts, contamination with foreign DNA, adaptor sequences) or sequencing (e.g., 3'/5' positional bias, repetitive sequences, cluster cross-talk). Diverse tools have been developed to evaluate library quality, which provide summary statistics on quality scores, GC content, sequence length distribution, duplication levels, and overrepresented sequences, to name a few. All previously mentioned criteria are important and help to assess library quality, unintended low-quality base-calls or repetitive sequences are trimmed. Commonly used tools for this are FastQC or PRINSEQ.

Alignment, or mapping, matches sequencing reads to a preexisting reference genome to determine from which region of the genome the read originates from. Examples for alignment programs are TopHat, Bowtie2, or STAR. Human reference genome assemblies are provided by the Human Genome Project (HGP), while diverse gene annotations are available via Ensembl, Havana, RefSeq, NCBI, Gencode, or the USCS Genome Browser. Reads mapping to rRNA, tRNA, or mtRNA are often removed for downstream analysis. After mapping, read coverage per gene is quantified to infer expression levels of each gene. One commonly used tool for this step is htseq, or the quantification option from STAR. Library size normalization is performed either by calculating FPKM (Fragments Per Kilobases of gene per Million reads) or TPM (Transcripts Per Million) values. After library size normalization it is recommended to determine the biological heterogeneity, i.e., the biological and technical variability of all samples in one condition. Commonly this is done by performing a principal-component analysis (PCA). Once expression levels per gene are calculated, statistical tests can be applied to compare expression values of genes between two or multiple conditions (e.g., disease vs. control). Heatmaps, scatter-, MA-, or volcano-plots are commonly ways to visualize differentially expressed genes (DEGs). Diverse methods for differential gene expression analysis have been introduced in the last years, from which edgeR, DESeq2, and limma are most often used.

Once the list of statistically significant DEGs has been generated, the next step is to interpret the biological meaning. For example, gene ontology or pathway enrichment analysis determines whether enriched biological processes or pathways may be of functional relevance to the underlying biological, pathological or therapeutical mechanism (i.e., drug action, safety and efficacy). For further details please see Reimand et al. 2019.

The following are examples of questions which are to be considered in downstream analysis:

- Which protein families are in the list of DEGs (e.g., transcription factors, G protein coupled receptors, kinases, secreted proteins)?
- Which pathways are dysregulated (over- or underrepresented) between conditions?
- How many different pathways are affected?
- Can a molecular mechanism be inferred from the list of DEGs?
- Are the de-regulated pathways tissue specific?
- How does the response mechanism to one compound differ to another compound with known or unknown pharmacological and toxicological properties?
- Is the response mechanism to a pharmacological intervention influenced by the genetic background?

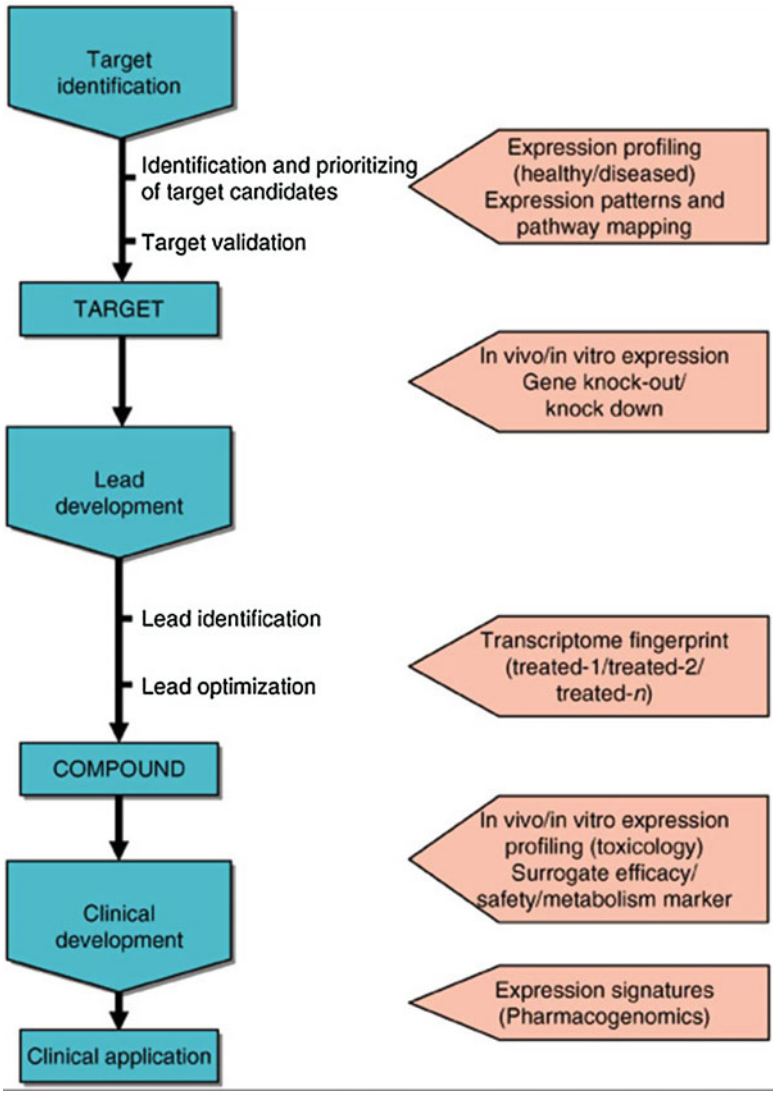
At present, there is no golden standard to address all these questions, but a combination of bioinformatic analysis should be used to address particular question of interest as thoroughly as possible. It is recommended to compare multiple databases and different evaluation pipelines and compare the results to further find support for findings.

Pharmacological Relevance

There are ~20,000 protein coding genes in the human genome, from which ~3000 are estimated to be part of the druggable genome. Approximately 700 proteins are currently targeted by FDA approved drugs, which are dominated by Ion channels, Kinases, G protein coupled receptors, and nuclear receptors (Santos et al. 2016).

Properly designed NGS-based studies, including whole genome sequencing and gene expression profiling of large patient cohorts, have not only an impact on therapeutic target identification, but also on many aspects on the drug discovery process and on drug efficacy. NGS sequencing is used at the following specified steps in the process of drug development:

- Target discovery and **biomarker** identification, to identify genes or pathways with altered expression in diseased human tissues or in animal model of disease which can be used either as a drug target or diagnostic or prognostic marker.



Gene Expression Analysis and Next-Generation Sequencing, Fig. 2 How NGS technologies contribute to the discovery of novel drugs

- Target validation, to determine that a gene product is causative of disease symptoms or that activation of the target protein ameliorates disease symptoms. Agonist/activator or an inhibitor which may be therapeutic can be validated by using NGS.
- **Compound optimization**, to screen a series of therapeutic drug candidates and determine on-target versus off-target effects.
- Toxicology (**toxicogenomics**), to identify potential human and environmental toxicants, and to find correlations between toxic responses to toxicants and changes in the genetic profiles of the objects exposed to such toxicants.
- **Drug metabolism**, determine whether multiple drug interfere with same pathways, or cause drug-drug interactions. This raised particular interest in recent years, as NGS data enable the analysis of variants in Phase I or II metabolizing enzymes such as the cytochrome P450 family, Thiopurine-Methyltransferase (TPMT) and UGT1A1.
- **Drug efficacy**, to identify the individual mode of action and whether drugs in a co-treatment interfere with the same pathways (Fig. 2).

Oncology is currently the biggest driver for drug discovery (Allen 2002). The emergence of diverse "omics" technologies supported this rapid development. One example here is the identification of the $BRAF^{V600}$ mutation. It was identified in ~50% of all melanomas and led to the development of a selective $BRAF^{V600}$ inhibitor, Vemurafenib. Nine years after discovery, the drug was FDA approved in 2011. Trametinib, a MEK inhibitor which targets the same signaling pathway, entered the market 2 years later.

NGS data generation in the clinics can furthermore help to make decisions for a personalized therapy. Some described side effects of chemotherapies are strongly correlating to the mutations of distinct genes. Examples here are the treatment of breast cancer, which depends on the gene expression profile and mutation status. Different molecular subgroups of breast cancer have been defined so far. While Alpelisib (Piqray) is used for HER2-negative breast cancer with PIK3CA mutation, Trastuzumab (Herceptin) is used to treat HER2-overexpressing cancer, just to name a few examples. For these examples, NGS provided additional information for targeted and personalized medicine.

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Gene Expression Profiling

▶ Gene Expression Analysis and Next-Generation Sequencing

Gene Medicine

▶ Gene Therapy Vectors

Gene Therapy

▶ Gene Therapy Vectors

Gene Therapy Vectors

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Synonyms

Gene medicine; Gene therapy; Gene transfer; Viral vectors

Definition

Gene therapy is, in a broad sense, the delivery of genetic information to a patient's cells in order to positively affect disease course and improve patient outcome. Typically, this is achieved through the introduction of a functional version of a disease-causing mutated gene or by correction of a defective gene by gene editing. Additionally, genes coding for therapeutically beneficial proteins can be introduced to target diseases that are not strictly etiologically genetic, such as infectious and cardiovascular disease. While the concept of gene therapy is fairly simple, the vehicle used to transfer the therapeutic gene is a critical factor for the success or failure of gene therapy trials.

Description

With the progress of the human genome project and functional genomics, the number of genes that could have a therapeutic impact is increasing at an astonishing pace. Advances in molecular biology have also yielded an array of sophisticated genetic tools for editing the genome of living cells, such as TALENs, zinc finger, and CRISPR/Cas9 nucleases that induce double-strand breaks (DSBs) and homology-directed repair. More recently, alternative strategies have been developed that are not based on DSBs including prime editing tools (Anzalone et al. 2019) that can perform search-and-edit or search-and-replace functions on the target genome without DSBs and introduce far fewer off-target instances. However, successful delivery of these therapeutic genes or gene-editing tools depends on the vehicle and is still a major obstacle.

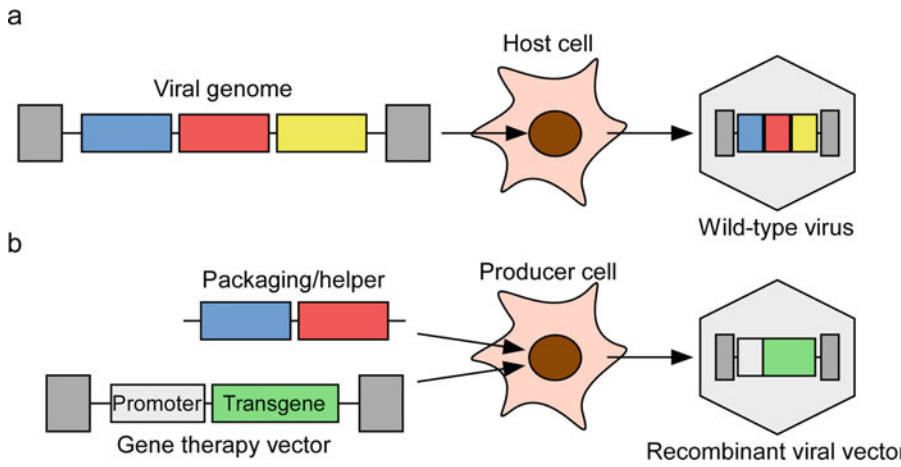
Design of Viral Vectors

Viral vector design focuses mainly on the efficacy of gene delivery and on the biosafety of the engineered viruses. A prerequisite for the use of a virus as gene therapy vehicle is to identify and eliminate pathological or toxic viral genes. Ideally, all viral genes are replaced by the gene of interest (also called transgene) (Fig. 1). The viral gene products required for the assembly of infectious particles and packaging of the vector into these particles are provided in *trans* by the so-called packaging cells, while necessary *cis*-acting factors like packaging signals are incorporated into the vector genome.

Types of Viral Vectors

Adenoviruses

Viral vectors based on adenoviruses (Ad) were among the first used in human clinical trials and are presently used in 19% of all clinical gene therapy trials. Ad contain a 36-kb double-stranded DNA genome and can infect a broad spectrum of cells, including nondividing cells like hepatocytes and neuronal cells. Ad vectors can be produced at high titers (~10^{14} particles/ml) and can efficiently express transgenes in many different cells and tissues. However, transgene expression is only short-lived, because the Ad chromosome does not integrate into the host genome and is maintained as an episome. In addition, adenoviral infection causes a humoral and cellular immune response in immune-competent hosts. This immune response is directed not only against the viral particles but also against the infected cells. An unbalanced, massive immune response directed against Ad can be life-threatening to the



Gene Therapy Vectors, Fig. 1 General design of gene therapy vectors. (**a**) Schematic drawing of wild-type virus genome and life cycle. (**b**) The viral genes required for the production of infectious particles are provided in *trans* and are not packaged. The vector itself carries the gene of interest (transgene) under the control of a specific promoter

patient, especially if a high concentration of Ad vectors is administered (Verma and Weitzman 2005). The strong humoral immune response directed at the viral particles themselves has also severely limited the use of repeated cycles of Ad vector-mediated gene therapy. To limit immune reactions against Ad vectors, gutless vectors have been developed that do not contain viral genes. Transgene expression from these gutless vectors has been reported to be more long-lasting and the host immune response less pronounced (see Pfeifer and Verma (2001)). Replication-competent Ad vectors are especially promising for oncolytic therapy. To this end, Ad vectors, which preferentially replicate in tumor cells, have been designed.

Adeno-associated viruses (AAV)

AAVs are parvoviruses that are nonpathogenic to humans and carry a small genome of only 4.7 kb, consisting of the genes *rep* and *cap*. Entry of AAV into the cell is mediated by binding to heparin sulfate proteoglycan. Integrin avb5 and the fibroblast growth factor receptor 1 act as coreceptors. In the absence of the so-called helper virus (Ad, herpes viruses, or vaccinia viruses), AAV integrates into the host genome (human chromosome 19) and establishes latent infection. Upon co-infection with a helper virus, transcription of the latent AAV genome is activated, and the virus enters the productive stage of its life cycle. To generate recombinant AAV (rAAV) vectors, the *rep* and *cap* genes are replaced by the transgene of interest, rendering the virus replication-deficient. The integration of wild-type AAV is site-specific and requires the presence of the viral Rep protein, which is normally absent from AAV vectors since Rep is provided in *trans* during vector production. Therefore, rAAVs lacking the *rep* gene exhibit no site-specific integration but are more likely than wild-type AAV to integrate at non-homologous sites in the host genome. Overall, rAAV integration rates are still exceedingly low, but long-term transgene expression can nevertheless be achieved, since extrachromosomal rAAV genomes can persist as episomes (Pfeifer and Verma 2001).

Another important issue is the size limitation of AAV vectors: the maximum size of rAAV genomes is ~4.7 kb. While this is sufficient for many applications of gene therapy, the delivery of advanced gene-editing tools with rAAVs may prove difficult. To overcome this size constraint, dual vector systems with split genomes have been developed (Pfeifer and Verma 2001): dual vectors are based on episomal circular multimers formed by the AAV vector genomes. However, the efficacy of the dual vector system has been questioned.

Presently, 8% of all clinical gene therapy trials use AAV vectors as gene-delivery vehicle (Table 1). Although no pathology is known to be associated with AAV, the high frequency of antibodies against AAV (80% of the human population are seropositive for the most common AAV subtype) could be a major limitation for gene therapy using AAV-based vectors (Manno et al. 2006).

Retroviral Vectors

Retroviruses are enveloped viruses carrying an RNA genome with a DNA intermediate, which randomly integrates in the host genome. All retroviruses contain a basic set of three genes: *gag* (the structural virion proteins), *pol* (essential viral enzymes), and *env* (the viral glycoprotein of the envelope). Prototypic retroviruses like murine leukemia virus (MLV) carry only this simple set of genes, while complex retroviruses like lentiviruses carry additional regulatory genes. The first gene therapy vectors were derived from prototypic retroviruses, and they were the first viral vectors to be used in clinical trials. Retroviral virions can accommodate ~7 kb of transgene after reverse transcription in the target cells, although recent attempts at restructuring the genome of lentiviral vectors suggest this could be increased to ~8 kb (Vink et al. 2017). Integration of the proviral DNA is of advantage if long-term (even life-long) vector expression is wanted. However, vector integration harbors the risk of insertional mutagenesis and activation of proto-oncogenes. To cope with the latter problem, self-inactivating (SIN) retroviral vectors have been developed which carry deletions of the essential viral promoter/enhancer sequences. However, incorporation of the SIN mutations can result in a significant reduction of retroviral titers (Pfeifer and Verma 2001; Verma and Weitzman 2005). Additionally, for applications where transient expression is desired (e.g., of gene-editing machinery), integration-deficient retroviral vectors have been developed (Wanisch and Yanez-Munoz 2009).

Gene Therapy Vectors, Table 1 Gene therapy – clinical trials

Vector	%
Adenovirus	19
AAV	8
Retrovirus	17.5
Lentivirus	9.5
Naked/plasmid DNA	15.4

Detailed information can be found at http://www.abedia.com/wiley/

A major drawback of vectors derived from prototypic retroviruses is that they can only transduce dividing cells. Therefore, these vectors cannot be used for gene transfer in many nondividing cells (e.g., muscle and brain cells).

To circumvent this problem, vectors that are based on lentiviruses have been developed. In contrast to prototypic retroviruses, lentiviruses do not require cell division for integration since the proviral DNA can be transported through the nuclear pores. Gene therapy vectors have been developed from a broad spectrum of lentiviruses including human immunodeficiency virus (HIV), simian and feline immunodeficiency virus, as well as visna/maedi virus. The most widely used lentiviral vector system is based on HIV-1. These vectors can efficiently transduce a wide range of dividing and nondividing cells including neurons, hepatocytes, muscle cells, and hematopoietic stem cells (see below) (Pfeifer and Verma 2001; Verma and Weitzman 2005).

To address biosafety concerns, self-inactivating lentiviral vectors and stable packaging cell lines have been developed, which greatly reduces the risk of generating replication-competent viral vectors.

Apart from being a promising tool for gene therapy, lentiviral vectors are also important tools for molecular biology. These vectors efficiently transduce a variety of nondividing cells in vitro. Lentiviral vectors can be used to generate clinically relevant transgenic animal models (lentiviral transgenesis) (for a detailed review, see Pfeifer (2004)). Importantly, lentiviral transgenesis is not restricted to rodents (mouse and rat) but can also be used to generate transgenic animals in medically relevant livestock species (pig, cattle, chicken).

Finally, lentiviral vectors have been shown to transduce human embryonic stem cells. Therefore, this type of gene therapy vector might also be used in stem cell-based therapies.

Retrovirus- and lentivirus-derived viral vectors are used in approximately 27% of all gene therapy trials (Table 1). The gene therapy trial in patients suffering from severe combined immunodeficiency-X1 (SCID-X1) led by Alain Fischer and colleagues initially reported the successful restoration of the immune systems of SCID patients (Verma and Weitzman 2005). In this trial, hematopoietic stem cells were transduced with vectors derived from simple retroviruses. Unfortunately, five of the patients subsequently developed leukemia. Detailed analysis of vector-integration sites revealed that the retroviral vector had integrated close to the proto-oncogenes *LMO2* or *CCND2*, which led to aberrant expression of these genes, presumably through the enhancer-promoter activity of the viral long terminal repeats (LTRs) (Verma and Weitzman 2005). Later trials abandoned the use of viral LTRs to drive transgene expression and instead used the less mutagenic elongation factor 1α promoter. This, together with SIN modifications to remove enhancer-promoter activity of the LTRs, might have reduced the oncogene potential of the vectors derived from simple retroviruses (Hacein-Bey-Abina et al. 2014). In even more recent trials for SCID-X1, the simple SIN retroviral vectors were substituted for SIN lentiviral vectors, which led to more efficient transduction of B-cells and restoration of humoral immunity (Mamcarz et al. 2019).

Delivery, Tropism, and Targeting

In clinical gene therapy, there are two distinct approaches for introducing therapeutic genes into patient cells. The most common approach to date is ex vivo gene therapy. Here, cells are extracted from the patient, treated with gene therapy vectors, and transplanted back into the patient. This type of therapy is typically applied to hematopoietic stem cells or blood cells, when the immune system is the target, e.g., for the treatment of SCID and for CAR-T cell therapy. Recently approved examples of this kind of gene therapy include Strimvelis® (for ADA-SCID) and Kymriah® (CAR-T for B-cell acute lymphoblastic leukemia). Conversely, in vivo gene therapy is applied in cases where the target cells must remain in situ. An example of this is the first clinically approved gene therapy, Glybera®, in which an adeno-associated viral vector carrying the lipoprotein lipase gene is directly injected into the patient's skeletal muscle tissue.

In some cases, the pathology of a genetic disease arises from a cell type that is not amenable to neither ex vivo gene therapy nor local injection of gene therapy vectors. For this, so-called targeted viral vectors are needed. The goal of this is to alter the tropism of viral vectors to enhance transduction efficiency of the target cells in vivo and to avoid off-target effects. This is achieved by changing the epitope of the viral particle that binds to the host cell receptor. A classic example is the modifications done to enhance the tropism of lentiviral vectors. The most used lentiviral vectors derive from the wild-type HIV-1 virus. This virus interacts with its host cells (CD4-positive cells) through the glycoprotein gp160, which resides in the virus lipid envelope. By generating HIV-derived viral vectors that express the glycoprotein from the vesicular stomatitis virus (VSV. G), the tropism of the vector is vastly expanded to virtually any type of cell (Burns et al. 1993). This process is known as pseudotyping. An analogous example for non-enveloped viruses such as AAVs is so-called cross-packaging, where the tropism of AAV vectors is altered by packing the AAV genome, e.g., from AAV-2, into the capsid of other AAV serotypes. Further modification of AAV tropism can also be achieved by shuffling the capsid gene DNA of different AAV serotypes to generate chimeras with novel targeting properties (Vandenberghe et al. 2009).

Finally, specific tropism can be engineered by rational design of antigen-binding epitopes on the viral surface. For example, incorporation of single-chain variable fragments or DARPINs targeted to a specific receptor can mediate highly

accurate targeting of a modified viral vector to a certain cell type (Hartmann et al. 2018).

Regulation of Gene Expression

An important issue is the regulation of gene expression in the target cells and tissues. Although steady-state expression over prolonged time intervals is desired in many diseases, controlled expression of the foreign gene in a reversible manner will be highly desirable (e.g., gene therapy for insulin-dependent diabetes mellitus). To achieve more physiological expression profiles, novel regulatable systems that are controlled by endogenous (e.g., hormones) and/or exogenous (e.g., pharmacological agents) factors have to be developed. In addition, regulation of transgene expression by the gene product itself via negative or positive feedback mechanisms would be an additional desired feature.

Other Viral Vectors

Several vectors based on viruses other than the ones mentioned above have also been designed and used for gene therapy in animal models and clinical trials (these have been reviewed in detail elsewhere) (Anguela and High 2019; Kotterman et al. 2015; Lundstrom 2018). Herpes simplex virus (HSV)-based vectors yield stable long-term expression of transgenes, have an extremely high insert capacity (up to 150 kb for amplicon vectors), and have been used in clinical trials to treat CNS disorders and malignant tumors (oncolytic HSV vectors). The measles virus is an enveloped virus that has been widely used to generate oncolytic viral vectors. The host cell entry mechanism of the measles virus is particularly amenable to engineered tropism, due to the fact that host cell receptor interaction and viral entry are mediated by two distinct viral proteins. This has allowed incorporation of antibody fragments greatly increasing the tropism to various cancer cells. Additionally, VSV vectors have been used, e.g., in preclinical studies to deliver the HIV-1 genes *gag* and *env* in order to elicit a humoral response against HIV-1 infection. Several vectors based on other viruses are also being investigated.

Potential Risks

An important safety issue of viral vectors is whether or not the recombinant viruses are able to replicate in the infected cells. Replication of viral vectors is unwanted in most gene therapy approaches. Therefore, replication-defective vectors have been designed, which are able to perform only one initial infectious cycle within the target cell. In addition, replication-competent vectors have been designed, which are able to productively infect the target cell and to spread in the target tissue, e.g., cancer. Immunological reactions of the host to either the delivery vehicle or its cargo are another concern. The host immune response might not only eliminate the vector particles before they reach the target cells/tissues; it can also be directed at the product(s) of the genes delivered by the vector, especially if a null mutation is replaced with a functional copy of the affected gene (in this case no immunological tolerance would exist for the product of the normal gene).

The development of leukemia in patients receiving retrovirally transduced cells clearly underlines that insertional mutagenesis is a major concern for integrating vectors, although careful engineering of the vectors has greatly reduced the risk of this occurrence. Nevertheless, improving safety profiles of viral vectors remains of critical importance in the development of gene therapies.

Pharmacological Relevance

Although pharmacology still relies heavily on small chemical substances, biologicals like recombinant proteins, cell-based therapies, and gene therapy approaches have great potential for the treatment of a broad spectrum of diseases. Gene therapy has been shown to be particularly versatile and has found application in the treatment of genetic diseases, infectious diseases, and cancer. Therefore, the focus of modern pharmacology should shift toward these innovative therapies.

Gene Therapy Vectors, Table 2 List of most common therapeutic genes used in gene therapy trials (viral and nonviral vectors)

Gene type	%
Antigen	18
Receptor	16.8
Cytokine	13.1
Deficiency	8.5
Growth factor	6.5
Tumor suppressor	6.2
Suicide	6.1

Detailed information can be found at http://www.abedia.com/wiley/

Presently, 2,930 gene therapy trials are underway worldwide (Tables 1 and 2). For details see http://www.abedia.com/wiley/. At present, two thirds of all gene therapy trials target cancer diseases, while 11.5% target monogenic diseases. The vast majority of nonviral gene therapy trials used naked/plasmid DNA (15.4% of all gene therapy trials). Almost three quarters of all trials are based on viral vectors.

Cross-References

► Cancer Immunotherapy

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Gene Transfer

► Gene Therapy Vectors

General Anesthetics

► GABAergic System

Genetics

► Rheumatoid Arthritis

Ghrelin

► Ghrelin, Physiological Roles and Clinical Relevance of

Ghrelin, Physiological Roles and Clinical Relevance of

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Synonyms

Ghrelin; GhrR; Pharmacology; Growth hormone; Homeostatic appetite; Hedonic appetite; Neuroprotection; Alcohol dependence; Gastric motility; Glucose metabolism; Heterodimerization; LEAP2

Definition

Ghrelin is a pleiotropic gastric hormone that has gained much attention in metabolic research in the past two decades. This is largely due to the fact that ghrelin is a gastrointestinal (GI) hormone that stimulates appetite and possibly weight gain in opposition to a wide array of well-characterized satiety-inducing hormones from the digestive system. Today, we understand that besides appetite regulation, ghrelin signaling plays a role in growth, reward, memory, gastric motility, glucose metabolism, cardiovascular functions, and possibly adiposity (Fig. 1). These functions have positioned the ghrelin receptor as a potential drug target for both agonists and antagonists.

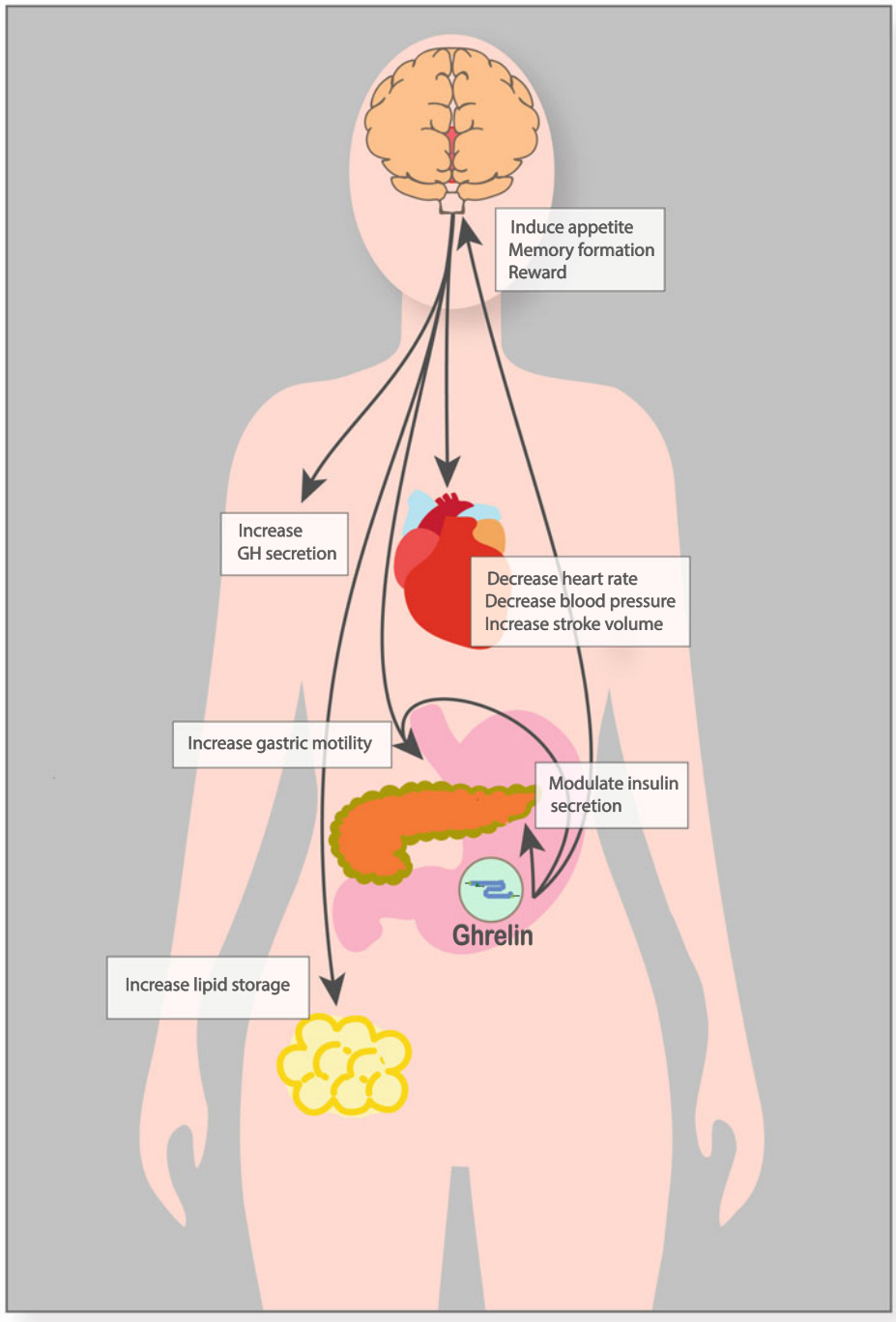
Basic Mechanism

Ghrelin Production and Acylation

In 1999, ghrelin was discovered as the endogenous ligand of the growth hormone secretagogue receptor, later known as the ghrelin receptor (GhrR). Circulating ghrelin is predominantly synthesized in the X/A-like cells in the gastric lumen, while the small intestine has the second largest expression of ghrelin mRNA in humans. Ghrelin consists of 28 amino acids; however, the molecular mass of the purified peptide is greater than the predicted value for a 28-amino acid peptide. This led to the discovery that ghrelin undergoes post-translational modification. A serine octanoylation occurs on the third hydroxyl-containing residue near the N-terminus. This acylation is required for ghrelin to be recognized by the GhrR. The enzyme ghrelin *O*-acyltransferase (GOAT) is responsible for this modification. In humans, GOAT mRNA is mainly expressed in the stomach and pancreatic tissue and has a high degree of co-localization to ghrelin-producing cells within the stomach. Furthermore, GOAT-deficient mice have an absence of plasma acyl ghrelin, demonstrating that GOAT is essential for this acylation process (Yanagi et al. 2018). Ghrelin is synthesized as a prohormone that is processed by prohormone convertase 1/3 to its mature form. The preproghrelin gene encodes ghrelin as well as another hormone, obestatin. Obestatin was initially suggested to act as an endogenous agonist for GPR39, a member of the GhrR family that decreases food intake, but subsequent studies have challenged this observation.

The Ghrelin Receptor and Its Signaling Pathways

The GhrR is the only known receptor for ghrelin, and expression of GhrR mRNA has been detected in several brain regions involved in energy



Ghrelin, Physiological Roles and Clinical Relevance of, Fig. 1 Ghrelin is secreted from the stomach and targets both the brain and peripheral organs. The arrows indicate direct and centrally mediated effects of ghrelin

homeostasis, learning, memory, and reward, as well as in several areas of the periphery, for example, the GI tract and pancreatic tissue (Müller et al. 2015). All ghrelin-mediated functions are dependent on the GhrR, which belongs to the seven-transmembrane G protein-coupled receptor

(GPCR) family. The receptor has been shown to be promiscuous in its signaling pathways, including $G\alpha_{q/11}$, $G\alpha_{i/o}$, $G\alpha_{12/13}$, and β-arrestin recruitment. The orexigenic effect of GhrR activation is thought to be mainly mediated via the $G\alpha_q$-coupled pathway through the phospholipase C-inositol trisphosphate cascade (Hedegaard and Holst 2020). The GhrR exhibits a uniquely high ligand-independent constitutive activity of approximately 50% of maximal capacity and is able to initiate downstream signaling events in the absence of ghrelin (Holst et al. 2003). This high basal activity suggests a ghrelin-independent role for the GhrR.

Role of Ghrelin in the Regulation of Growth Hormone

Since its discovery, ghrelin has been described as a hormone involved in growth hormone (GH) secretion. GhrR mRNA expression has been found in the pituitary gland, and both central and peripheral administrations of ghrelin increase both the basal level and the physiological pulsatility of GH released from pituitary somatotroph cells (Müller et al. 2015).

Role of Ghrelin in the Homeostatic Regulation of Appetite

Appetite is regulated by both physiological demands for nutrients and motivation to eat formed by the sense of reward from former experiences. These two components are classically termed the homeostatic and hedonic regulation of appetite. The homeostatic regulation of appetite balances energy intake and expenditure. Sensory inputs are relayed from the GI tract via both peripheral hormones and the vagal nerve, which are then integrated in the central nervous system, primarily via the arcuate nucleus (ARC) in the mediobasal hypothalamus. Within the ARC, there are two distinct neuronal populations with opposing effects on food intake. Neurons that express pro-opiomelanocortin (POMC) suppress feeding and increase energy expenditure, and neurons that co-express neuropeptide Y (NPY) and agouti-related peptide (AgRP) stimulate food intake and decrease energy expenditure. In the hypothalamus, ghrelin primarily binds and activates GhrRs on the presynaptic terminals of NPY/AgRP neurons and thereby stimulates food intake.

NPY/AgRP and POMC neurons innervate melanocortin-4 receptor (MC4R)-expressing neurons in the paraventricular nucleus (PVN) of the hypothalamus, which then regulate appetite and energy expenditure by projecting to neurons of the lateral parabrachial nucleus in the brainstem, which are commonly referred to as "satiety" neurons. Activation of ARC POMC neurons increases release of α-melanocyte-stimulating hormone (α-MSH), which binds to and activates MC4Rs to decrease food intake. When activated, the terminals of NPY/AgRP axons increase secretion of γ-aminobutyric acid (GABA) that inhibits the activity of postsynaptic POMC neurons to inhibit their anorexigenic effect. Additionally, the AgRP peptide antagonizes MC4Rs, while the NPY peptide acts on Y1 and Y5 receptors in the PVN to stimulate appetite (Yanagi et al. 2018). In rodents, fasting increases plasma ghrelin levels, ghrelin mRNA levels in the stomach, and GhrR mRNA in the hypothalamus and pituitary gland, suggesting a role for ghrelin under calorie deficit. GhrR expression in NPY/AgRP neurons is required for ghrelin's stimulatory effects on acute food intake and GH secretion. It has been shown that the selective reintroduction of the GhrR into AgRP neurons of GhrR knockout (KO) mice partially restores the orexigenic effects of ghrelin (Müller et al. 2015). Additionally, the NPY and AgRP neuropeptides are essential for ghrelin's orexigenic effects as peripheral administration of ghrelin failed to increase food intake in *Agrp:Npy* double KO mice (Müller et al. 2015).

The ARC also contains a population of tyrosine hydroxylase (TH) neurons that mainly release dopamine (DA) as well as GABA and have been shown to have orexigenic effects when activated. Optogenetic stimulation of ARC TH neurons in mice increases food intake through local inhibitory projections onto POMC neurons and inhibitory projections to the PVN and through indirect activation of NPY/AgRP neurons (Hedegaard and Holst 2020). Ghrelin has a direct excitatory action on ARC TH neurons, suggesting that it may also act on this population of neurons to regulate energy homeostasis. Additionally, when ras homolog gene family member A

(RhoA), an intracellular signaling molecule downstream of the $G\alpha_{12/13}$ pathway of the GhrR, was specifically knocked out in TH neurons in mice, there was a prolonged increase in food intake compared to wild-type (WT) mice when peripherally administered with ghrelin. The RhoA KO mice were also prone to overeating and obesity (Skov et al. 2019), suggesting the importance of the $G\alpha_{12/13}$ pathway of the GhrR in this population of neurons.

The lateral hypothalamus (LH) shares direct connections with the ARC and PVN, and there is evidence that ghrelin communicates with hippocampal neurons to regulate appetite as the LH also receives inputs from the ventral temporal subregion of the hippocampus (vHP). Administration of ghrelin directly into the vHP was shown to stimulate feeding by activating LH orexin neurons, indicating that the LH is downstream of vHP ghrelin-mediated orexigenic effects (Yanagi et al. 2018).

Role of Ghrelin in the Hedonic Regulation of Appetite

While the GhrR is present in hypothalamic centers, it is also expressed in the ventral tegmental area (VTA) of the midbrain. The VTA, a key node in the mesolimbic DA motive system, plays an important role in mediating motivational behaviors and the hedonic consumption of highly palatable foods. NPY/AgRP neurons in the ARC project to the LH, where second-order neurons project to the VTA to modulate activity in the mesolimbic DA system (Yanagi et al. 2018). The VTA is largely composed of DA neurons that project to the nucleus accumbens (NAc), ventral pallidum (VP), prefrontal cortex (PFC), amygdala, and hippocampus to regulate hedonic food intake, motivation to obtain food, and cue associations. Direct ghrelin administration into the VTA of mice causes a significant increase in the intake of standard chow, palatable food, and sucrose, while GhrR KO mice have a reduced intake of rewarding food compared to chow (Edwards and Abizaid 2017). Additionally, GhrR signaling in the VTA has been shown to be important for reward-motivated locomotor behaviors (Skov et al. 2017). GhrR immunoreactivity was found in a subset of VTA DA neurons in both mice and rats, and patch clamp recordings showed an increase in the frequency of action potentials of VTA DA neurons in brain slices after the application of ghrelin (Edwards and Abizaid 2017).

In summary, the GhrR has been identified in hypothalamic regions, including the ARC, an important region for controlling appetite, and the peripheral and central administration of ghrelin has been shown to increase appetite. Furthermore, the GhrR is expressed in the VTA, and ghrelin has been shown to play a role in hedonic food intake (Fig. 2).

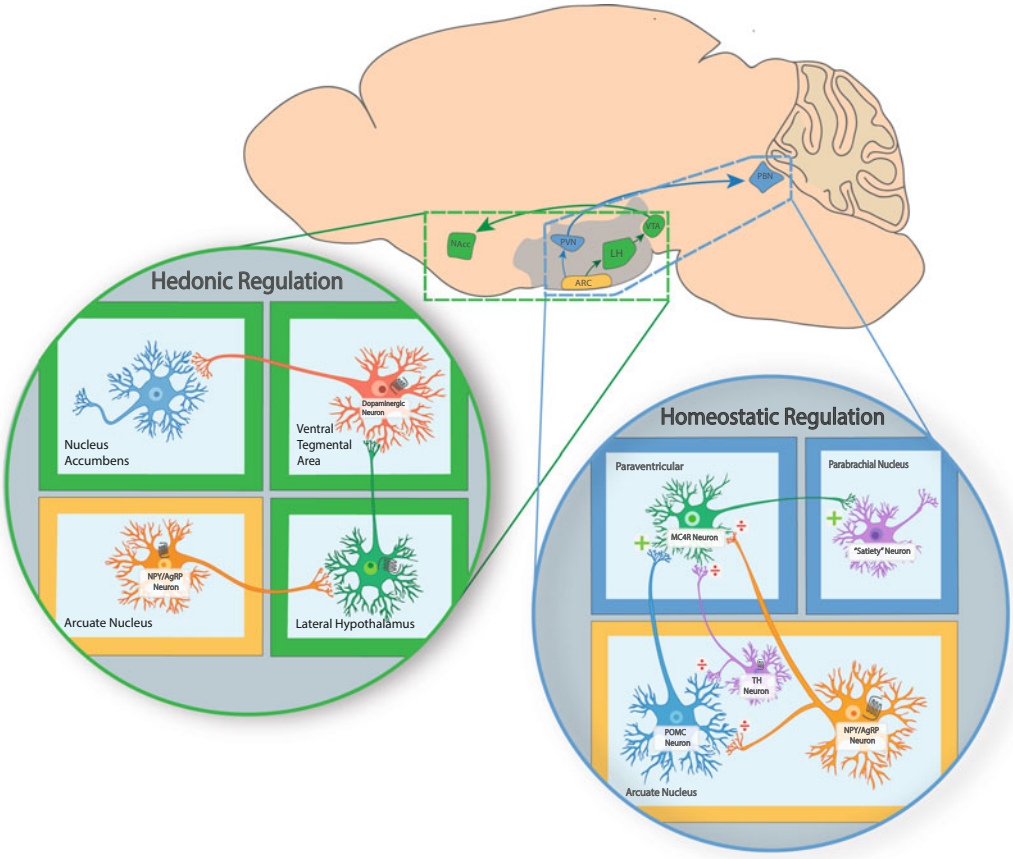
Role of Ghrelin in Stress and Anxiety

Studies have shown that ghrelin is secreted in response to acute stressors, such as fasting, physical restraint, and psychological and social stress, possibly to counter anxiety and depression. Administration of ghrelin induced antidepressant-like and anxiolytic-like responses in mice during acute stress tests compared to untreated mice. Conversely, GhrR KO mice displayed more anxiogenic-like behavior when exposed to the same stress compared to WT mice.

Both epinephrine and norepinephrine along with other β-adrenergic receptor agonists are some of the most potent stimulators of ghrelin secretion, indicating an important role of stress in the regulation of the ghrelin system. When GhrR KO and WT mice were subjugated to acute restraint stress, KO mice were more anxious during the following behavioral tests but had an attenuated corticosterone response compared to WT mice, indicating a dysfunctional glucocorticoid response in the absence of ghrelin. Additionally, when mice were subjugated to chronic social defeat stress, a paradigm that induces a depressive-like phenotype, plasma ghrelin levels were elevated, resulting in long-lasting increase in food intake and body weight (Wittekind and Kluge 2015).

Role of Ghrelin in Memory and Neuroprotection

The GhrR plays a role in enhancing synaptic plasticity and hippocampal neurogenesis. It has been shown that the GhrR is expressed in CA3



Ghrelin, Physiological Roles and Clinical Relevance of, Fig. 2 Ghrelin and the GhrR are involved in hedonic and homeostatic regulation of appetite. The homeostatic regulation of appetite is partially framed with a blue color: Activation of GhrR on the orexigenic NPY/AgRP neurons in the arcuate nucleus (ARC) inhibits the satiety-inducing network, which consists of POMC neuron stimulation of MC4R neurons in the paraventricular nucleus (PVN) that in turn stimulates "satiety" neurons in the parabrachial nucleus (PBN) in the brainstem. TH neurons in the ARC inhibits POMC and MC4R neurons and indirectly activates NPY/AgRP neurons (not illustrated). The hedonic regulation of appetite is marked with green: GhrR activation of NPY/AgRP neurons projects to the lateral hypothalamus (LH) that in turn stimulates the mesolimbic reward system by activating dopaminergic neurons in the ventral tegmental area (VTA). These in turn activate neurons in the nucleus accumbens (NAc). The receptors on the neurons symbolize the 7TM G-protein-coupled ghrelin receptor, GhrR

and CA1 hippocampal neurons, and its activation promotes dendritic spine synapse formation and long-term potentiation. Peripheral administration of ghrelin to rats has been demonstrated to improve spatial memory compared to controls. Additionally, it has been shown that repeated peripheral ghrelin administration stimulates neurogenesis in the adult mouse hippocampus.

Huntington's disease (HD) is a neurodegenerative disease that also causes metabolic disturbances such as weight loss and muscle wasting. Using a mouse model of HD, it was found that in the late stage of HD, mice had reduced circulating ghrelin levels as well as downregulation of the GhrR in the hypothalamus and an attenuated response to peripheral ghrelin administration compared to WT mice (Rudenko et al. 2019). However, the response to ghrelin was not dysfunctional, while the mice were still pre-symptomatic and chronic peripheral administration of ghrelin was able to increase lean body mass while decreasing energy expenditure and fat

utilization during the early stage of HD (Rudenko et al. 2019).

There is increasing evidence that the ghrelin system is impaired in Parkinson's disease (PD), as patients with PD have reduced plasma ghrelin levels and a diminished pre-prandial peak response and post-prandial ghrelin suppression compared to healthy controls. The degeneration of DA neurons in the substantia nigra pars compacta (SNc) leads to PD, and this population of neurons also expresses the GhrR (Yanagi et al. 2018). Studies have demonstrated that peripheral administration of ghrelin can protect against neurodegeneration and decrease SNc DA neuron loss in rodent models of PD.

Effects of Ghrelin on the Autonomic Nervous System

While there is not consistent evidence that the GhrR is expressed in white adipose tissue, it has been demonstrated that central ghrelin administration increases food intake and adiposity in rodents. This is at least in part mediated via altered sympathetic activity, as demonstrated by an abolished effect on body weight when ghrelin is administered to β-adrenergic receptor KO rodents (Theander-Carrillo et al. 2006).

The Current Understanding of the Spatial Separation Between Ghrelin and the GhrR in the Brain

It is apparent that ghrelin has the ability to regulate GH release, appetite, stress, and body weight when administered directly into specific brain areas or the lateral ventricle and that neurons in various brain regions express the GhrR and respond to ghrelin. It has also been demonstrated that peripheral ghrelin is able to directly or indirectly activate central targets. However, evidence has shown that ghrelin produced in the periphery has a limited ability to permeate the blood-brain barrier to reach extrahypothalamic regions, such as the VTA (Edwards and Abizaid 2017). Therefore, one of the most persistent unanswered questions in the research of central ghrelin functions is the function of GhrRs in brain regions that are not readily accessible to circulating ghrelin. Studies in rodents and humans indicate that ghrelin reaches the hypothalamus via the afferent fibers of the vagal nerve. Blocking of the vagal afferent pathway attenuated ghrelin-induced food intake, and following the administration of peripheral ghrelin, vagal afferents that travel to the NTS increased norepinephrine in the ARC. However, several other studies have suggested that ghrelin may also reach the ARC directly via the fenestrated capillaries or tanocytes in the median eminence (Edwards and Abizaid 2017).

The formation of heterodimers and the high constitutive activity of the GhrR point to a ghrelin-independent function that may explain the expression of the GhrR in brain areas not accessible to peripheral ghrelin. The G protein α-subunits activated following ghrelin binding to the GhrR are varied and influenced by many factors such as cell type, tissue, and heterodimer partner (Edwards and Abizaid 2017). Heterodimerization is the interaction of two receptors in a heteromeric complex. Heterodimerization between GPCRs can modify the strength and/or nature of downstream signaling pathways. Evidence indicates that the GhrR can homodimerize with itself or heterodimerize with other GPCRs, including dopamine receptors 1 and 2 (D1R, D2R), serotonin 2C receptor (5-HT_{2c}), melanocortin 3 receptor (MC3-R), and somatostatin receptor 5 (SST5) in order to modulate the downstream signaling of these receptors (Hedegaard and Holst 2020). For example, D1R has been shown to form heterodimers with the GhrR in hippocampal cells, and the heterodimer changes the signaling pathway of the D1R from Gαs-coupling to $G\alpha_q$-coupling. This modification in signaling pathway results in an increase in early markers of synaptic plasticity, suggesting that the D1R-GhrR heterodimer plays a role in memory formation in the hippocampus (Hedegaard and Holst 2020). Interestingly, beta-amyloid deposits, which are a characteristic of AD, have been shown to disrupt the formation of D1R-GhrR heterodimers in the human brain. This points to a local disruption of GhrR signaling in the pathophysiology of this type of dementia.

Role of Ghrelin in Gastric Motility

The GhrR is sparsely expressed in peripheral tissues compared to the brain, but receptor

expression has been identified in the GI tract and pancreas. In the periphery, ghrelin specifically regulates gastric motility and glucose metabolism. Pharmacological doses of ghrelin increase gastric tone and gastric emptying in rodents and humans. Ghrelin exerts its effect on gastric motility both directly on the enteric nerves of the GI tract and indirectly via central modulation of autonomic nerve activity. Ghrelin signaling via afferent vagal fibers projects to the NTS and the dorsal motor nucleus in the brain stem, which causes the release of acetylcholine and substance P from efferent vagal fibers resulting in contraction of gastric smooth muscle (Peeters 2013).

Role of Ghrelin in Glucose Metabolism

Peripheral ghrelin administration increases blood glucose, lowers insulin levels, and decreases insulin sensitivity in rodents and humans (Müller et al. 2015). In isolated rat pancreatic islet cell cultures, ghrelin suppressed insulin release, while a blockade of the GhrR increased cytosolic calcium (Ca^{2+}) in β-cells and increased glucose-stimulated insulin secretion. Furthermore, the inhibitory effects of ghrelin on insulin secretion and Ca^{2+} release were absent when β-cells were treated with an antisense oligonucleotide for the $G\alpha_{i2}$-subunit of the GhrR, indicating that in β-cells, the GhrR mediates suppression of insulin release via the $G\alpha_i$ pathway. Additionally, evidence shows that GhrR heterodimerization is important in modulating insulin secretion as the heterodimer formation of the GhrR with somatostatin receptor 5 is required to initiate the $G\alpha_i$ signaling pathway (Hedegaard and Holst 2020).

While ghrelin KO and GhrR KO mice showed no difference in phenotype compared to WT littermates under basal conditions, mice with KO of either ghrelin, the GhrR, or GOAT develop fatal hypoglycemia during prolonged caloric restriction. This has led to the hypothesis that ghrelin acts as a survival hormone that maintains glucose levels during starvation. In rodents, it has been shown that ghrelin levels increase during caloric restriction, and the associated increase in GH prevents fatal hypoglycemia (Mani and Zigman 2017).

Pharmacological Intervention

Clinical Findings of Ghrelin's Effect on Growth Hormone

A missense mutation that abolishes the constitutive activity of the GhrR has been found to result in short stature, pointing to a role of GhrR signaling in normal growth in humans (Yanagi et al. 2018). Due to their potency in stimulating somatotroph cells, synthetic GhrR agonists are in clinical use for diagnosing GH deficiency.

Clinical Findings of Ghrelin's Effect on Appetite and Body Weight

Plasma levels of ghrelin increase shortly before a meal, with the lowest values post-prandially. Clinical studies show that an administration of ghrelin increases energy intake, and long-term administration has been reported to cause an increase in the sense of appetite (Yanagi et al. 2018). Despite the appetite-stimulating effect of ghrelin in humans, clinical studies with GhrR agonists do not consistently lead to increased adiposity. A number of studies have investigated weight changes after long-term administration of ghrelin analogs and found they tend to cause a slight increase in body weight in patients with chronic illness, as well as in normal-weight individuals. Studies that report on body composition have found that the increased weight is caused by an increase in lean body mass (Garin et al. 2013).

Total ghrelin plasma levels are inversely correlated with nutritional status, such that obese individuals have lower levels and lean individuals have higher levels (Müller et al. 2015). Total ghrelin increases after diet- or exercise-induced weight loss. Additionally, ghrelin decreases after bariatric surgery, especially after sleeve gastrectomy. This difference in plasma levels between the two causes of weight loss is hypothesized to be an important contributing factor to the relative stability of the weight loss achieved after bariatric surgery (Svane et al. 2019).

Clinical Findings of Ghrelin's Effect on Anxiety and Alcohol Dependence

In clinical studies, reports on the relation between physiological and pharmacological levels of

ghrelin to anxiety have not been consistent. One study found that a majority of patients with malignancy-related cachexia preferred ghrelin to placebo, which could indicate a positive effect on well-being. In summary, more research is needed in order to understand if ghrelin influences mood and anxiety in humans.

Several clinical trials and rodent studies have investigated ghrelin levels in relation to alcohol dependence. Ghrelin infusion increases alcohol craving compared to placebo and has been shown to significantly increase the frequency of alcohol self-administration in humans. Significant correlation has been found between plasma ghrelin and mesolimbic activation in response to alcohol cues in individuals with alcohol dependence. A GhrR inverse agonist has recently been tested as a potential treatment for alcohol dependence in a phase I trial, with an acceptable safety profile and a promising finding of decreased alcohol craving at the highest dosage (Lee et al. 2020).

Clinical Findings of Ghrelin's Effect on Neuroprotection

There is evidence that ghrelin activity is dysfunctional in brain areas involved in memory formation in Alzheimer's disease (AD). Patients with AD have unaltered levels of plasma ghrelin. However, expression of the GhrR has been shown to increase in proportion to the pathognomonic accumulation of beta-amyloid deposits in the hippocampus, and amyloid beta impairs ghrelin signaling.!!Expression of the GhrR in human inferior temporal lobe is significantly lower in diseased tissue than in control specimen from non-demented individuals. In a mouse model of AD, peripheral administration of a synthetic GhrR agonist reduced beta-amyloid accumulation and inflammation. However, one multi-center randomized clinical trial has tested the same drug in more than 400 patients with AD and, disappointingly, found no effect on disease progression after 1 year of treatment (Sevigny et al. 2008).

Clinical Findings of Ghrelin's Effect on the Autonomic Nervous System

Ghrelin decreases sympathetic and increases parasympathetic activity, possibly by acting on GhrRs in the hypothalamus and the nucleus tractus solitarius (NTS), thereby causing beneficial effects on the cardiovascular system. Ghrelin administration decreases blood pressure and heart rate in healthy individuals, and several studies found that ghrelin increases ventricular contractility (Garin et al. 2013). However, this last finding is inconsistent in the literature. There are reports that the GhrR is expressed in myocardial tissues and blood vessels, but these findings are not consistent. Therefore, the impact of ghrelin on the cardiovascular system is currently perceived as being centrally mediated. In patients with chronic heart disease, 3 weeks of ghrelin treatment improved contraction of the left ventricle and physical performance. In contrast, GhrR antagonism may have a potential negative effect on the autonomic regulation of the heart and vasculature. Preclinical data show that antagonism of the GhrR causes increase in blood pressure and heart rate, and more research in the cardiac safety profile of GhrR antagonists is warranted.

Clinical Findings of Ghrelin's Effect on Gastric Motility

In clinical trials, ghrelin increases gastric emptying in healthy volunteers as well as in patients with neurogenic gastroparesis. Two recently published systematic meta-analyses have documented that parenteral administration of ghrelin mimetics improves gastric emptying in patients with neurogenic gastroparesis and in a mixed population of patients receiving pro-motility agents. There is a concern for the increase in plasma glucose when treating patients with diabetic gastroparesis with ghrelin mimetics, but overall, the potential benefits of treatment have led one of the GhrR agonist, relamorelin, being accepted for fast track designation for diabetic gastroparesis by the FDA in 2017.

Clinical Findings of Ghrelin's Effect on Glucose Metabolism

In order to assess the effect of ghrelin itself on blood glucose in humans, one study investigated the effect of ghrelin infusion in individuals with GH deficiency. In these individuals, there was no significant effect on plasma glucose by ghrelin. However, they

did find that ghrelin induced peripheral insulin resistance measured during a hyperinsulinemic euglycemic clamp (Vestergaard et al. 2017).

GhrR Antagonism

An inverse agonist for the GhrR that is able to eliminate its high constitutive activity has been highly sought after as a potential anti-obesity agent. In 2003, a known low-potency GhrR antagonist, [D-Arg1,D-Phe5,D-Trp7,9,Leu11]-substance P (SPA), was found to be a high-potency inverse agonist of the receptor. Truncation analysis of this compound showed that the inverse agonist property is located in the C-terminal pentapeptide, and mutational analysis of the GhrR binding sites showed that, in contrast to ghrelin, the inverse agonist binds to an intracellular part of the receptor (Holst et al. 2003).

Liver-expressed antimicrobial peptide 2 (LEAP2) was recently found to be the endogenous antagonist for GhrR (Ge et al. 2018). LEAP2 is a 40-amino-acid-long peptide with two disulfide bridges that is primarily secreted by cells in the small intestine and liver. LEAP2 has been shown to be both an inverse agonist of the GhrR and a competitive antagonist to ghrelin at the GhrR (M'Kadmi et al. 2019). Whole-cell patch-clamp recording on mouse hypothalamus showed that ghrelin depolarizes NPY neurons and that this is inhibited by LEAP2, which further hyperpolarizes the neuronal membrane, likely due to a decrease in the constitutive activity of the GhrR (Mani et al. 2019). LEAP2 and the N-terminal part of LEAP2 have been found to inhibit both ghrelin's orexigenic effect and the stimulation of GH release (Ge et al. 2018; M'Kadmi et al. 2019). The development of LEAP2 analogs that are able to diminish the constitutive activity of the GhrR could be a potential therapeutic anti-obesity target in the future. A recent study examined the plasma levels of LEAP2 compared to ghrelin in different nutritional states and found that LEAP2 has an opposite pattern of secretion to ghrelin in that it increases post-prandially in obese individuals and positively correlates with BMI and fat mass in both mice and humans. LEAP2 plasma levels were found to decrease during 24 h fasting and increased in response to an oral glucose load and after streptozotocin-induced diabetes mellitus in mice. It was also shown that gastric bypass and gastric sleeve surgery decreased circulating LEAP2 levels in a follow-up period of up to 2 years (Mani et al. 2019). More studies are needed to confirm the regulation of LEAP2 in obesity.

Cross-References

- ▶ Anti-obesity Drugs
- ▶ Appetite Control
- ▶ Neuropeptide Y

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GhrR

► Ghrelin, Physiological Roles and Clinical Relevance of

Glucocorticoid Agonists

► Glucocorticoids

Glucocorticoids

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Synonyms

Asthma controllers; Corticosteroids; Glucocorticoid agonists; Steroids

Definition

Both external and internal stress cause the release of glucocorticoids (cortisol) from the zona fasciculata of the adrenal gland. Heightened levels of glucocorticoids result in muscle catabolism, which causes the release of amino acids which can be used by the liver to enhance glucose synthesis (gluconeogenesis). Another key function of glucocorticoids, particularly in acute and chronic disease, is the downregulation of inflammation and immune responses that occur in response to exogenous infections or other stimuli. This aspect of glucocorticoid function has been utilized clinically for over 70 years, and topical and systemic glucocorticoids are the most effective anti-inflammatory agents used to treat most immunoinflammatory diseases.

Regulation of Inflammation

Many chronic diseases such as bronchial asthma are driven by excess activation of inflammatory and immune responses. Each disease will have precise inflammatory and immune features associated with their site of inflammation, but they all require infiltrating immune and inflammatory cells to be recruited and both infiltrating and tissue resident cells to be activated. In asthma, airway structural cells are also important inflammatory cells. Chronic inflammatory diseases feature

enhanced mediator expression including cytokines, chemokines, growth factors, enzymes, receptors, and adhesion molecules often under the control of the pro-inflammatory transcription factor nuclear factor-κB (NF-κB). For example, asthma is characterized by exaggerated NF-κB activation in immune and structural cells such as airway epithelial cells, and upregulation of NF-κB in these cells drives the abnormal inflammatory gene profile observed in bronchial asthma (Adcock and Mumby 2017; Wadhwa et al. 2019). Recent advances in high-throughput and genome-wide technologies have revealed unforeseen complexities in both inflammatory gene expression and how glucocorticoids regulate this expression. Many of these insights are summarized in the sections below.

Mechanism of Gene Induction by NF-κB

Virtually all of the stimuli that drive asthma or asthma exacerbations can enhance NF-κB activity including allergen, pollution, and rhinovirus exposures. Cytokines, such as tumor necrosis factor-α (TNFα), interleukin (IL)-1β, and key asthma cytokines such as IL-13, also activate NF-κB within airway cells. Importantly, NF-κB is also a key target for glucocorticoid actions (Adcock and Mumby 2017). There is ubiquitous expression of NF-κB in immune and inflammatory cells and it alone can induce the expression of numerous inflammatory and immune genes although its actions are often amplified by cooperation/interaction with cell- and signal-specific transcription factors such as CAAT/enhancer-binding protein (C/EBP)α and interferon response factor (IRF)-1 (Taniguchi and Karin 2018). Within a resting inflammatory cell, NF-κB is localized in the cytoplasm in an inactive state by interaction with an inhibitor protein I-κBα. Stimulation of the inflammatory cells activates a receptor-driven phosphorylation cascade that leads to IκBα phosphorylation and dissociation of IκBα from NF-κB. Released activated NF-κB is then able to translocate into the nucleus where it binds to specific *regulatory regions* of DNA within the promoters of NF-κB-responsive inflammatory genes (Taniguchi and Karin 2018). However, it is important to note that other transcription factors such as activator protein (AP)-1 and members of the signal transducers and activators of the transcription (STAT) family are also important in regulating inflammation in airway cells under various stimuli (Adcock and Mumby 2017).

The several meters of DNA within each nucleus are carefully packaged around basic histone proteins, which form a structure relatively inaccessible to the enzyme RNA polymerase II that stimulates the formation of mRNA. This chromatin structure is described as "closed" and is generally linked with limited gene expression. Heightened inflammatory gene expression following NF-κB activation is linked to enzymatic modification of these basic histones, which results in an alteration in local and also more widespread chromatin structure (Miranda et al. 2013; Pope and Medzhitov 2018; Vandewalle et al. 2018). Activated NF-κB that is bound to DNA recruits transcriptional coactivator complexes such as CREB-binding protein (CBP) and p300/CBP-associated factor (PCAF), which possess intrinsic histone acetyltransferase (HAT) activity. Acetylation of the σ-group on lysine residues within the N-terminal tail of histones close to the site of NF-κB DNA binding generates a "mark" or "tag" (Miranda et al. 2013; Pope and Medzhitov 2018; Vandewalle et al. 2018).

This tag results in mutually opposing charges on DNA and histones, which, together with the ability of coactivator enzymes and chromatin remodeling complexes being able to recognize this tag, promotes a more relaxed local DNA structure that enables the further recruitment of RNA polymerase II and other components of the basal transcriptional machinery. Inflammatory stimuli including NF-κB activating cytokines such as TNFα and IL-1β are able to enhance histone acetylation in a time- and concentration-dependent manner. The precise sequence of the NF-κB DNA-binding sequence in each inflammatory gene tends to differ, as does the chromatin

status in different cell types. NF-κB may also interact with other transcription factors in a cell-specific manner. As a result, the composition and time course of the NF-κB-induced transcriptomic profile varies with the precise cell and stimulus examined. Recent data using RNA sequencing have identified a range of time courses for NF-κB-directed gene expression, and interestingly a similar number of genes are also repressed by NF-κB (Miranda et al. 2013; Oh et al. 2017; Pope and Medzhitov 2018; Vandewalle et al. 2018).

Suppression of gene expression has been linked to a reversal of this process under the action of histone deacetylases (HDACs). Deacetylation of acetylated histones removes the charge differences between DNA and histones which results in enhanced winding of DNA around histone residues. Thus, we end with a dense chromatin structure, which produces a barrier to transcription factor access to their binding sites and suppression of inflammatory gene expression (Adcock and Mumby 2017; Wadhwa et al. 2019).

Mechanisms of Glucocorticoid Functions

Dexamethasone-induced transcriptomic profiles vary considerably across cell types and only a few genes are regulated across many cells. This indicates that identifying a key set of glucocorticoid-induced anti-inflammatory genes and mechanisms will be probably cell-specific (Adcock and Mumby 2017).

Gene Induction

Glucocorticoids generally mediate their effects by binding to a cytoplasmic glucocorticoid receptor (GR) present in virtually all cells. The unliganded GR is retained within the cytoplasm in an inactive complex containing two heat shock protein (HSP) 90 proteins. Hsp90 binding to GR blocks the nuclear localization signal within the GR preventing its activation. Glucocorticoids are lipophilic molecules that freely diffuse across the plasma membrane to enable binding to the cytoplasmic GR. Ligand binding alters the structure of GR complex with dissociation of HSP90 allowing activated GR to translocate into the nucleus. Within the nucleus, activated GR seeks out and binds specific DNA sequences (GREs, GGTACAnnnTGTTCT) as a homodimer and interacts with coactivator complexes. Classical GREs consist of inverted repeats of hexameric half sites separated by a sequence of three base pairs. GR can also transactivate genes by binding to a GRE half site as a monomer by binding to other transcription factors through tethering or by binding to composite elements (Miranda et al. 2013; Oh et al. 2017).

Recent research has highlighted the diversity in the sequence of GREs and how this affects the overall strength of the transcriptional response acting in a manner synonymous with ligand binding to GR. Furthermore, cryptic GREs are found within NF-κB- and AP-1-binding sites, and GR has been found to occupy AP-1 response elements in the absence of AP-1 to influence its activity (Miranda et al. 2013; Oh et al. 2017). In addition, RNA sequencing identified that GR can also repress gene expression by binding to inverted repeat GR-binding sequences (IR-GBS) following recruitment of corepressors such as HDACs (Vandewalle et al. 2018).

Inflammatory Gene Suppression by Glucocorticoids

Glucocorticoids can either directly modulate the expression of genes by GR acting as a transcription factor in a similar way to that described above for NF-κB with the target genes identified by the upstream GREs in a process known as transactivation. Initial studies suggested that the activated GR *directly* upregulated between 10 and 100 genes depending upon the cell type studied including the expression of anti-inflammatory proteins, such as annexin-1, DUSP1, IL-10, and the inhibitor of NF-κB, IκB-α (Table 1) (Adcock and Mumby 2017; Ronchetti et al. 2018). Again, RNA sequencing analysis has greatly amplified the number of GR-regulated genes that are

Glucocorticoids, Table 1 Glucocorticoid-sensitive genes

Increased transcription	Decreased transcription
Lipocortin-1/annexin-1 (phospholipase A_2 inhibitor)	Cytokines (IL-1, 2, 3, 4, 5, 6, 9, 11, 12, 13, 16, 17, 18, TNFα, GM-CSF, SCF)
β-Adrenoceptor	Chemokines (IL-8, RANTES, MIP-1α, MCP-1, MCP-3, MCP-4, eotaxin)
Secretory leukocyte inhibitory protein (SLPI)	Inducible nitric oxide synthase (iNOS)
Clara cell protein (CC10, phospholipase A_2 inhibitor)	Inducible cyclooxygenase (COX-2)
IL-1 receptor antagonist	Cytoplasmic phospholipase A_2 (cPLA $_2$)
IL-1R2 (decoy receptor)	Endothelin-1
IκBα (inhibitor of NF-κB)	NK_1-receptors, NK_2-receptors
CD163 (scavenger receptor)	Adhesion molecules (ICAM-1, E-selectin)

upregulated to differing extents and over distinct time courses and identified genes that are repressed following direct GR-DNA interaction (Miranda et al. 2013; Oh et al. 2017; Van Moortel et al. 2020; Vandewalle et al. 2018).

Alternatively, activated GR may downregulate inflammatory gene expression driven by NF-κB following protein-protein interaction with activated NF-κB within the nucleus or while DNA associated known as transrepression. This may occur through a process known as tethering with NF-κB and GR interacting while on DNA. However, the concept of tethering as a mechanism for suppression of NF-κB function does not occur in all cases. Bulk RNA sequencing and chromatin-binding analysis in mouse bone marrow-derived macrophages stimulated with LPS prior to exposure to dexamethasone to mimic the clinical situation gave a similar anti-inflammatory transcriptomic profile as dexamethasone pretreatment. Chromatin occupancy of GR did not predict dexamethasone suppression of gene expression rather GR-induced suppressors of NF-κB and AP-1 chromatin interaction (Adcock and Mumby 2017; Miranda et al. 2013; Oh et al. 2017). However, although representing a simplification, it is likely that many of the anti-inflammatory effects of glucocorticoids in suppressing inflammation occur via transrepression while glucocorticoid side effects, such as osteoporosis, cataracts, skin fragility, and hypothalamic-preoptic-adrenal axis suppression, may reflect transactivation by GR (Wadhwa et al. 2019).

Transcription factors including GR and NF-κB regulate transcription through altered chromatin modifications, DNA unwinding, and local recruitment of basal transcriptional complexes including RNA polymerase II. GR interacts with coactivator proteins such as CBP, PCAF, and steroid receptor coactivator-1 (SRC-1), which all are able to promote HAT activity. How do we explain how GR or NF-κB interacts with its specific DNA-binding site when DNA is compacted? For GR, this may result from GR binding to a GRE whose residues face outward from the compacted DNA-chromatin structure. GRE interaction may modify the local chromatin structure, further enabling GR access to other GREs (Adcock and Mumby 2017; Miranda et al. 2013; Oh et al. 2017; Vandewalle et al. 2018).

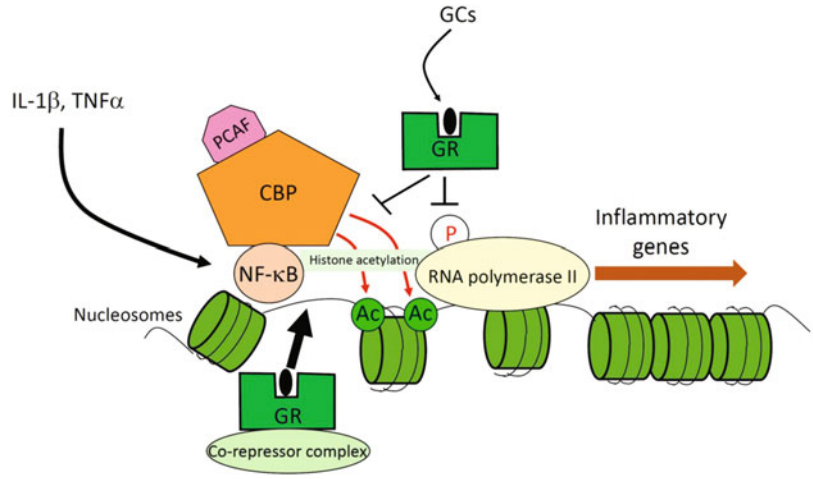
GR only remains bound to DNA for a short time (<10 s) and so it must utilize a "hit-and-run" mechanism of action. Activated GR tracks along DNA until it encounters an accessible GRE, it is quickly ejected, which allows replacement by another GR or by regulatory HAT-containing factors that enhance gene transcription. This has also been implicated in GR feedback mechanisms and subsequent GR proteasomal degradation (Adcock and Mumby 2017; Miranda et al. 2013; Oh et al. 2017; Vandewalle et al. 2018).

Although glucocorticoids are very efficient at inducing gene transcription, their main major anti-inflammatory effects are through repression of inflammatory and immune genes. This is due mainly via and interaction between the activated GR and pro-inflammatory transcription factors including NF-κB and AP-1 (a heterodimer of Fos and Jun proteins). The precise interplay

between these pro-inflammatory transcription factors and GR differs between immune and structural, which may reflect different NF-κB/transcription factor/coactivator complexes activated by differing stimuli between the cell types and the distinct inflammatory gene and chromatin profiles within the cells (Adcock and Mumby 2017; Vandewalle et al. 2018; Wadhwa et al. 2019). Thus, the synthetic glucocorticoid dexamethasone attenuates NF-κB-enhanced histone acetylation in lung epithelial cells and macrophages following stimulation with IL-1β. This is due to a combination of direct inhibition of NF-κB-associated HAT activity and by active recruitment of HDAC-containing corepressor complexes (Fig. 1). The overall effect is a localized loss of pro-inflammatory transcription factor function linked to histone deacetylation and chromatin remodeling resulting in suppression of inflammatory gene expression (Adcock and Mumby 2017; Vandewalle et al. 2018; Wadhwa et al. 2019).

Other Mechanisms of GR Regulation of Inflammation

Inflammatory gene expression is also regulated by mitogen-activated protein kinases (MAPKs) and their ability to activate pro-inflammatory transcription factors. MAPK pathways are also targets for glucocorticoid actions. The expression of cyclooxygenase-2 (COX-2), an important pro-inflammatory gene, is repressed by glucocorticoids acting to reduce COX-2 mRNA stability downstream of suppressing p38 MAP kinase activity. This effect is driven by the upregulation of p38 MAPK phosphatase (MKP-1) with a subsequent dephosphorylation of active phospho-p38 MAPK. Furthermore, activated GR prevents c-Jun phosphorylation and AP-1 activation by attenuating the Jun N-terminal kinase (JNK) signaling cascade. Glucocorticoids also prevent the activity of other JNK-stimulated transcription factors such as ETS-like kinase 1 (Elk-1) and activating transcription factor 2 (ATF-2). This effect



Glucocorticoids, Fig. 1 How glucocorticoids switch off inflammatory genes. Inflammatory genes are activated by inflammatory stimuli, such as interleukin (IL)-1β or tumor necrosis factor (TNF)α, resulting in activation of the transcription factor nuclear factor κB (NF-κB). NF-κB translocates to the nucleus and binds to specific κB recognition sites and also to *transcriptional coactivators*, such as CREB-binding protein (CBP) or p300/CBP-activating factor (PCAF), which have intrinsic *histone* acetyltransferase (HAT) activity. This results in acetylation of lysines in *histone* proteins, alteration in the charge between histones and DNA resulting in chromatin remodeling and recruitment of *RNA polymerase II* resulting in enhanced inflammatory gene expression. Binding of glucocorticoids (GCs) to glucocorticoid receptors (GR) results in their activation, nuclear translocation, and interaction with *corepressor* proteins to inhibit NF-κB-associated gene expression. Activated GR is also able to recruit corepressor proteins including *histone* deacetylases (HDAC) to the NF-κB complex further enabling suppression of inflammatory genes. GR may also affect the phosphorylation of *RNA polymerase II* to block inflammatory gene expression or directly induce repressors of NF-κB activation

is mutual since JNK attenuates GR function by direct phosphorylation (Adcock and Mumby 2017; Vandewalle et al. 2018; Wadhwa et al. 2019).

The construction of a GR dimerization-deficient mutant mice in which the ability of GR to form homodimers and bind DNA has provided insights into glucocorticoid actions. These mice were initially thought to show a complete separation of GR DNA binding (transactivation) from transrepression since dexamethasone suppressed AP-1- and NF-κB-mediated inflammation with a reduced ability to induce cortisol suppression and T-cell apoptosis (Adcock and Mumby 2017). These early data indicated that the development of glucocorticoids with a greater therapeutic window is possible if DNA binding was responsible for the detrimental side effects of glucocorticoids. However, the separation of transactivation from transrepression in these mice is not complete and that these mice also suffer glucocorticoid-induced side effects (Rogliani et al. 2020; Ronchetti et al. 2018; Van Moortel et al. 2020).

Nongenomic Actions of Glucocorticoids

The GR may also act rapidly within minutes through nongenomic actions that do not require transcription or protein synthesis. These actions include glucocorticoid effects on membrane lipids, changing their physicochemical properties and the presence of a membrane-bound GR, either related or unrelated to the classical GR (Panettieri et al. 2019). A recent report has indicated that one of these unrelated receptors may be the adhesion G-protein-coupled receptor G3 (ADGRG3; also known as GPR97) (Ping et al. 2021). Glucocorticoids have very rapid effects on intracellular calcium ([Ca2+]i) levels, but whether calcium levels are up- or downregulated depend upon the target cell type. For example, short-term (<20 min) treatment of airway smooth muscle (ASM) cells with dexamethasone reduced contraction and enhanced the bronchodilator effects of β_2-agonists. This effect may be a result of nonspecific interactions of dexamethasone with the cell membrane (Panettieri et al. 2019). Furthermore, the effect of dexamethasone on phosphorylation of the cell surface caveolin-1 protein also occurs rapidly (within 2 min), but in this case, it probably reflects the existence of a membrane-localized GR (Panettieri et al. 2019; Vandewalle et al. 2018).

An additional nongenomic action of GR results from GR translocation into mitochondria where it can rapidly regulate cellular metabolism and apoptosis via direct effects on mitochondrial membrane potential and the release of proapoptotic factors such as cytochrome C for example. These effects of mitochondrial GR probably vary according to cell type with the proapoptotic action of glucocorticoids being more important in the treatment of lymphoproliferative disorders (Kokkinopoulou and Moutsatsou 2021).

Clinical Use (Including Side Effects)

A wide variety of immune and inflammatory diseases have been treated with glucocorticoids for the past 70 years since their beneficial effects were first discovered in the treatment of rheumatoid arthritis. In asthma, glucocorticoids have been the mainstay of therapy since the recognition of the critical role of airway inflammation in driving the disease even in patients with mild asthma. Indeed, inhaled glucocorticoids are the first-line anti-inflammatory treatment in adults and children with persistent asthma, the most common chronic inflammatory disease of the airway (Rogliani et al. 2020; Ronchetti et al. 2018; Vandewalle et al. 2018; Wadhwa et al. 2019).

Inhaled glucocorticoids have profound suppressive effects on most airway cells particularly on infiltrating mast cells, macrophages, and T-lymphocytes. Glucocorticoids drive differentiation of monocytes/macrophages toward an anti-inflammatory phenotype and regulate granulocyte trafficking. They reduce the expression of selectins on neutrophils, and of integrin receptors on endothelial cells, to prevent extravasation. This effect is amplified by the ability of glucocorticoids to upregulate annexin-1 (Anxa1), thereby reducing migration but counteracted by the fact that glucocorticoids protect them from apoptosis, which helps the fight against infections. In contrast, eosinophils and basophils undergo apoptosis in response to glucocorticoids (Rogliani et al.

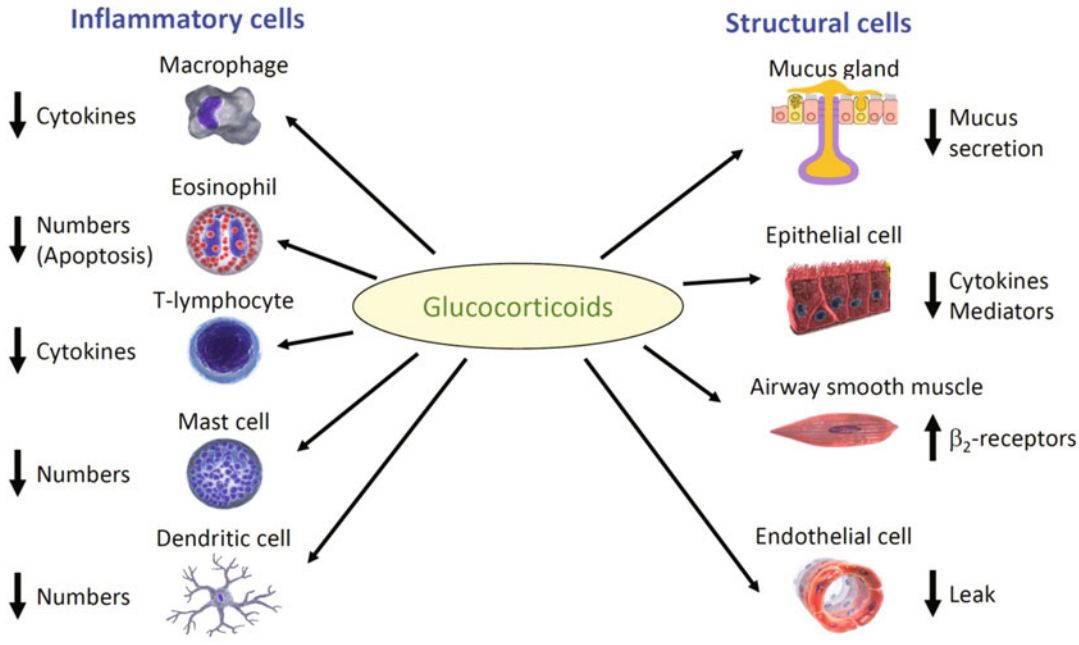
2020; Ronchetti et al. 2018; Vandewalle et al. 2018; Wadhwa et al. 2019).

Furthermore, glucocorticoids have important effects on airway remodeling by reversing the shedding of epithelial cells and the goblet-cell hyperplasia and reducing airway smooth hypertrophy, which are characteristic features of asthma (Fig. 2). Inhaled glucocorticoids also reduce airway hyperresponsiveness in asthmatic adults and children probably indirectly by reducing airway inflammation (Adcock and Mumby 2017; Rogliani et al. 2020; Ronchetti et al. 2018; Vandewalle et al. 2018; Wadhwa et al. 2019).

Combination Therapy in Asthma

Asthma guidelines indicate that rather than increasing the dose of inhaled glucocorticoid when a subject loses control of their asthma, the addition of a long-acting β_2-agonist should be considered. Indeed, the new Global Initiative for Asthma (GINA) guidelines (https://ginasthma.org/) suggest that a combination of inhaled glucocorticoid with a fast-acting, long-duration β2-agonist should be preferentially used as needed for mild and moderate asthmatics. The rationale being that (i) long-acting β_2-agonists can enhance GR activation and inflammatory gene suppression without affecting the side-effect profile and that (ii) inhaled glucocorticoids can prevent downregulation of β_2-agonist efficacy. Thus, a fast-acting, long-duration β_2-agonists in combination with an inhaled glucocorticoid will provide immediate relief when a patient feels that they require additional help while delivering a sufficient anti-inflammatory dose to control further deterioration in disease (Adcock and Mumby 2017; Rogliani et al. 2020; Ronchetti et al. 2018; Vandewalle et al. 2018; Wadhwa et al. 2019).

Glucocorticoids, particularly when delivered in combination with a fast-acting, long-duration β_2-agonist, are extremely effective in controlling asthma. They are similarly effective in many other chronic immune or inflammatory diseases. However, a small proportion of patients with a



Glucocorticoids, Fig. 2 Cellular effect of glucocorticoids. Glucocorticoids can affect the activation of most resident and infiltrating cells with the airway suppressing either cell number or mediator release or both. In addition, glucocorticoids are able to decrease vascular permeability (leak) within the airways that causes edema and increases the expression of β_2-receptors in smooth muscle cells

chronic immune or inflammatory disease including asthma do not respond well to high doses of topical or even systemic or oral glucocorticoids. A relative insensitivity or resistance to the beneficial effects of glucocorticoid therapy is across most chronic inflammatory and immune diseases such as inflammatory bowel disease, atopic dermatitis, and rheumatoid arthritis. These patients with a relative glucocorticoid insensitivity constitute ~5% of severe asthmatics, with greater numbers in other diseases, and present considerable management problems. Absolute glucocorticoid resistance is extremely rare and often reflects genetic mutations in GR and is associated with an abnormal hypothalamic-pituitary-adrenal (HPA) axis (Adcock and Mumby 2017; Rogliani et al. 2020; Ronchetti et al. 2018; Vandewalle et al. 2018; Wadhwa et al. 2019).

However, relative insensitivity is part of a spectrum with asthmatic patients with relative resistance seen in patients who require high doses of inhaled and oral glucocorticoids (glucocorticoid-dependent asthma). A number of mechanisms have been proposed to account for this relative glucocorticoid insensitivity including defective GR nuclear translation and/or association with transcriptional cofactors or repressors, elevated inflammation driven by p38 MAPK, STAT, IL-17, and AP-1 pathways, oxidative stress, and infections. Recently, the expression of a long non-coding RNA (lncRNA) GAS5, which binds to the DNA-binding domain of GR, was found to be upregulated was found to be upregulated in patients with severe asthma as well as children with glucocorticoid-resistant inflammatory bowel disease (Adcock and Mumby 2017; Rogliani et al. 2020; Ronchetti et al. 2018; Vandewalle et al. 2018; Wadhwa et al. 2019).

The detrimental side effects seen with high-dose glucocorticoid therapy prevent the use of ever- increasing doses of glucocorticoids in these subjects. However, the advent of antibodies directed against Th2 targets such as IL-5 and IL-4Rα is providing an alternative therapeutic approach for some of these patients with T2-high asthma, but no similar treatments are available in severe asthmatics whose disease is not driven by T2 mechanisms.

Glucocorticoid-Induced Adverse Effects

Topical or inhaled glucocorticoids in the case of asthma have few side effects and these depend upon the dose, the frequency of administration, and the delivery system used. The most common side-effects, which affects one-third of asthmatics on inhaled glucocorticoids, is dysphonia or hoarseness. Oropharyngeal candidiasis or thrush is seen in some elderly patients when the drug is taken more often than twice daily. There were initial concerns that children taking inhaled glucocorticoids were at risk of growth suppression or stunting, but this has not proved to be an issue even in children treated with higher doses of inhaled glucocorticoids for a long period since they "catch up" by the age of 18. Skin thinning, telangiectasia, and bruising are classic side effects of glucocorticoids, and elderly asthmatic patients on inhaled glucocorticoids are more susceptible to skin bruising. In addition, long-term therapy of COPD patients with inhaled glucocorticoids may increase the susceptibility to opportunistic infections (Adcock and Mumby 2017; Rogliani et al. 2020; Ronchetti et al. 2018; Vandewalle et al. 2018; Wadhwa et al. 2019).

The use of oral glucocorticoids in the treatment of severe asthmatics, however, presents a far greater risk of adverse side effects. Of major importance is the hypothalamic-pituitary-adrenal axis, which is suppressed by glucocorticoids resulting in reduced release of cortisol by the adrenal glands. The impact on the adrenocorticotrophic hormone (ACTH) system gives rise to profound withdrawal symptoms after discontinuation and is a major clinical problem with patients on high-dose oral or systemic glucocorticoids. The degree of suppression depends upon the dose, duration, frequency, and timing of glucocorticoid administration. Prolonged treatment with 5 mg prednisone equivalent per day is generally linked with a low risk of adverse effects, while >10 mg/day is associated with a high frequency of adverse glucocorticoid side effects (Adcock and Mumby 2017; Bourdin et al. 2020).

Oral glucocorticoid therapy also causes osteoporosis with an increased risk of vertebral and rib fractures, particularly in the elderly, and results from effects of glucocorticoids on

phospho-calcium metabolism and a subsequent reduction in bone mineral density and mass. However, there is no evidence that long-term treatment with inhaled glucocorticoids increases the risk of bone fractures. Mechanistically, glucocorticoids act to impair osteoblast proliferation and reduce their ability to produce bone matrix proteins, while high doses cause osteocyte apoptosis. In GRdim/dim mice, skeletal side effects of glucocorticoids remain suggesting that these events are mediated through glucocorticoid receptor transrepression. Long-term glucocorticoid use is also associated with reduced muscle mass and strength that contributes to the increased numbers of falls seen in these patients (Adcock and Mumby 2017; Bourdin et al. 2020).

Furthermore, high-dose oral steroids affect the growth hormone system leading to growth impairment as well as having a major impact on behavioral changes in children. Glucocorticoid-induced diabetes and glucose fluctuations occur in patients due to effects on carbohydrate metabolism leading to insulin resistance. In addition, long-term treatment with oral glucocorticoids increases the risk of posterior subcapsular cataracts; this may be a problem in some patients taking inhaled glucocorticoids (Adcock and Mumby 2017; Bourdin et al. 2020).

Approaches for Reducing Glucocorticoid-Induced Side Effects

Due to the presence of adverse side effects observed particularly with oral glucocorticoids and occasionally in patients on high doses of inhaled glucocorticoids with a high degree of systemic absorption, researchers have strived to obtain safer glucocorticoids for inhalation and oral administration. As described above, a major mechanism of the anti-inflammatory effect of glucocorticoids is *trans*repression, while many endocrine and metabolic effects of glucocorticoids are mediated by *trans*activation. This has resulted in a search for novel glucocorticoids that selectively *trans*repress without significant *trans*activation, thereby reducing the risk of systemic side effects (Wadhwa et al. 2019).

Several compounds with either a steroidal or nonsteroidal structure have been developed that bind to GR which possess dissociated properties although none have reached the clinic (Van Moortel et al. 2020). In a Phase II study, 12 weeks treatment of rheumatoid arthritis patients with fosdagrocorat attenuated disease activity to a similar extent as prednisolone (10 mg daily) but with reduced impairment of glycemic control, a side effect known to be driven by transactivation (Buttgereit et al. 2019). In addition, a recent chemical systems biology approach has been used to generate GR ligands with distinct muscle-sparing effects and reduced mitochondrial potential while retaining an anti-inflammatory function both in vitro and in vivo, although these need to be tested in humans (Bruno et al. 2021). Of interest, deflazacort, a glucocorticoid developed in 1986, appears to possess dissociated qualities in patients with a number of chronic inflammatory diseases since it has a similar anti-inflammatory profile as prednisolone and hydrocortisone but with a much reduced impact on the ACTH/HPA axis, osteoporosis, glycaemia, and growth impairment in children. No studies have been performed to address the molecular mechanisms of deflazacort actions in key cells to determine the reasons for these effects (Joseph et al. 2019). Further studies are needed with these and similar steroids in patients with severe asthma who currently require high-dose oral glucocorticoid therapy.

Other approaches that have been used to reduce glucocorticoid side effects are linked rro the glucocorticoid ligand to an antibody or drug to provide targeted delivery to a specific cell type using, for example, anti-CD163 to target activated macrophages or glucagon-like peptide-1 (GLP1). The latter being developed for metabolic disease (Uchinuma et al. 2020). Encapsulation of glucocorticoids within liposomes has also been proposed for targeted delivery to inflamed tissues in rheumatoid arthritis, Crohn's disease, and atherosclerosis (Vandewalle et al. 2018).

Despite the huge amount of work undertaken in examining the molecular mechanisms of glucocorticoids, there are still major gaps that need to be addressed with respect to mechanisms in cells and tissues from patients with disease. Currently, the data suggest that there is the potential to

develop novel nonsteroidal anti-inflammatory treatments, which mimic the anti-inflammatory actions of glucocorticoids independent of GR (Panettieri et al. 2019; Vandewalle et al. 2018). It has proved difficult to develop effective inhibitors of NF-κB or to specific HATs that are linked to NF-κB activation, but selective bromodomain mimics such as RVX000222 have been produced that show benefit in phase II clinical studies in chronic kidney disease, cardiovascular disease, and chronic metabolic diseases (Wadhwa et al. 2019). Glucocorticoids target NF-κB, and this is an obvious target as a novel anti-inflammatory approach. Initial studies using small molecule inhibitors of IkB kinase-2 (IKK2), which is an upstream kinase that activates NF-κB, were developed but failed to take into consideration the complex feedback control processes that regulate IKK2. Other approaches that target NF-κB such as proteasome inhibitors have some success in clinical trials (Adcock and Mumby 2017; Taniguchi and Karin 2018). Other treatments that may have therapeutic potential as glucocorticoid-sparing agents include kinase inhibitors such as those directed against p38 MAP kinase and Janus kinases (Wadhwa et al. 2019). The clinical benefit of monoclonal antibody therapy in selected patients with severe asthma highlights the potential for novel therapeutic agents when the disease driver mechanisms have been elucidated and a good biomarker developed (Chung and Adcock 2019).

Cross-References

► Inflammation

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Glucose Metabolism

► Ghrelin, Physiological Roles and Clinical Relevance of

Glucose-Lowering Drugs Other than Insulin

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Synonyms

Antihyperglycaemics; Blood glucose-lowering drugs; Hypoglycaemic agents; Insulin secretagogues

Definition

This section is focused on glucose-lowering drugs other than insulin that are used in the treatment of hyperglycemia in type 2 (non-insulin-dependent) diabetes mellitus and occasionally as adjuncts to insulin in the treatment of type 1 (insulin-dependent) diabetes mellitus. They are used in conjunction with non-pharmacological interventions involving diet, exercise, and health education. Insulin therapy is essential for all type 1 patients and is often used to treat more advanced stages of type 2 diabetes. Type 2 diabetes accounts for more than 85% of all cases of diabetes. The classes of glucose-lowering drugs other than insulin are the biguanide metformin, sulphonylureas, prandial insulin releasers (also termed meglitinides), dipeptidyl peptidase-4 (DPP-4) inhibitors (also termed gliptins), glucagon-like peptide-1 receptor agonists (GLP-1RAs), sodium-glucose co-transporter-2 (SGLT-2) inhibitors, thiazolidinediones (TZDs), and α-glucosidase inhibitors. Pramlintide, bromocriptine, and colesevelam are also approved in some countries for glucose-lowering in type 2 diabetes (Table 1) (Bailey 2015; Tahrani et al. 2016).

Mechanism of Action

Type 2 diabetes is a heterogeneous and progressive endocrine disorder involving hyperglycemia and widespread metabolic disturbances. Key pathogenic factors are insulin resistance (impaired insulin action) and defective function of the insulin-secreting β-cells in the pancreatic islets of Langerhans. Obesity aggravates the pathogenic process alongside many other contributing factors including excess glucagon secretion, an impaired incretin effect, and abnormalities of the gluco-regulatory functions of adipose tissue, liver, kidney, and the central nervous system (DeFronzo et al. 2015). Hence a variety of differently acting glucose-lowering agents has emerged with capability to address different pathogenic factors. The present classes of glucose-lowering medicines other than insulin offer a variety of actions that can improve the effects of insulin, increase insulin secretion, suppress glucagon secretion, mimic incretin hormones, increase urinary glucose elimination, slow the rate of intestinal carbohydrate digestion, or slow gastric emptying.

Biguanide

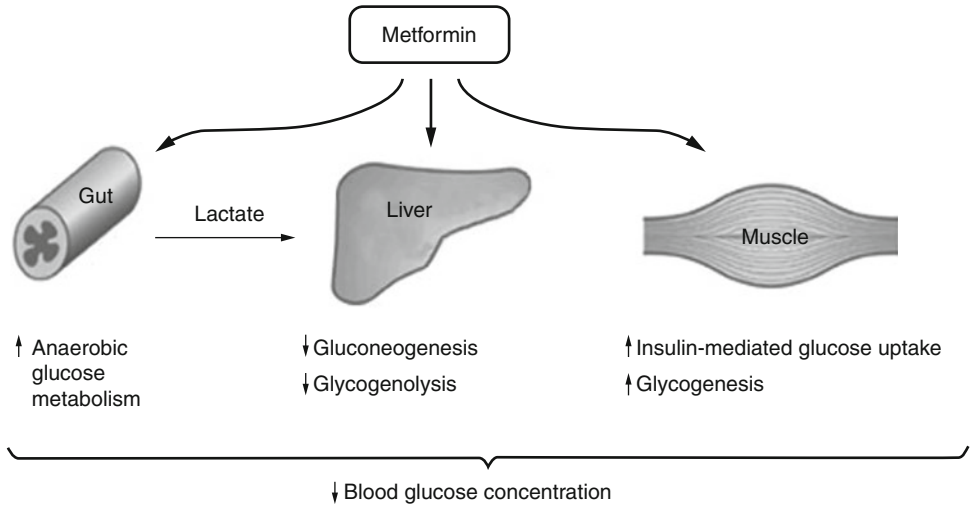
Metformin is now the only compound in this class in most countries: other biguanides, namely, phenformin and buformin have been discontinued.

Glucose-Lowering Drugs Other than Insulin, Table 1 Classes of glucose-lowering drugs other than insulin and their main mechanisms of action

Class	Examples[a]	Main mechanism of action	Route
Biguanide	Metformin	Counter insulin resistance	Oral
Sulphonylureas	Chlorpropamide, glibenclamide[b] gliclazide, glimepiride glipizide tolbutamide	Stimulate insulin secretion (typically 6–24 h)	Oral
Prandial insulin releasers (meglitinides)	Repaglinide, nateglinide	Stimulate insulin secretion (rapid and short acting <6 h)	Oral
DPP-4 inhibitors (gliptins)	Sitagliptin Vildagliptin Saxagliptin Linagliptin Alogliptin	Inhibit DPP-4[c]: enhance prandial insulin secretion	Oral
Glucagon-like peptide-1 receptor agonists (GLP-1RAs)	Exenatide Liraglutide Lixisenatide, Dulaglutide Semaglutide	Mimic GLP-1[d]: enhance prandial insulin secretion	SC injection[e]
Sodium-glucose cotransporter-2 inhibitors (SGLT-2 inhibitors)	Canagliflozin Dapagliflozin Empagliflozin Ertugliflozin Sotagliflozin[f]	Increase glucose elimination in the urine	Oral
Thiazolidinediones (TZDs)	Pioglitazone, Rosiglitazone[g]	Improve insulin action via PPARγ agonism[h]	Oral
α-Glucosidase inhibitors	Acarbose Miglitol, Voglibose	Slow rate of carbohydrate digestion	Oral
Amylin analogue	Pramlintide	Suppress glucagon secretion and slow gastric emptying	SC injection[e]
Dopamine agonist	Bromocriptine	Improve circadian glycemic control	Oral
Bile sequestrant	Colesevelam	Alter release of incretin hormones	Oral

[a]Availability of agents and prescribing information vary between countries
[b]Glibenclamide is called glyburide in some countries
[c]Dipeptidyl peptidase-4
[d]Glucagon-like peptide-1
[e]Subcutaneous injection
[f]Sotagliflozin is a dual inhibitor of SGLT-2 and SGLT-1
[g]Rosiglitazone has been withdrawn in many countries
[h]PPARγ, peroxisome proliferator-activated receptor-γ

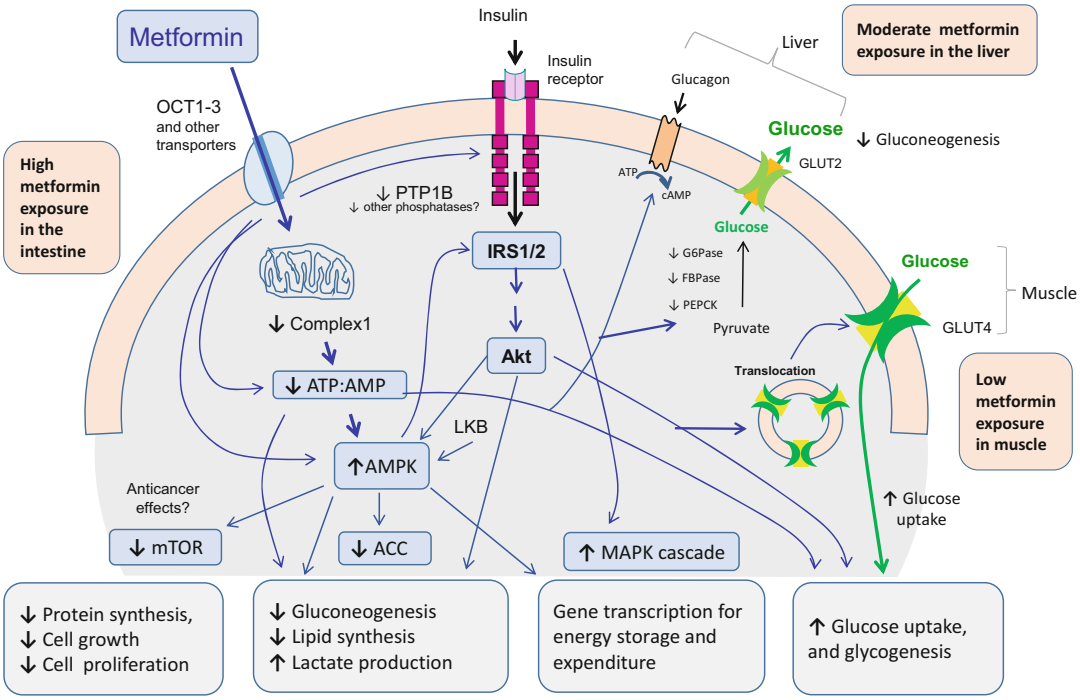
Metformin was introduced in the late 1950s and is now the preferred initial pharmacological glucose-lowering therapy in most guidelines for the treatment of hyperglycemia in type 2 diabetes. This reflects its antihyperglycemic efficacy without weight gain, negligible risk of hypoglycemia, reduced long-term cardiovascular risk, low cost, and a mode of action that counters insulin resistance. The metabolic effects of metformin are produced by effects on the intestine, liver, and muscle (Fig. 1). At a cellular level, metformin exerts several different effects depending on the drug concentration within the tissue and the metabolic pathways operative within that tissue. Some of these effects are independent of insulin, and some are dependent on insulin, but the blood glucose-lowering efficacy of metformin requires a presence of insulin. The drug does not stimulate insulin release and is often associated with a small decrease in basal insulin concentrations in hyperinsulinemic patients (Metformin Special Issue 2017).



Glucose-Lowering Drugs Other than Insulin, Fig. 1 The glucose-lowering effect of metformin involves increased anaerobic glucose metabolism by the intestine, suppression of glucose production by the liver and increased glucose uptake by muscle. ↑, increase; ↓ decrease

Metformin enters cells via organic cation transporters (OCT1-3), the plasma membrane monoamine transporter (PMAT), and other transporters (Fig. 2). Very high concentrations of metformin that accumulate in enterocytes increase anaerobic glucose metabolism by suppression of the respiratory chain at complex I, reducing ATP availability and giving rise to increased lactate production. This lactate is recycled to glucose by the liver, thus increasing glucose turnover. Metformin can also interfere with mitochondrial function by reducing the mitochondrial glycerophosphate dehydrogenase (mGPD) shuttle. Depletion of ATP raises ADP and AMP and activates AMP-activated protein kinase (AMPK): metformin can also enhance LKB-mediated activation of AMPK. Increased activity of AMPK decreases the activity of acetyl-CoA carboxylase, reducing lipogenesis and allowing increased fatty acid oxidation. Activation of AMPK also inhibits the mammalian target of rapamycin mTOR, and high concentrations of metformin may additionally exert a direct inhibitory effect on mTOR. Suppression of mIOR reduces cell growth and division and could contribute to reduced neoplasm in the intestine. It is likely that high concentrations of metformin in the intestinal lumen alter the microbiome, but such effects are unclear at this time. There is also evidence that metformin can increase secretion of glucagon-like peptide-1 and peptide YY from enteroendocrine L-cells.

Metformin reduces hepatic gluconeogenesis and glycogenolysis. The concentration (and accordingly the activity) of metformin achieved in the liver is less than in enterocytes. Gluconeogenesis is reduced through effects on three key enzymes: raised AMP in hepatocytes reduces the activity of fructose-1,6-bisphosphatase, while increased activity of AMPK reduces expression of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase. Metformin also appears to reduce the effectiveness of glucagon in the liver, possibly related to a decrease in the ATP:AMP ratio, reducing glucagon-stimulated adenylate cyclase activity. In consequence, reduced cAMP could further reduce expression of gluconeogenic enzymes. It is noted that the collective effect of these mechanisms produces only a modest reduction of gluconeogenesis (typically 10–30%) which is not sufficient to allow interprandial blood glucose concentrations to fall into hypoglycemia. Metformin has been shown to acutely reduce hepatic glucose output, and there is some evidence that this involves a decrease in the rate of glucagon-induced glycogenolysis. Metformin has also been reported to promote some



Glucose-Lowering Drugs Other than Insulin, Fig. 2 The cellular mechanisms of action of metformin vary with drug exposure and metabolic pathways in individual tissues. Metformin reduces ATP production by suppression of the mitochondrial respiratory chain at complex 1, increases activity of AMPK, reduces activity of mTOR, and alters the expression and activity of key enzymes affecting hepatic glucose production and glucose uptake by muscle. ↑, increase; ↓ decrease. ACC, acetyl-CoA carboxylase; Akt, protein kinase B; AMPK, adenosine monophosphate-activated protein kinase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; FBPase, fructose-1,6-bisphosphatase; G6Pase, glucose-6-phosphatase; GLUT, glucose transporter isoform; GPD, glycerophosphate dehydrogenase; IRS, insulin receptor substrate; IR-TKA, insulin receptor tyrosine kinase activity; LKB, serine/threonine kinase 11; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; OCT, organic cation transporter; PEPCK, phosphoenolpyruvate carboxykinase; PTP-1B, protein tyrosine phosphatase-1B

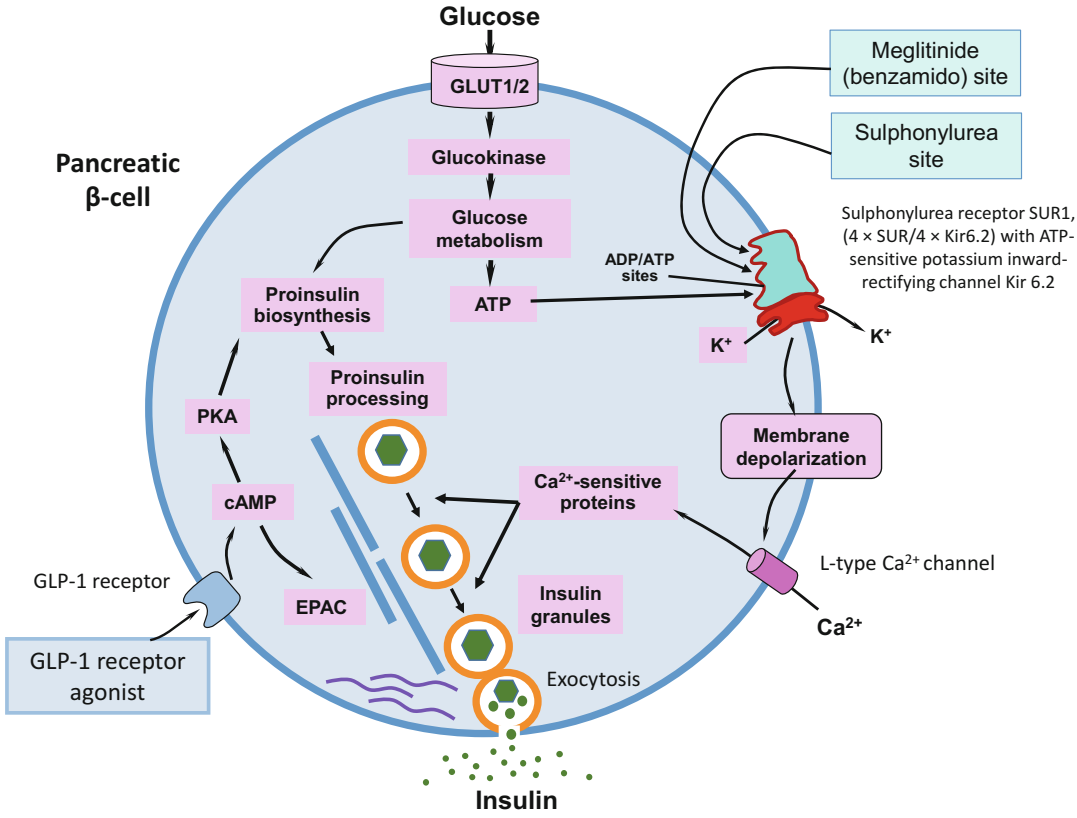
actions of insulin in the liver, partly by reducing the activity of protein-tyrosine phosphatases that dephosphorylate (and so deactivate) the tyrosine kinase activity of the insulin receptor. Increased activity of several post-receptor steps of insulin action has been associated with metformin exposure to the liver, including increased activation of insulin receptor substrates and downstream signaling intermediates, but mechanisms have not been determined.

In muscle, metformin increases glucose uptake by enhancing translocation of insulin-sensitive glucose transporters (GLUT4) into the plasma membrane partly via pathways involving AMPK, c-Jun N-terminal kinase, and Akt. Concentrations of metformin in the muscle, which are lower than in liver, do not preclude an increase in glucose oxidation and promote glycogenesis.

Further effects of metformin have been reported, although their cellular and molecular mechanisms have not been fully delineated. These include reduced platelet aggregation, altered fibrin that assists clot dispersal, increased endothelium-mediated vasodilatation, reduced atherogenesis, improved cardiac contractility, reduced neoplasms (see comments on mTOR), reduced androgen production in polycystic ovarian syndrome, and general antiaging effects.

Sulphonylureas

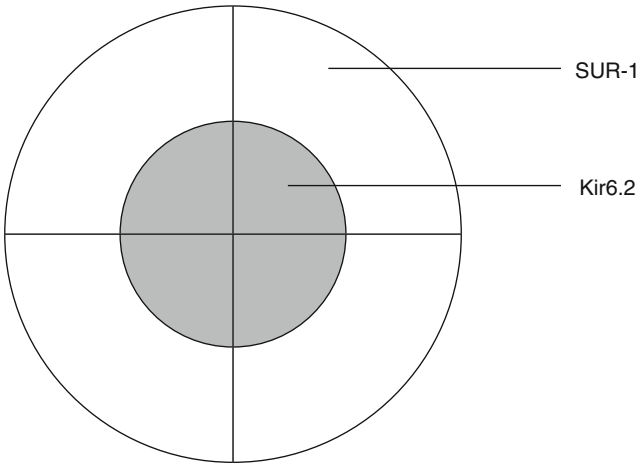
The first sulphonylureas were introduced in the 1950s (Lebovitz and Melander 2015). They



Glucose-Lowering Drugs Other than Insulin, Fig. 3 Sulphonylureas stimulate insulin release by pancreatic β-cells. They bind to the sulphonylurea receptor-1 (SUR-1), which closes the Kir6.2 (ATP-sensitive) potassium channel. This promotes local depolarization, voltage-dependent calcium influx, and activation of calcium-sensitive proteins that control exocytotic release of insulin. EPAC, exchange protein activated by cAMP; GLP-1, glucagon-like peptide-1; GLUT, glucose transporter isoform; PKA, cAMP-dependent protein kinase A

stimulate insulin secretion by a direct effect on pancreatic β-cells. The insulin then increases glucose uptake and metabolism by muscle and reduces glucose output by the liver (insulin, ► insulin receptor). Sulphonylureas enter the β-cell and bind to a site at the cytosolic face of the sulphonylurea receptor-1 (SUR-1). SUR-1 forms part of a transmembranal complex that makes up the ATP-sensitive Kir6.2 potassium efflux channel (K-ATP channel). The binding of a sulphonylurea to SUR-1 produces a conformational change that closes the K-ATP channel, favoring local depolarization of the plasma membrane. This opens local voltage-dependent L-type calcium channels, which increase calcium influx and raise the cytosolic free calcium concentration. In turn, this activates calcium-dependent signaling proteins controlling the contractile activities of microtubules and microfilaments that mediate exocytosis of insulin granules. Preformed insulin granules adjacent to the plasma membrane are released first (first-phase insulin release). Newly formed granules contribute to the secretory pool within 1 h of continued stimulation. Increased insulin release is sustained as long as drug stimulation is maintained, provided the β-cells are functionally competent (Fig. 3).

The SUR–Kir6.2 complex is a non-covalently bonded octamer (4 × SUR/4 × Kir6.2), with a central pore-forming Kir6.2 channel (Fig. 4). SUR molecules are members of the ATP-binding cassette proteins (ABC proteins). Each SUR-1 molecule comprises 17 transmembrane domains, 2 cytosolic nucleotide-binding domains, and



Glucose-Lowering Drugs Other than Insulin, Fig. 4 Octameric structure (4 × SUR/4 × Kir6.2) of the SUR–Kir6.2 complex

cytosolic-binding domains for a sulphonylurea, a benzamido compound, and other ligands. The Kir6.2 channel also has cytosolic binding regions, including one for ADP/ATP. Sulphonylureas bind to the sulphonylurea site with high affinity (e.g., *Ki* for glibenclamide in the low nanomolar range), being dependent on a "U" shape to the ligand with 5.5 Å between the hydrophobic rings.

By closing K-ATP channels, sulphonylureas induce insulin release by activating a step along the normal pathway of glucose-induced insulin secretion. Activation of insulin secretion is therefore independent of glucose, provided there is sufficient glucose metabolism to stimulate proinsulin biosynthesis and service the energy requirements for the cellular processing and exocytosis of insulin. Hence sulphonylureas can stimulate insulin secretion at low glucose concentrations, creating the risk of hypoglycemia. Sulphonylureas will also increase the amount of insulin secreted at any level of stimulation by glucose, subject to adequate β-cell function. Additionally, sulphonylureas may potentiate insulin release that is stimulated by glucose and other nutrients. This may involve SUR molecules located within the membranes of insulin granules and activation of certain isoforms of protein kinase C.

Although the main therapeutic effect of sulphonylureas is increased insulin secretion, there are reports that these drugs exert weak extra-pancreatic effects. The latter effects include suppression of hepatic gluconeogenesis, possibly by suppression of a kinase which leads to increased formation of fructose-2, 6-bisphosphate. This stimulates phosphofructokinase and suppresses fructose-1, 6-bisphosphatase, thereby increasing glycolytic flux and suppressing gluconeogenic flux. Sulphonylureas might also enhance insulin-stimulated glucose transport by increasing translocation of GLUT4 glucose transporters to the plasma membrane in adipocytes and muscle. However, these effects appear to require supra-therapeutic concentrations of sulphonylureas and are probably not therapeutically relevant. Sulphonylureas have been reported to reduce the hepatic extraction of insulin and to act on pancreatic α-cells to transiently stimulate and then suppress glucagon secretion.

Prandial Insulin Releasers (Meglitinides)

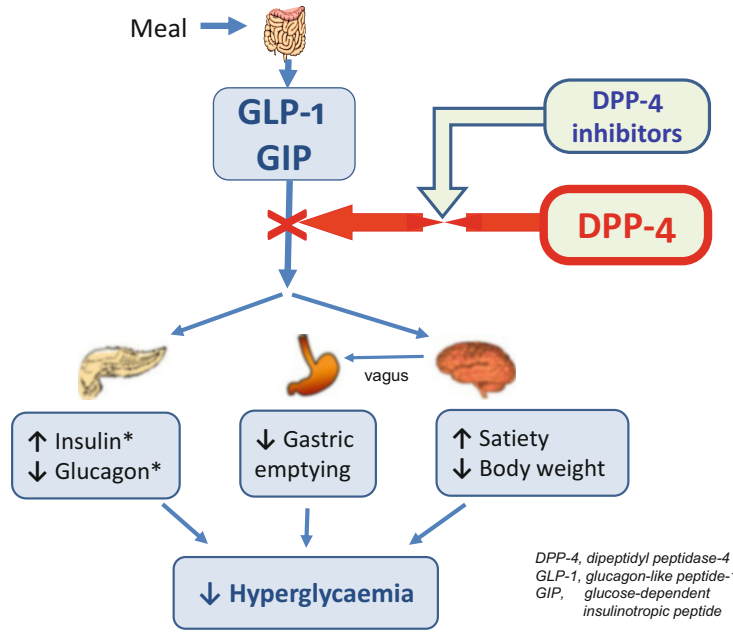
This class comprises the meglitinide analogue repaglinide (introduced in 1998) and the structurally related D-phenylalanine analogue nateglinide (introduced in 2001) (Lebovitz and Melander 2015). These agents have a benzamido group that binds to a site on SUR-1 that is distinct from the sulphonylurea site but is probably in close proximity and capable of binding interference. Some sulphonylureas also have a benzamido moiety (e.g., glibenclamide, glimepiride, glipizide), but the binding affinity for the sulphonylurea site has a higher affinity. Binding of repaglinide or nateglinide to the benzamido site closes the K-ATP channel and induces insulin secretion via the same pathway described for sulphonylureas (Fig. 3).

Repaglinide and nateglinide are rapidly absorbed; their binding durations to SUR-1 are much shorter than sulphonylurea binding, and their hepatic metabolism and subsequent elimination are faster. Consequently, repaglinide and nateglinide are faster-acting and shorter-acting insulin releasers than sulphonylureas. They can be taken immediately before a meal and quickly stimulate insulin secretion to coincide approximately with the period of meal digestion, hence their categorization as "prandial insulin releasers."

Dipeptidyl Peptidase-4 Inhibitors

Dipeptidyl peptidase-4 (DPP-4) inhibitors (also termed gliptins) selectively inhibit the cell surface and extracellular enzyme DPP-4 (EC 3.4.14.5) which inactivates the incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP, also termed gastric inhibitory peptide). GLP-1 (from enteroendocrine L-cells) and GIP (from enteroendocine K-cells) are normally released in response to a meal and act on the pancreatic islets directly and via neural pathways to potentiate nutrient-induced insulin secretion. GLP-1 also reduces glucagon secretion in a glucose-dependent manner. Accordingly, DPP-4 inhibitors enhance the incretin effect by slowing the degradation of endogenous GLP-1 and GIP, thereby enhancing the potentiation of nutrient-induced insulin secretion and further suppressing glucagon secretion. These effects lower blood glucose concentrations, especially after a meal (Fig. 5). The first DPP-4 inhibitor, sitagliptin, became available in 2007 (Deacon 2019).

DPP-4 cleaves the dipeptides X-alanine or X-proline from the N-terminus. In addition to GLP-1 and GIP, there are many other circulating substrates for DPP-4 including neuropeptide Y (NPY), peptide YY (PYY), gastrin-releasing polypeptide (GRP), substance P, insulin-like growth factor-1 (IGF-1), vasostatin-1, and several chemokines. Thus, DPP-4 inhibitors have the potential to influence the hunger-satiety system, gastrointestinal motility, growth, vascular reactivity, and immune mechanisms, although reports of any clinically significant adverse effects have been rare. DPP-4 is also the T-cell activation molecule CD26, but its role in immune regulation appears to be unaffected by the DPP-4 inhibitors used in the treatment of type 2 diabetes. DPP-4 is closely related to DPP-8, DPP-9, fibroblast activation protein-α, and quiescent cell proline dipeptidase, but DPP-4 inhibitors are sufficiently



Glucose-Lowering Drugs Other than Insulin, Fig. 5 Dipeptidyl peptidase-4 (DPP-4) inhibitors prevent the enzymatic degradation of incretin hormones GLP-1 and GIP by DPP-4. * Glucose-dependent effects. GLP-1, glucagon-like peptide-1; GIP, glucose-dependent insulinotropic peptide

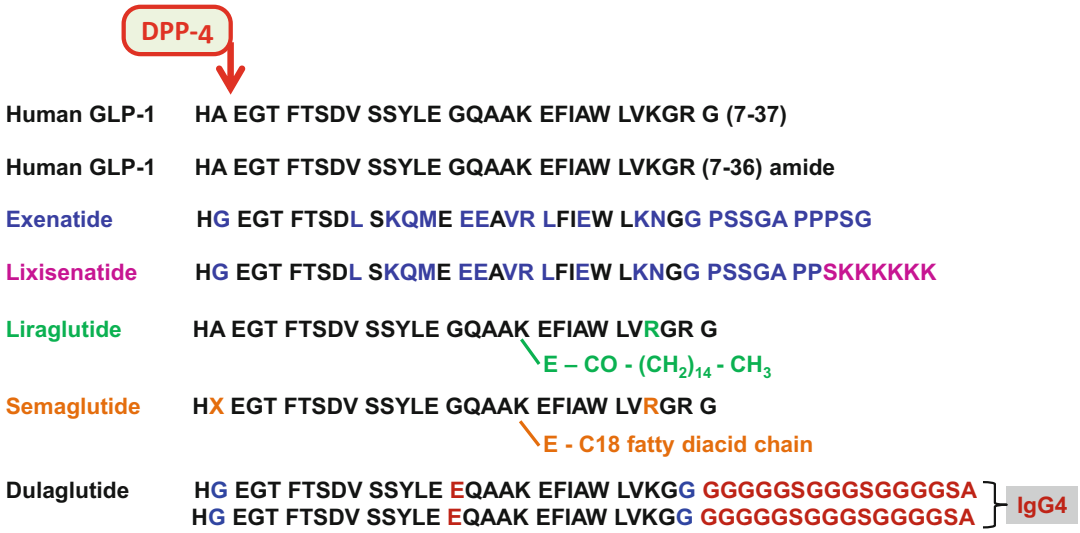
selective for DPP-4 that this has not been problematic for clinical use.

In clinical studies, DPP-4 inhibition has almost completely inhibited the peptidase function of DPP-4 for 12–20 h and has increased active circulating concentrations of endogenous GLP-1 and GIP by twofold to threefold. This was associated with increased glucose-induced insulin secretion and suppression of glucagon secretion, but since incretin hormones are not effective at low glucose concentrations, there is little risk of hypoglycemia. DPP-4 inhibitors do not cause weight gain and may assist modest weight loss, although they are not associated with measurable changes in satiety or gastric emptying as seen with high concentrations of exogenous GLP-1.

Glucagon-like Peptide-1 Receptor Agonists

Glucagon-like peptide-1 receptor agonists (GLP-1RAs), sometimes referred to as incretin mimetics, are analogues of the incretin hormone glucagon-like peptide-1 (GLP-1). Because GLP-1 is rapidly degraded by DPP-4, native GLP-1 is not suitable for routine clinical purposes, so GLP-1RAs have modified amino acid sequences and formulation features that protect against degradation by DPP-4 (Fig. 6). The first GLP-1RA, exenatide, was introduced in 2005. It is a synthetic form of exendin-4, a peptide discovered in the saliva of a lizard from Arizona – the Gila monster (*Heloderma suspectum*) (Drucker 2018).

As peptides, GLP-1RAs are administered by subcutaneous injection, although an oral formulation of one GLP-1RA (semaglutide) is advanced in development. Therapeutic doses of GLP-1RAs typically achieve more than ten times the concentration of active endogenous GLP-1, substantially boosting the normal incretin effect with enhanced potentiation of nutrient-induced insulin secretion and suppression of glucagon secretion. Additionally, GLP-1RAs delay gastric emptying and exert a satiety effect. Collectively these actions reduce prandial blood glucose excursions and improve basal glycemic control with negligible risk of hypoglycemia, and they also assist a reduction in body weight. Although GIP makes an important contribution alongside GLP-1 to generate the normal incretin effect, the insulin-releasing effect of GIP becomes considerably reduced in type 2 diabetes, which has favored use of GLP-1 (rather than GIP) as a therapeutic template. There is also mounting evidence that GLP-1RAs produce beneficial effects on the vasculature to reduce atherosclerotic cardiovascular disease, and preliminary evidence suggests these agents may reduce albuminuria in diabetes patients. Further effects of



Glucose-Lowering Drugs Other than Insulin, Fig. 6 Amino acid sequences of GLP-1 receptor agonists. DPP-4, dipeptidyl peptidase-4; IgG4, immunoglobulin-4; X, aminoisobutyric acid

GLP-1RAs to improve cognitive function and bone mineral density have also been reported.

Most evidence relating to the cellular mechanism of action of GLP-1 and GLP-1RAs derives from their effect on pancreatic β-cells, where they bind to a G protein-coupled receptor. Binding to the receptor brings about a conformational change that activates the $G\alpha_s$ subunit which in turn activates adenylate cyclase to produce cAMP from ATP. The increased cytosolic concentration of cAMP activates cAMP-dependent protein kinase A (PKA) and the exchange protein activated by cAMP (EPAC). PKA improves insulin biosynthesis by increasing the rate of transcription of the insulin gene and decreasing the rate of breakdown of insulin mRNA and may also promote distal steps in the pathway of insulin granule exocytosis. Transcriptional effects that are independent of PKA have also been reported. EPAC activates ryanodine receptors to liberate calcium from intracellular stores, raise cytosolic calcium, and activate early steps in the exocytotic pathway.

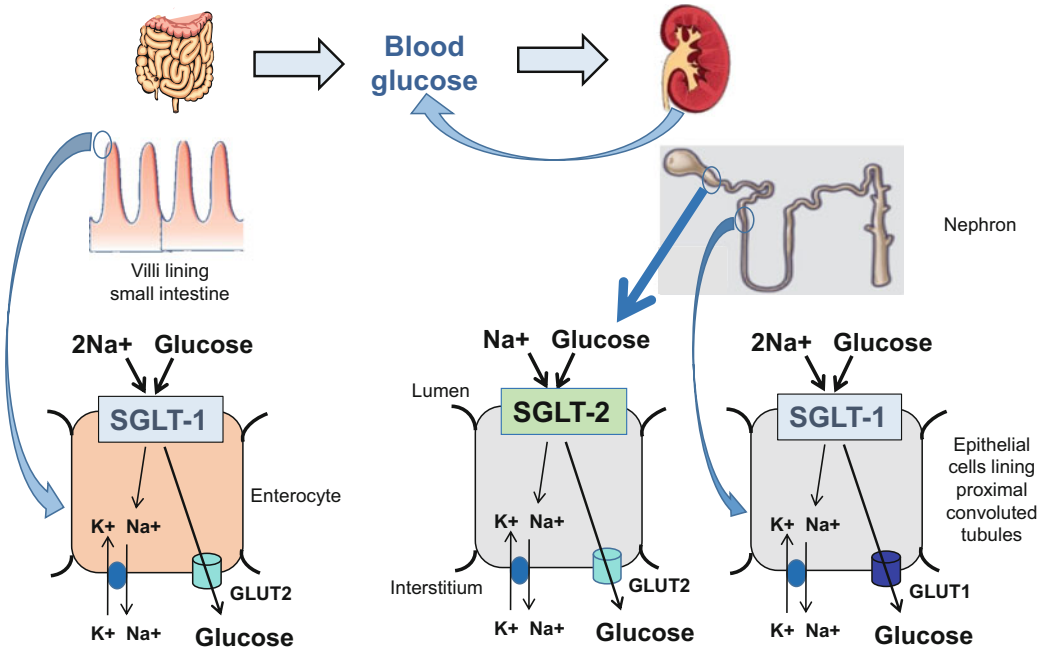
In addition to amplifying nutrient-stimulated insulin release, animal studies and in vitro studies have indicated that GLP-1RAs can increase β-cell mass through increased neogenesis and proliferation, as well as reduced apoptosis, although effects have varied considerably between models. In vitro and animal studies indicate that GLP-1 can initiate neogenesis of β-cells, possibly from exocrine ductal cells and by transdifferentiation of other islet endocrine cells. GLP-1 can also promote proliferation of existing β-cells in part via PKA and PDX-1, but mechanisms are unclear. Moreover, it is uncertain whether GLP-1RAs can instigate these effects to preserve β-cell mass in advancing stages of human type 2 diabetes when β-cell numbers and functional capacity are already significantly compromised.

Mechanisms through which GLP-1RAs suppress glucagon secretion appear to include a direct effect on islet α-cells as well as an inhibitory effect of somatostatin released from islet δ-cells in response to a direct effect of GLP-1RAs and via vago-vagal pathways. Central effects of GLP-1RAs to promote satiety are mediated partly via vagal afferents triggered by GLP-1RAs in the intestinal mucosa and portal circulation. However, some circulating GLP-1RAs may be able to cross the blood-brain barrier. GLP-1 receptors are located in many regions of the brain including the limbic system and lateral hypothalamus, but it has been difficult to distinguish effects mediated by GLP-1 and GLP-1RAs entering from the circulation compared with GLP-1 that is generated by many central neurons.

Sodium-Glucose Cotransporter-2 Inhibitors

Sodium-glucose cotransporter-2 (SGLT-2) inhibitors, sometimes termed flozins, were introduced in 2013 as agents to lower blood glucose by eliminating excess glucose in the urine. The template for these agents was the glucoside phlorizin which was isolated from the bark of apple trees and has long been known to cause glucosuria (Tahrani et al. 2013).

SGLT-2 (SLC5A2) is one of several members of the sodium substrate symporter family. It is located in the apical (luminal) membrane of epithelial cells lining the first segment of the proximal tubules of the kidney where it is responsible for the reuptake of about 90% of the glucose filtered into the tubule at the glomerulus. Glucose passing further along the tubule is taken up by SGLT-1 (SLC5A1) located in apical membranes along the third segment of the proximal tubules. SGLT-2 is a low-affinity high-capacity cotransporter with a 1:1 stoichiometry of sodium to glucose, whereas SGLT-1 is a high-affinity low-capacity cotransporter with a sodium to glucose stoichiometry of 2:1. Both cotransporters are secondary active, being driven by the Na+/K+-ATPase pump in the basolateral membrane. Glucose taken into the tubular cells by these transporters moves out across the basolateral membrane via the facilitative transporters GLUT1 and GLUT2 (Fig. 7). SGLT-2 inhibitors act through competitive inhibition, and although they are highly selective for SGLT-2, some of these agents can also exert a weak inhibitory effect at SGLT-1. Circulating concentrations are insufficient for an effect on renal SGLT-1, but oral dosing of agents with the strongest inhibitory effect on SGLT-1 (sotagliflozin and canagliflozin) can achieve concentrations in the upper small intestine that interfere with glucose absorption by SGLT-1. Deferring glucose absorption to more distal regions of the intestine helps to



Glucose-Lowering Drugs Other than Insulin, Fig. 7 Sodium-glucose cotransporter-2 (SGLT-2) inhibitors act mainly by competitive inhibition of SGLT-2 cotransporters in the renal proximal tubules. Members of the class that can also exert an inhibitory effect on SGLT-1 cotransporters can reduce glucose uptake in the upper intestinal tract

reduce prandial blood glucose excursions and deliver more glucose to ileal L-cells resulting in increased release of GLP-1 and peptide YY, which further assist blood glucose lowering.

With regard to the treatment of type 2 diabetes, a key feature of the mechanism of action of SGLT-2 inhibition is that it is independent of insulin, i.e., it will lower plasma glucose concentrations irrespective of insulin resistance or hypoinsulinemia provided there are adequate basal levels of endogenous insulin to sustain life. Indeed, some SGLT-2 inhibitors can be used as an adjunct to insulin in type 1 diabetes, and their mechanism of action is complementary to that of other glucose-lowering therapies with which they can be combined in the treatment of type 2 diabetes.

In individuals with a good to moderate glomerular filtration rate (eg GFR >60 ml/min/1.73m^2) and substantial hyperglycemia (e.g., an average daily plasma glucose concentration exceeding >150 mg/dl), an SGLT-2 inhibitor can create a glucosuria of 30–100 g glucose/day. The loss of calories associated with the glucosuria is typically associated with weight loss. A reduction in blood pressure is also commonly noted in patients receiving an SGLT-2 inhibitor: this is attributed at least in part to a reduction in plasma volume due to the osmotic diuresis of glucosuria.

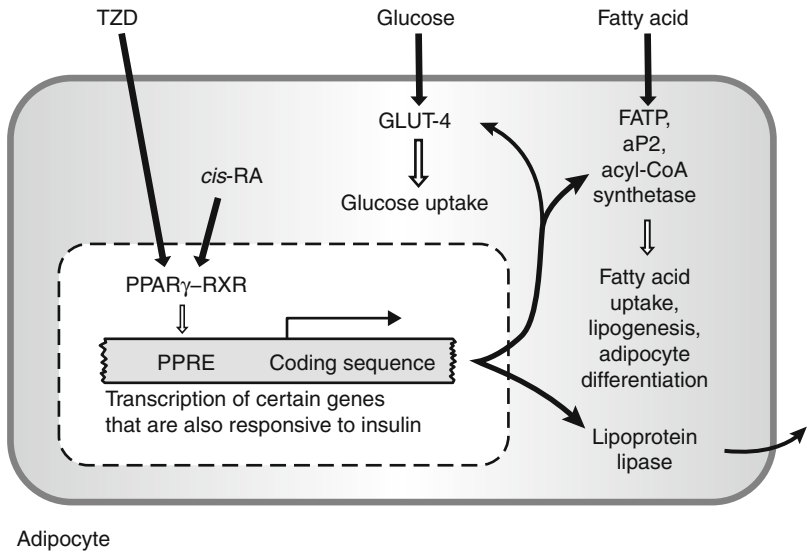
Recent studies have noted that SGLT-2 inhibitors reduce the onset and progression of heart failure which is likely to reflect a lowering of body weight and blood pressure, although several other mechanisms have been indicated that are independent of glucose lowering. These include inhibition of the cardiac sodium-hydrogen exchanger which increases mitochondrial calcium, adjustments to electrolyte balance and hemodynamics, a reduction in uric acid, and increased production of ketones. SGLT-2 inhibition also reduces albuminuria and, in the long-term, slows the rate of decline in GFR. This is explained in part by the retention of additional sodium in the tubules during SGLT-2 inhibition which is sensed by the macula densa to increase juxtaglomerular feedback, constricting afferent

glomerular arterioles and reducing intraglomerular pressure.

Thiazolidinediones

Two thiazolidinediones (TZDs), pioglitazone and rosiglitazone, were introduced in 1999: pioglitazone remains available in most countries, but rosiglitazone was withdrawn in Europe and many other countries in 2010. The use of rosiglitazone was restricted in the USA for 2010–2015, and it has received little use thereafter. Another TZD, troglitazone was briefly introduced and withdrawn. TZDs improve insulin sensitivity, and their principal mechanism of action is stimulation of the nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ). PPARγ (PPARs) is a member of the nuclear receptor superfamily for retinoid, steroid, and thyroid hormones. PPARγ exists as a heterodimer with the retinoid X receptor (RXR). Binding of a TZD to PPARγ together with binding of *cis*-retinoic acid to the RXR moiety produces a conformational change that prompts dissociation of corepressors. The activated heterodimer then binds to the peroxisome proliferator response element (PPRE), which is a sequence (AGGTCAXAGGTCA) located in the promoter region of the responsive genes. Recruitment of co-activators including PGC-1 and assembly of the RNA polymerase complex follows, initiating transcription (Fig. 8). Many of the responsive genes are also activated by insulin or encode for proteins that enhance actions of insulin, hence the ability of TZDs to improve insulin sensitivity (Cariou et al. 2012).

PPARγ is strongly expressed in adipocytes, and stimulation by TZDs promotes adipogenesis, predominantly in preadipocytes from subcutaneous depots. Increased transcription of transporters and enzymes involved in fatty acid uptake, and lipogenesis increases the deposition of lipid in these adipocytes (Table 2). This appears to facilitate a reduction in hyperglycemia by reducing circulating concentrations of nonesterified (free) fatty acids and triglycerides. The consequent effect on the glucose-fatty acid (Randle) cycle is to reduce the availability of fatty acids as an energy source, thereby favoring the utilization of glucose. Additionally, TZDs increase transcription of GLUT-4 glucose transporters that facilitate



Glucose-Lowering Drugs Other than Insulin, Fig. 8 Thiazolidinediones stimulate the PPARγ moiety of the PPARγ–RXR nuclear receptor complex, which then binds to a response element, leading to transcription of certain genes some of which are also responsive to insulin. These facilitate increased uptake of fatty acids, lipogenesis and adipogenesis. PPARγ, peroxisome proliferator-activated receptor-γ; RXR, retinoid X receptor; PPRE, peroxisome proliferator response element; TZD, thiazolidinedione; *cis*-RA, *cis*-retinoic acid; GLUT-4, glucose transporter isoform-4; FATP, fatty acid transporter protein; aP2, adipocyte fatty acid-binding protein

Glucose-Lowering Drugs Other than Insulin, Table 2 Tissue expression, ligands, genes activated, and biological actions of the peroxisome proliferator-activated receptor-γ (PPARγ)

Tissue expression	Mainly white and brown adipose tissue; weak expression in liver, muscle, gut, macrophages, pancreatic β-cells and hemopoietic tissues
Natural ligands	Certain unsaturated fatty acids and prostaglandin metabolites
Synthetic ligands	Thiazolidinediones and some non-steroidal anti-inflammatory drugs
Genes activated	Lipoprotein lipase; fatty acid transporter protein; adipocyte fatty acid binding protein; acyl-CoA synthetase; malic enzyme; GLUT-4 glucose transporter; phosphoenolpyruvate carboxykinase
Biological actions	Adipocyte differentiation; fatty acid uptake; lipogenesis; glucose uptake; other effects on nutrient metabolism which lower hepatic glucose production

glucose uptake. Reducing circulating free fatty acid concentrations reduces ectopic fat which in turn reduces the production of lipid metabolites, which would suppress early postreceptor steps in the insulin-signaling pathway. TZDs may further improve insulin signaling by increasing production of the adipocyte hormone adiponectin, decreasing production of the adipocyte cytokine tumor necrosis factor-α (TNFα), and decreasing production of the adipocyte hormone resistin (and possibly leptin), which have been implicated in the pathogenesis of insulin resistance.

There is weak expression of PPARγ in the liver, muscle, and other tissues, enabling TZDs to support the effects of insulin in these tissues, notably reduced glucose production by the liver and increased glucose uptake by muscle.

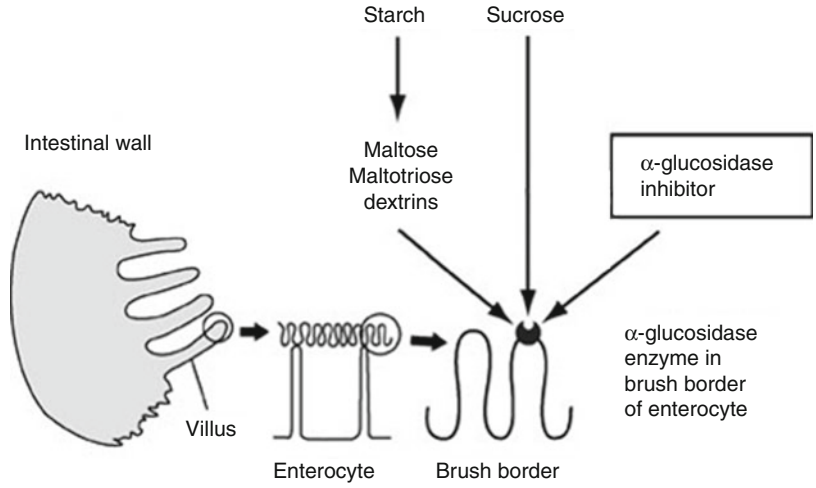
α-Glucosidase Inhibitors

The first member of this class, acarbose, was introduced in the early 1990s. α-Glucosidase inhibitors slow the intestinal process of carbohydrate digestion by competitive inhibition of the activity of α-glucosidase enzymes located in the brush border of the enterocytes (Fig. 9). Acarbose also causes a modest inhibition of pancreatic α-amylase activity. The principal α-Glucosidase enzymes are glucoamylase, sucrase, maltase, and dextrinase. The inhibitors bind to these enzymes with much higher affinity than their natural disaccharide and oligosaccharide substrates. Hence, when bound to the inhibitor, the enzyme fails to cleave the disaccharides and oligosaccharides into their absorbable monosaccharides. The available α-glucosidase inhibitors, acarbose, miglitol, and voglibose, show different binding affinities for the enzymes, giving them different activity profiles. For example, the affinity profile of acarbose is glycoamylase > sucrase > maltase > dextrinase. Miglitol is a more potent inhibitor of sucrase, and voglibose of other α-glucosidases (Lebovitz 2004).

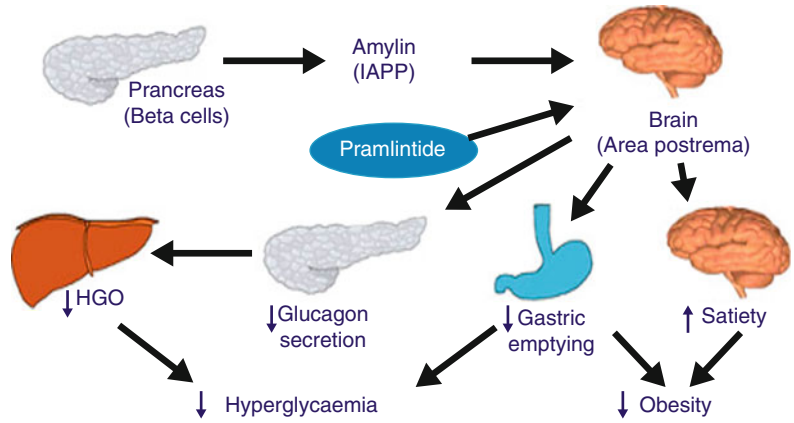
When α-glucosidase activity is inhibited, carbohydrate digestion is prolonged and takes place further along the intestinal tract. This in turn delays and spreads the period of glucose absorption, which reduces the extent of the postprandial rise in blood glucose concentrations. The effectiveness of α-glucosidase inhibitors is dependent on the consumption of a meal rich in complex carbohydrate.

Pramlintide

Pramlintide was introduced in the USA in 2005 as an adjunct to insulin therapy. It is a soluble analogue of the islet hormone amylin (islet amyloid polypeptide, IAPP) that is normally co-secreted from the pancreatic β-cells with insulin and C-peptide in response to nutrient stimuli. Paradoxically, amylin has been a suspect in the demise of β-cells in type 2 diabetes due to its accumulation and polymerization to form insoluble fibrils in the islets. However, normal amylin secretion appears to contribute to glucose homeostasis. Amylin acts centrally, via receptors in the area postrema (where there is no blood-brain barrier), dorsal raphe, and nucleus accumbens. The central effects induce satiety and initiate a vagally mediated suppression of prandial glucagon secretion and a slowing of gastric emptying (Fig. 10). In type 1 diabetes and advanced stages of type 2 diabetes, there is a lack or substantial reduction of amylin. Thus this non-aggregating analogue of amylin can be used to complement insulin therapy in type 1 and advanced type 2 diabetic patients (Messer and Green 2009).



Glucose-Lowering Drugs Other than Insulin, Fig. 9 α-Glucosidase inhibitors slow the rate of intestinal carbohydrate digestion by competitive inhibition of α-glucosidase enzymes in the brush border of enterocytes. The α-glucosidase inhibitors have a higher affinity for the α-glucosidase enzymes than the natural disaccharide and oligosaccharide substrates.



Glucose-Lowering Drugs Other than Insulin, Fig. 10 Mechanisms of action of the amylin analogue pramlintide

The structure of pramlintide (Fig. 11) differs from human amylin by the substitution of three residues with proline residues, retaining biological potency but preventing self-aggregation. Pramlintide is administered by subcutaneous injection and always as an adjunct to insulin therapy. Since pramlintide requires a more acidic pH than insulin, it has to be given as a separate injection to insulin, usually just before the main meals. The suppression of glucagon secretion and to a lesser extent the slowing of gastric emptying are the main immediate actions of pramlintide that reduce blood glucose. The satiety effect is typically associated with a long-term reduction of food intake and body weight: reduced adiposity in obese type 2 diabetes generally improves metabolic control. It is advised to reduce the mealtime insulin dose during initiation of pramlintide therapy to reduce the risk of interprandial hypoglycemia. Antibodies to pramlintide have been identified in some patients although these do not appear to affect biological activity.

Bromocriptine

Bromocriptine is a dopamine D2 receptor agonist that is well established as a treatment for Parkinson's disease and pituitary tumors. In 2009, a low-dose rapid release formulation was approved for glucose lowering in type 2 diabetes, based on studies showing improved glucose tolerance without increased insulin. It is suggested

Amylin	KCNTA TCATQ RLANF LVHSS NNFGA ILSST NVGSNT
Pramlintide	KCNTA TCATQ RLANF LVHSS NNFGP ILPPT NVGSNTY

Glucose-Lowering Drugs Other than Insulin, Fig. 11 Structure of human amylin and its soluble analogue pramlintide

that increased dopaminergic activity in the morning assists the normal circadian role of the hypothalamus in glucoregulation, particularly to reduce sympathetic activity and suppress hepatic glucose production. Bromocriptine is reported to improve the blood lipid profile and reduce cardiovascular risk (Raskin and Cincotta 2016).

Colesevelam

Colesevelam is a bile acid sequestrant that acquired an additional approval in 2008 to improve glycemic control in type 2 diabetes. The glucose-lowering effect is modest, and the mechanism is uncertain. There is evidence that when bile acids are carried by colesevelam to more distal regions along the intestine, they interact with the bile acid receptor-1 (TGR5) on enteroendocrine L-cells in the ileum and colon to promote GLP-1 secretion and thereby increase the incretin effect. Also, by reducing the enterohepatic circulation of bile acids, colesevelam reduces the activity the farnesoid X receptor (FXR), which could affect hepatic glucose metabolism (Beysen et al. 2012).

Clinical Use

Type 2 (non-insulin-dependent) diabetes typically emerges in middle or later life. Unlike type 1 diabetes in which there is total loss of pancreatic β-cells and a critical need for exogenous insulin administration, type 2 diabetes is associated with a continued presence of β-cells and continued insulin production. However, insulin resistance often develops as a prelude to type 2 diabetes and creates a demand for a compensatory increase in insulin secretion. Eventually, the β-cells are unable to produce sufficient extra insulin to overcome the insulin resistance. This results in impaired insulin-mediated glucose uptake by the muscle, failure of insulin to suppress hepatic glucose production, and consequently hyperglycemia. Pancreatic β-cells of type 2 diabetic patients become increasingly sluggish in their responsiveness to raised glucose concentrations, and eventually β-cell function becomes severely impaired, leading to a state of hypoinsulinemia and greater hyperglycemia. Obesogenic disturbances and other contributing factors mentioned earlier (in the section "Mechanism of Action") collectively conspire to create metabolic abnormalities in most body tissues. The toxic effects of hyperglycemia on the functional integrity of small blood vessels and nerves result in the long-term microvascular and neuropathic complications of diabetes (retinopathy, nephropathy, and neuropathy). The additional effects of dyslipidemia, hypertension, and other cardiovascular risks associated with insulin resistance are largely responsible for the long-term cardiovascular complications of type 2 diabetes (DeFronzo et al. 2015).

Achieving and maintaining blood glucose concentrations close to normal reduce the morbidity and premature mortality of the long-term complications of type 2 diabetes. Treatment begins with non-pharmacological measures (diet, exercise, and healthy living), and some patients can achieve remission, but the typical scenario of a gradual deterioration in glycemic control eventually requires add-on pharmacological therapy for the majority of patients. This is usually started with one oral glucose-lowering agent. Most guidelines and consensus reports favor metformin as the initial agent because it offers glucose-lowering efficacy without overswings into hypoglycemia, does not cause weight gain, counters insulin resistance, reduces long-term risk of cardiovascular complications, and may reduce risk of some neoplasms (Davies et al. 2018; Buse et al. 2019).

In some treatment algorithms, a sulphonylurea or a prandial insulin releaser is favored as the first oral glucose-lowering agent if substantial β-cell failure is suspected. A prandial insulin releaser would be preferred for individuals with either mainly postprandial hyperglycemia or irregular meal patterns which would predispose to interprandial hypoglycemia if taking a sulphonylurea. A sulphonylurea or prandial insulin releaser is often recommended as a complementary add-on to metformin as the disease progresses, although add-on therapy with a DPP-4 inhibitor has been adopted widely as a strategy for continued avoidance of hypoglycemia and weight gain. Recent guidance has suggested that add-on therapy to metformin should take particular account of the presence of cardiovascular and renal complications or very high risk of these complications, favoring use of a GLP-1RA or SGLT-2 inhibitor (Davies et al. 2018; Buse et al. 2019). The use of TZDs has receded since the emergence of the DPP-4 inhibitors, GLP-1RAs, and SGLT-2 inhibitors, largely due to the regulatory issues with

Glucose-Lowering Drugs Other than Insulin, Table 3 Main exclusions, adverse events, and precautionary monitoring required for clinical use of glucose-lowering drugs other than insulin

Class[a]	Main exclusions	Main adverse events	Monitoring[b]
Metformin	Renal or liver disease; any predisposition to hypoxia	Gastro intestinal upsets; risk of lactic acidosis if severely impaired renal function	GFR, creatinine, Hb, or Vit B12[b]
Sulphonylureas	Severe liver or renal disease[c]	Hypoglycemia	–[b]
Prandial insulin releasers (meglitinides)	Severe liver or renal disease[c]	Hypoglycemia[d]	–[b]
DPP-4 inhibitors (gliptins)	Caution if history of acute pancreatitis	Possible increased risk of pancreatitis	–[b]
Glucagon-like peptide-1 receptor agonists (GLP-1RAs)	Caution if history of acute pancreatitis	Initial nausea, possible increased risk of pancreatitis	–[b]
Sodium-glucose cotransporter-2 inhibitors (SGLT-2 inhibitors)	Poor renal function	Genital mycotic infection, dehydration, euglycemic ketoacidosis	GFR[b]
Thiazolidinediones (TZDs)	Heart failure; liver disease	Edema, anemia, heart failure, bone fractures	LFT[b]
α-Glucosidase inhibitors	Chronic intestinal disease	Gastrointestinal upsets	LFT[b,e]
Pramlintide	Gastroparesis	Hypoglycemia, nausea	–[b]
Bromocriptine	Hypertension, significant cardiovascular disease	Nausea; dizziness	Blood pressure[b]
Colesevelam	Intestinal disease or obstruction, biliary obstruction	Gastrointestinal upsets, increased triglyceride	LFT[b]

GFR, glomerular filtration rate; Hb, hemoglobin; Vit B12, vitamin B12; LFT, liver function test

[a]The dosage of each antidiabetic drug should be increased until either the target level of glycemia is achieved or the last dosage increment produces no additional effect

[b]Appropriate monitoring of glycemic control may involve self-measurement or laboratory measurement of fasting or random blood/plasma glucose, interstitial glucose monitoring, glycated hemoglobin (HbA1c) or fructosamine (glycated albumin)

[c]Depending upon pathways of metabolism and elimination of individual members of the class

[d]Prandial insulin releasers are less likely to produce severe or prolonged episodes of hypoglycemia than sulphonylureas

[e]Liver function should be checked in patients on high dose acarbose

rosiglitazone, weight gain, and concerns about possible edema and risk of heart failure for some patients. However, pioglitazone can be used in impaired renal function. The use of α-glucosidase inhibitors is more common in Asia and can be helpful if the diet is high in complex carbohydrate, but glucose-lowering efficacy may be modest and predominantly restricted to postprandial periods.

Insulin therapy in type 2 diabetes tends to be reserved until combinations of two or sometimes three differently acting classes of agents are no longer effective (insulin, ▶ insulin receptor). Insulin may be used earlier if hyperglycemia is extensive or patients are symptomatic, and rapid disease progression to require insulin may prompt enquiry to exclude late onset type 1 diabetes. Metformin is usually retained with insulin in type 2 diabetes: other oral glucose-lowering agents as well as GLP-1RAs may be indicated together with insulin to address concerns about weight, cardio-renal complications, or insulin dose.

It is noted that not all agents are available in all countries, and indications for use may vary between countries and between localities. Pramlintide, bromocriptine, and colesevelam have limited availability as glucose-lowering agents and are not approved treatments for type 2 diabetes in Europe. There are further agents used for glucose-lowering in parts of Asia, the Middle East, Africa, and South America that are not considered in the present account.

Precautions associated with the use of glucose-lowering agents, including restrictions in patients with chronic kidney disease, cardiovascular, and other morbidities, are itemized in the summary of product characteristics (also termed product label) for each agent, and may vary between formularies in different countries. The main limitations and cautions for use of glucose-lowering drugs are listed in Table 3.

Cross-References

▶ ATP-Dependent K^+ Channels
▶ Diabetes Mellitus
▶ Insulin Receptor

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Glycine Receptors

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Synonyms

Glycine-gated chloride channel; Inhibitory glycine receptor; Strychnine-sensitive glycine receptor

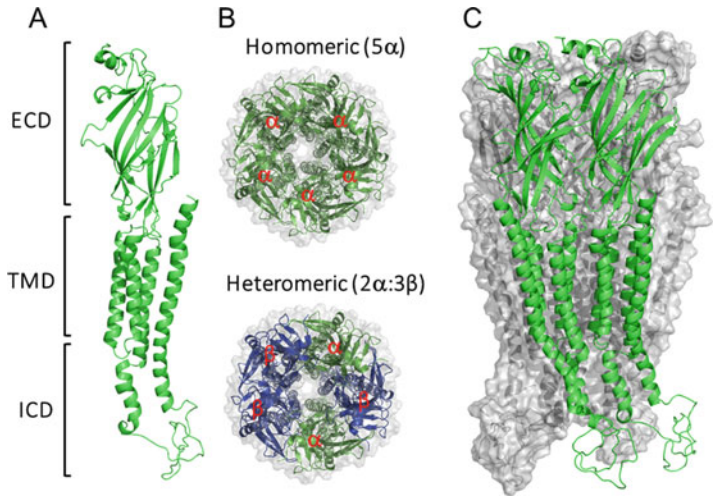
Definition

Glycine receptors (GlyRs) are from the family of ligand-gated pentameric ion channels (pLGICs) which also includes inhibitory gamma-aminobutyric acid receptors ($GABA_AR$) and excitatory nicotinic acetylcholine (nAChR) and 5-hydroxytryptamine receptors ($5\text{-}HT_3R$) (Burgos et al. 2016; Lynch et al. 2017a; Zeilhofer et al. 2018). The binding of the neurotransmitter glycine to the alpha (α) receptor subunit opens a chloride-permeable ion channel, allowing rapid hyperpolarization of the membrane potential and control of neuronal excitability in a millisecond time course. This effect is blocked by nanomolar concentrations of strychnine. Each α GlyR subunit consists of a large extracellular N-terminal domain (ECD) which contains the orthosteric binding site, four membrane-spanning helices (M1–M4; TMD) where the TM2 helices from each subunit contribute to shape the central pore, and a large intracellular domain (ICD) connecting the TM3 and TM4 (Fig. 1a). Adding to the receptor complexity, GlyRs are protein complexes composed of α- and β-subunits. Molecular cloning studies have identified four isoforms of the α-subunits (α1–4) and one β-subunit. The four α-subunits show a high degree of amino acid sequence identity but differ in their developmental regulation and regional expression, as well as in their biophysical properties. GlyRs have important physiological/pharmacological roles throughout the entire nervous system, contributing to the control of muscle tone, respiration, motor coordination, sensory processing, and higher brain functions. Impaired GlyR function underlies many neurological diseases, including hyperekplexia, chronic pain, autism, epilepsy, and spectrum disorders. Therefore, there is an active search for subunit selective allosteric modulators that can alter the course of these diseases (Burgos et al. 2016; Lynch et al. 2017b).

Basic Characteristics

Glycinergic Synapses

Besides the expression in the spinal cord and brainstem, synaptic GlyRs are also expressed in other areas such as the neocortex, amygdala, nucleus accumbens, and retina (Ogino and Hirata 2016; Muñoz et al. 2018). In these regions, functional GlyRs are pentameric complexes which are formed by five α-subunits (homomeric) or by a mixture of α- and β-subunits with a 2α:3β stoichiometry (Fig. 1b). During CNS development, GlyRs are predominantly expressed as heteromeric α2β conformations but then later switch to α1β in the adult state. However, α2 remains at rather high levels into adulthood in the cerebral cortex and striatum. The α3 subunit is also expressed as a heteropentamer in the adult CNS, but its expression is restricted mainly to the dorsal horn layer of the spinal cord. In humans, functional GlyRs containing the α4 subunit cannot be expressed because a premature stop codon is present within the fourth membrane-spanning domain (for this reason α4 is often considered as a pseudogene in humans) (Ogino and Hirata 2016; Patrizio et al. 2017). In contrast to the α-subunits, the β-subunit alone cannot form functional receptors. However, the β-subunit is essential for postsynaptic organization of the GlyRs via their interaction with the scaffold protein gephyrin. Therefore, whereas the heteropentameric GlyRs are accumulated at the postsynaptic sites, it is believed that homomeric GlyRs are mainly distributed in the extrasynaptic area or presynaptic terminal (Patrizio et al. 2017). These



Glycine Receptors, Fig. 1 General topology and architecture of the glycine receptor. (**a**). Homology model of the α1 GlyR subunit. The model was constructed from the electron cryo-microscopy structure (PDB 3JAE) and shows the ECD, four TMDs, and the large intracellular domain between TM3 and TM4. The ICD was added to the 3JAE structure. (**b**) Stoichiometry of the GlyRs. (**c**) Pentameric conformation of GlyRs. The model was constructed from the 3JAE structure. The ICD was added to the 3JAE structure

extrasynaptic GlyRs are activated by local glycine (or taurine) producing glycinergic currents that contribute to the tonic regulation of neuronal excitability. This tonic current response is likely controlled by the biosynthesis of glycine that is catalyzed by the enzyme serine hydroxymethyltransferase from the precursor serine. Glycine is incorporated into synaptic vesicles by the vesicular inhibitory amino acid transporter (VIAAT). Since VIAAT also mediates the uptake of GABA, a combined inhibitory glycinergic/GABAergic synapse frequently occurs. Glycinergic neurotransmission is terminated by clearance of glycine from the extracellular space, which is achieved by two high-affinity sodium-dependent glycine transporter subtypes: glycine transporter 1 (GlyT1) and glycine transporter 2 (GlyT2). While GlyT1 is predominantly expressed in astrocytes (controlling the allosteric modulation of NMDARs by glycine), GlyT2 is expressed in glycine-releasing neurons in the spinal cord and in the brain stem where it drives the reuptake of glycine into presynaptic terminals. It is feasible that the discovery of new potent and selective reuptake inhibitors of glycine might provide compounds with interesting pharmacological properties that affect GlyRs (Erdem et al. 2019).

Excitatory Role of GlyRs in Immature Neurons

Contrary to the role of GlyRs in the mature CNS, the activation of GlyRs in immature neurons induces depolarizing responses (at this stage the chloride gradient is reversed to that of mature neurons) which elicit the excitation of the neuronal network (Lynch 2009). The inverted chloride gradient during this developmental phase is originated by action of the Na^{+}-K^{+}-$_2Cl^{-}$ cotransporter (NKCC1) that transports chloride ions inwardly. During early development of the CNS, the activity of KCC2 increases thereby establishing a low intracellular chloride concentration. Thus, KCC2 activity is necessary for glycinergic synapse maturation. Indeed, deletion of KCC2 impaired cluster formation of α1 subunits and gephyrin in cultured spinal neurons. On the other hand, cluster formation composed by α2 subunits was not affected by KCC2 knockdown (Schwale et al. 2016).

Glycine Receptor Structure-Function Relationship

The architecture of GlyRs has been confirmed by recent high-resolution structures using

cryoelectron microscopy (cryo-EM) and X-ray crystallography. The studies have provided atomistic models for open and closed states. To date, structures for human α3 GlyR and zebra fish α1 GlyR subunits have been solved (Du et al. 2015; Huang et al. 2017). Functional GlyRs adopt a pentameric cylinder-shape assembly in which the five subunits are arranged around a central fivefold axis (Fig. 1b,c). The data show that the structural features and the amino acid identity of the ECD and the TMDs of the different GlyRs subunits are highly conserved. The ECD adopts a barrel-like structure formed by β-strands (β1–β10) accompanied by two α-helices at the N-terminal. The glycine binding site is located between two neighboring subunits and is formed by three loops (A, B, C) of the principal subunit (+) along with three β-strands (D, E, F) from the complementary subunit (−). In addition, residues R65, T204, Y202, S129, F159, and P207 interact with glycine. The TMD consists of four amphipathic α-helices (TM1–4), where TM2 helices form the channel pore while TM4s are located on the external face corresponding to the region that interacts with lipid components of the neuronal membrane. On the other hand, the large ICD connecting the TM3 and TM4 is the least conserved domain in length and amino acid composition among the GlyR subunits. Unfortunately, all the structural data available on GlyRs to date has been obtained from ion channels lacking an important portion of the ICD (Du et al. 2015; Huang et al. 2017).

With regard to the structure-function relationship, it has historically been thought that GlyRs transition between three discrete global conformational states: (1) **resting**, a non-conductive conformation unbound to the agonist; (2) **active**, a conductive state bound to the agonist; and (3) **desensitized**, a non-conductive conformation bound to the agonist. However, more recent structural and functional studies have proposed that the activation of GlyRs (a process known as gating) occurs through numerous intermediate states, with a conformational sequence moving from the ECD to the TMD during gating. These experimental and structural data have suggested that the gating process is mainly determined by the ECD and TMDs. Binding of the agonist to the orthosteric site triggers a rapid isomerization that results in a transient structural rearrangement of the TM2 and TM3 leading to the opening of a transmembrane pore that allows the diffusion of anions. If the binding of the agonist is prolonged, the channel adopts a closed-state conformation (distinct from the agonist-unbound state) or desensitized state. Clearance of the agonist from the synaptic cleft causes the receptor to return to the inactive-closed configuration (Du et al. 2015; Huang et al. 2017). However, despite significant advances, the mechanisms underlying the gating process in pLGICs are not fully understood. Structural data from the cryo-EM reconstruction of GlyRs in complex with glycine captured an open channel configuration with an active pore configuration larger than those obtained in complex with strychnine (a competitive antagonist of GlyR) or with glycine and ivermectin (an allosteric modulator of GlyR) (Du et al. 2015; Huang et al. 2017). These structures also differ from open configurations obtained from other members of the pLGICs family. Thus, the structural changes occurring during the gating process are still a matter of debate.

In this context, the contribution of the ICD to the gating process is also a debatable issue, especially considering the lack of structural data. For instance, studies showing that the inclusion of the GlyRs α1 ICD into the bacterial GLIC receptor (which does not have an ICD) did not produce noticeable functional changes in the proton-activated currents of this receptor (Jansen et al. 2008; Goyal et al. 2011). Nevertheless, functional evidence (based on site-directed mutagenesis) showed that the desensitization of α1 GlyRs is influenced by lysine residues located in the ICD. Moreover, a short splicing variant of the α3 GlyR, α3K, shows a faster desensitization compared with the α3LGlyR wild type. In addition, mutagenesis performed on arginine residues within the ICD of α1 GlyRs showed a contribution to ion channel conductance (Carland et al. 2009). Thus, key questions regarding the functional and structural relevance of the ICD in the context of functional GlyRs remain to be solved.

Interaction of GlyRs with Intracellular Proteins

Compared with GABAergic synapses, glycinergic-mediated neurotransmission has restricted molecular variants and fewer subunit combinations to influence the neurotransmitter content, channel kinetics, and pharmacology at both pre- and postsynaptic levels. Nonetheless, glycinergic synapses show neuroplasticity that is achieved by the interaction with anchoring proteins which have been described as essential for the synaptic localization of the receptor. At inhibitory synapses, gephyrin is the major scaffold protein for the transient immobilization of GlyRs, as well as $GABA_A$Rs. Mutagenesis and electrophysiological experiments have shown that the ICD of the GlyR β-subunit contains critical residues for the functional interaction with gephyrin (Tyagarajan and Fritschy 2014). Additional to the components of the postsynaptic protein scaffold, other interacting proteins have been identified, including collybistin, profiling, Gβγ heterodimer, the vesicular trafficking factor SEC8, and the ubiquitination protein HUWE. It is likely that these components contribute to distinct levels of synaptic plasticity.

Physiological Consequences of Impaired Glycinergic Function

Glycinergic malfunction is associated mainly with motor disorders. Hyperekplexia (startle disease, stiff baby syndrome), a rare neurological disorder characterized by neonatal hypertonia and exaggerated startle responses, is caused by hereditary mutations that disrupt the functioning of inhibitory glycinergic synapses in neuromotor pathways of the spinal cord and brainstem (Bode and Lynch 2014). So far at least 51 mutations in the α1 subunit and 18 in the β-subunit have been described. Mutations in the α1 subunit include **(a)** single nucleotide exchange, **(b)** nonsense mutation, and **(c)** large deletion. These mutations can be inherited in both an autosomal dominant and recessive fashion. Autosomal dominant mutations, which cannot be rescued by a wild-type allele, produce deleterious effects in glycinergic neurotransmission though different molecular mechanisms: **(a)** spontaneous activation (e.g., Y128C, Q226E, V280M, and R414H) which can displace the chloride equilibrium potential to more positive values leading to a further reduction in the inhibitory efficacy; **(b)** impaired channel gating where mutant GlyRs carrying the mutation R271Q or R271L showed a reduced channel conductance; **(c)** increased sensitization rate (e.g., P250T mutation reduces glycine-activated current amplitudes and induces fast desensitization); and **(d)** reduced cell surface expression (e.g., S231R, I244N, R252H, R392H). All these mutations exhibited low cell surface expression and reduced glycine sensitivity. In addition to hyperekplexia, altered glycinergic neurotransmission is also associated with inflammatory chronic pain (Harvey et al. 2004). The proposed mechanism of α3 GlyR-dependent pain sensitization involves the loss of glycinergic inhibition following the activation of neuronal EP2 receptors (EP2-R) by prostaglandin E2 (PGE2). EP2-R stimulation increases cAMP and subsequently promotes the PKA-dependent phosphorylation of the α3 GlyR at the S346 residue within the ICD, decreasing the amplitude of glycinergic currents. This loss of inhibitory control enhances the excitability of dorsal horn excitatory neurons, leading to the development and maintenance of chronic pain (Harvey et al. 2004). Recently, compelling evidence have shown that mutations affecting the function of α2 containing GlyRs are related to the occurrence of autism disorders (Zhang et al. 2017).

Allosteric Pharmacological Modulation

The function of GlyRs is modulated by several compounds having distinct chemical entities. Unfortunately, these ligands do not show specificity and can modulate α1, α2, and α3 containing GlyRs with similar potencies. Moreover, many of these modulators can also affect the function of related receptors such as $GABA_AR$ or $5HT_3R$. The spectrum of these modulators includes bivalent cations, general anesthetics, cannabinoids, tropeines, colchicine, ginkgolic acids,

ivermectins, synthetic neurosteroids, and n-alcohols. Mutagenesis, electrophysiological, and structural studies have proposed that the effect of many of these modulators is mediated by residues located mainly in the TMD (S267) and TM3 (A288, A296) that form an intrasubunit cavity able to accommodate compounds of different chemical characteristics (Burgos et al. 2016). Thus, mutations of these residues reduced the potentiation of glycine-evoked currents by ethanol, propofol, isoflurane, alphaxolone, THC, and cannabidiol (CBD). On the other hand, more recent studies have suggested that the ICD also has an important role on the sensitivity of GlyRs to these modulators. For example, electrophysiological studies using chimeric receptors with and without ICDs showed that the ICD is essential for the modulation of GlyRs by ethanol, propofol, and CDB. Mutagenesis and electrophysiological studies have also shown that residues in the ICD are critical for the effects of ethanol (α1 GlyRs 385KK386), propofol (α1 GlyRs F380), and the propofol derivative 2,6-DTBP (α3 GlyRs F388) (Yévenes and Zeilhofer 2011). Interestingly, KI mice having this 385KK386 mutation were less sensitive to ethanol-induced sedation and drank more ethanol that their WT counterpart (Muñoz et al. 2019).

More recently, the study of α3 GlyRs structure with a novel class of tricyclic sulfonamides (AM-1488) described a novel allosteric site (Huang et al. 2017). Interestingly, the compound was able to reduce allodynia in mice with spared nerve injury-induced neuropathic pain. Therefore, even though the pharmacology of GlyRs has surely advanced in the last decade, there are no pharmacological drugs available for treatment of glycinergic failures.

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Glycine-Gated Chloride Channel

▶ Glycine Receptors

GPCR

▶ Dopamine System

GPR4

▶ Proton-Sensing GPCRs

G-Protein-Coupled Receptors

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Definition

G-protein-coupled receptors (GPCRs) are a large family of heptahelical membrane proteins able to translate the binding of extracellular molecules into modulation of intracellular signaling pathways. This process involves in most cases activation of heterotrimeric G-proteins, but may also occur in a G-protein-independent manner.

Basic Characteristics

GPCRs are the largest family of transmembrane receptors in eukaryotes. Due to their typical seven transmembrane structure, they are also known as seven-(pass)-transmembrane domain receptors, 7TM receptors, heptahelical receptors, or serpentine receptors. GPCRs mediate cellular responses to a wide variety of signals, including neurotransmitters, hormones, local mediators, metabolic or olfactory cues, and light (Rosenbaum et al. 2009). The human genome encodes about 800 GPCRs, the majority of them sensory (olfaction, vision, taste) receptors. The approximately 360 nonsensory GPCRs have central roles in cell-cell communication in all organ systems, and constitute a highly successful group of drug targets. However, for more than 100 nonsensory GPCR, endogenous ligand and function are still unknown, so-called orphan GPCRs (Ngo et al. 2016; Wise et al. 2004).

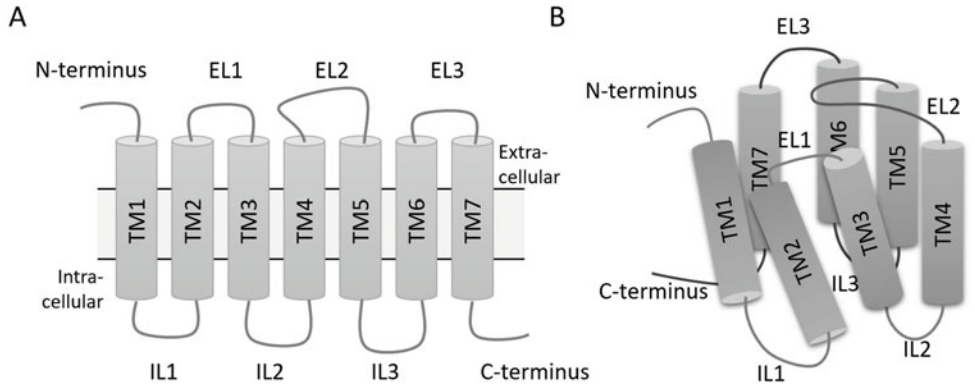
Structure and Function

All GPCRs consist of an extracellular N-terminus, followed by seven transmembrane (7-TM) α-helices (TM-1 to TM-7) connected by three intracellular (IL1 to IL3) and three extracellular loops (EL1 to EL3), and finally an intracellular C-

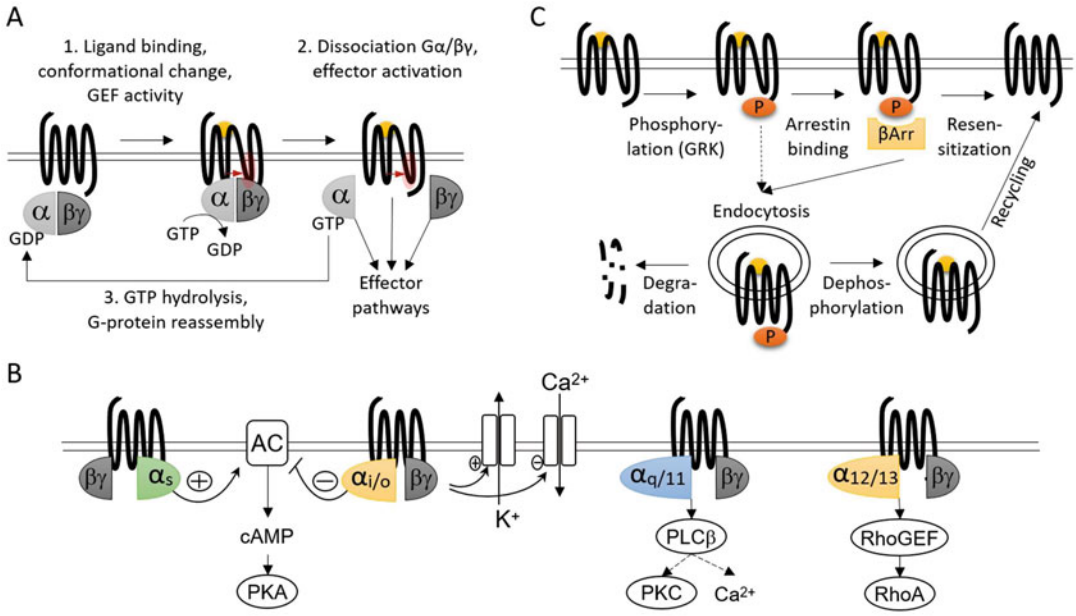
terminus, which in some GPCR contains an eighth helical region termed helix 8 (Fig. 1a). The N-terminus is in many GPCRs glycosylated, and the C-terminus is often tethered to the plasma membrane by a lipid modification. The GPCR arranges itself into a tertiary structure resembling a barrel, with the seven transmembrane helices forming a cavity within the plasma membrane (Fig. 1b). This cavity serves in many GPCRs as the ligand-binding domain and is often covered by EL2. Ligands may also bind elsewhere, especially in case of bulkier ligands (e.g., proteins or large peptides), which instead interact with the extracellular loops, or, as illustrated by the class C metabotropic glutamate receptors, the large N-terminal tail (see also Fig. 3) (Culhane et al. 2015).

Regardless of where ligand binding occurs, the consequence is a change in the relative orientation of the TM helices, for example, an outward movement of transmembrane helices TM5 and TM6 (Rasmussen et al. 2011; Weis and Kobilka 2018), resulting in a wider intracellular surface and better accessibility of residues crucial for signal transduction. This conformational change allows the GPCR to act as a guanine nucleotide exchange factor (GEF) towards the heterotrimeric G-protein, i.e., it facilitates the release of GDP from and consecutive binding of GTP to the G-protein α subunit (Fig. 2a). After successful GDP/GTP exchange, α and β/γ subunit of the heterotrimeric G-protein dissociate and are free to interact with their respective downstream effectors. β/γ subunits regulate ion channels such as G protein-gated inward rectifier channels or calcium channels, other potential β/γ effectors are phospholipase C (PLC) β isoforms and adenylyl cyclases (AC) (Wettschureck and Offermanns 2005). Responses elicited by the α subunit depend on the type of G-protein: activation of $G\alpha_s$ results in activation of AC and consecutive cAMP-dependent PKA activation, activation of $G\alpha_{i/o}$ inhibits AC activity, stimulation of $G\alpha_{q/11}$ induces phospholipase C β-dependent calcium mobilization and PKC activation, and $G\alpha_{12/13}$ finally mediates RhoGEF-dependent activation of RhoA (Fig. 3b) (Wettschureck and Offermanns 2005). G-protein activation is terminated by the intrinsic GTPase activity, which returns the G-protein into the inactive, GDP-bound state. Intrinsic GTPase activity is slow, but can be greatly enhanced by RGS proteins. In addition to signaling cascades controlled by heterotrimeric G-protein activation, GPCRs may signal independently of G-protein, for example, through β-arrestins (Shukla et al. 2011).

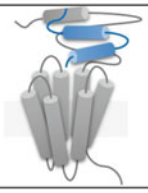
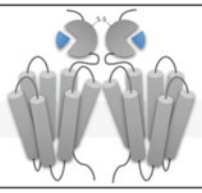
GPCR activation itself is not only terminated by disappearance of the ligand, but also by the process of densitization. The main underlying mechanism is GPCR phosphorylation by G protein-coupled receptors kinases (GRKs) and other kinases, resulting in β-arrestin recruitment. β-arrestin binding results in uncoupling of the G-protein α subunit from the receptor and clathrin-mediated internalization of the receptor (Shukla et al. 2011). In addition, also



G-Protein-Coupled Receptors, Fig. 1 GPCR structure and function. (**a**, **b**) Diagrams showing structural features of GPCRs (lateral view): secondary structure (**a**) and three-dimensional structure (**b**). EL, extracellular loop; IL, intracellular loop; TM, transmembrane domain



G-Protein-Coupled Receptors, Fig. 2 Principles of GPCR signaling. (**a**) Diagram depicting the events initiated by agonist binding (red arrow indicates outward movement of TM5/6). (**b**) Overview of canonical G-protein-dependent effector pathways. (**c**) Cartoon illustrating the process of GPCR desensitization. AC, adenylyl cyclase; βArr, βarrestin; cAMP, cyclic adenosine monophosphate; GRK, G protein-coupled receptor kinase; PCRPKA/PKC, protein kinase A/C, PLCβ, phospholipase C β; RhoGEF, Rho-specific guanine nucleotide exchange factor

Human GPCRs: classification and characteristics					
GRAFS system	**Rhodopsin family**	**Secretin**	**Adhesion**	**Glutamate**	**Frizzled**
A-F system	**≈class A**	**≈class B**		**≈class C**	**≈class F**
Total GPCRs	**721**	**15**	**33**	**22**	**11**
Sensory	440 (403 olfactory, 7 vision, 25 taste, 5 VN)	0	0	3 (taste)	0
Known ligand	194	15	0	11	11
orphan	87 (54)*	0	33 (10)*	8 (1)*	0
Ligands	From light and small molecules to proteins	Peptide hormones	Mostly unknown	Ions and small molecules	Wnt proteins
Structure					

G-Protein-Coupled Receptors, Fig. 3 Human GPCRs: classification and characteristics. Classes D & E not listed, since not present in vertebrates. VNO, vomeronasal receptors for pheromones. *Numbers in brackets refer to orphan receptors for which an endogenous ligand has been proposed in at least one publication. Ligands (or in the case of adhesion GPCRs, the proteolytic cleavage site) are indicated in red.VN, vomeronasal pheromone receptors (for details, see https://www.guidetopharmacology.org/GRAC/ReceptorFamiliesForward?type=GPCR).

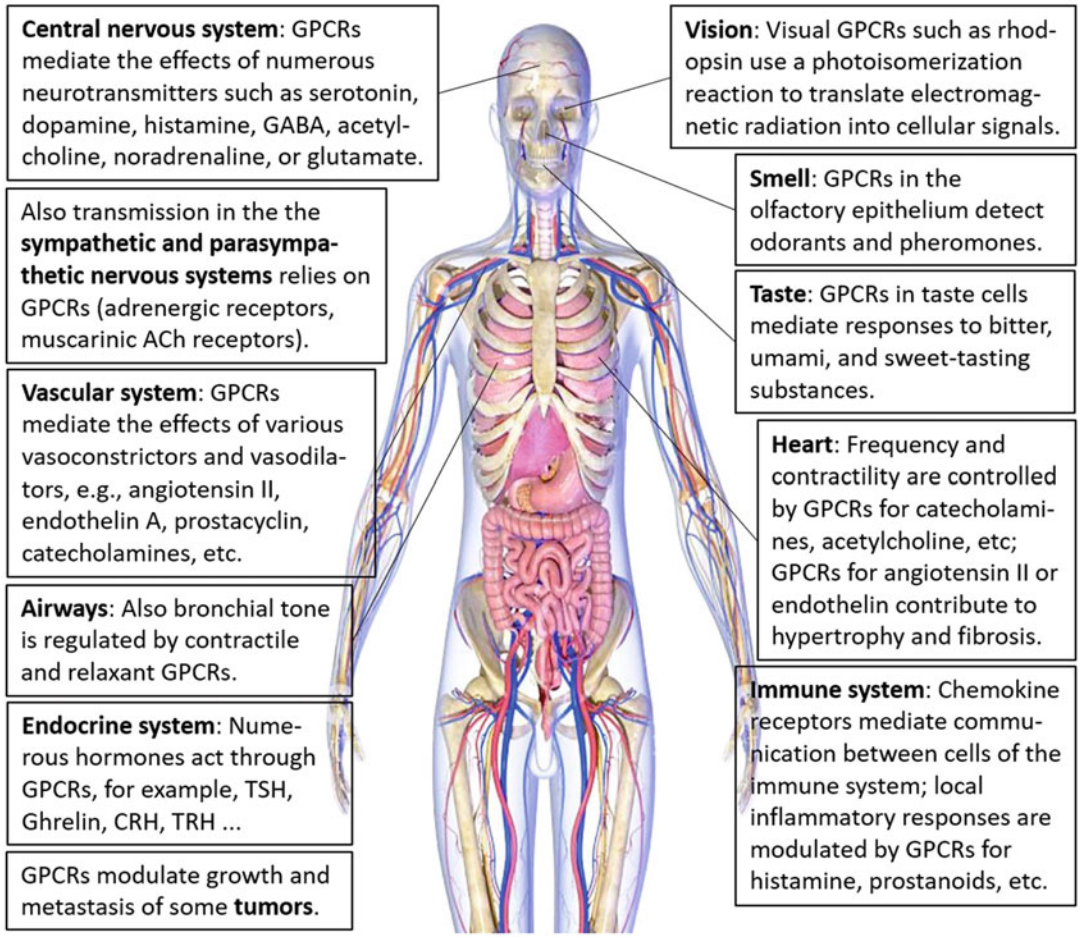
β-arrestin-independent internalization may occur. This process is not only observed in ligand-activated GPCR (homologous densitization), but can also affect nonactivated receptors (heterologous desensitization).

Classification

Based on sequence homology and functional similarity, different classification systems have been proposed for GPCRs, for example, the A-F system (A: Rhodopsin-like, B Secretin receptor family, C: Metabotropic glutamate/pheromone; D Fungal mating pheromone receptors, E: Cyclic AMP receptors, F: Frizzled/Smoothened) or the GRAFS system (Glutamate, Rhodopsin, *Adhesion*, Frizzled/Taste2, Secretin) (Bjarnadóttir et al. 2006). Figure 3 summarizes main features of GPCR classes.

Dimerization

Numerous co-immunoprecipitation studies using tagged receptors indicated that GPCRs can form homo- or heterodimers, and it was suggested that dimerization may affect both signaling and trafficking (Gurevich and Gurevich 2008; Milligan 2009). While for most class A receptors the functional relevance of dimerization is unclear, there is no doubt that class C receptors exist and function as stable dimers: metabotropic glutamate receptors (mGluR) and the calcium-sensing receptor (CASR) form homodimers, whereas $GABA_B$ and sweet and umami taste receptors are



G-Protein-Coupled Receptors, Fig. 4 Physiological functions of GPCRs (selection)

G-Protein-Coupled Receptors, Table 1 Data are based on Sriram and Insel (2018) and https://www.guidetopharmacology.org/. Some drugs have effects on multiple targets (including various GCPRs), but not all these interactions are necessarily of therapeutic relevance. In these cases, the respective drug is only listed for those GPCRs whose modulation is most likely underlying the therapeutic effect. For example, estradiol is an agonist at the G-protein-coupled estrogen receptor GPER1, but its main action is mediated via nuclear estrogen receptors; Estradiol is therefore not listed for GPER1

Receptor	Mode of action	Clinical use (example)	Approved drug (example)
Cardiovascular system			
Adenosine A1 receptor	Agonist	Supraventricular tachycardia	Adenosine
Adenosine A2a receptor	Agonist	Diagnostic agent	Regadenoson
Adrenoceptor alpha 1A, 1B, 1D	Antagonist	Arterial hypertension	Prazosin, Doxazosin, Terazosin, . . .
Adrenoceptor alpha 1A, 2A	Agonist	Nasal congestion	Oxymetazoline
Adrenoceptor Beta 1	Antagonist	Hypertension, angina pectoris, arrhythmia, . . .	Atenolol, Carvedilol, Metoprolol, . . .
Bradykinin receptor B2	Antagonist	Hereditary angioedema	Icatibant
Angiotensin II receptor AT1A	Antagonist	Hypertension	Candesartan, Irbesartan, Losartan, . . .
Arginine vasopressin receptor 2	Agonist	Central diabetes insipidus	Vasopressin, Desmopressin
Arginine vasopressin receptor 1A, 2	Antagonist	Euvolemic/hypervolemic hyponatremia	Conivaptan
Dopamine receptors D1-D5	Agonist	Shock syndrome, kidney failure	Dopamine
Endothelin receptor type A, ETA	Antagonist	Pulmonary arterial hypertension	Ambrisentan, Macitentan
Endothelin receptor type B, ETB	Antagonist	Pulmonary arterial hypertension	Bosentan
Prostaglandin I2 receptor	Agonist	Art. hypertension, scleroderma, Raynaud's	Iloprost, Treprostinil
Proteinase-activated receptor 1, PAR1	Antagonist	Prevention of platelet activation	Vorapaxar
Purinergic receptor P2Y12	Antagonist	Prevention of platelet activation	Ticagrelor
Endocrine/metabolic system			
Calcitonin receptor	Agonist	Osteoporosis	Calcitonin
Calcium sensing receptor	Agonist	Sec. hyperparathyreoidism	Etelcalcetide
Cholecystokinin B receptor, CCK_2	Agonist	Diagnostic agent	Pentagastrin
Corticotropin releasing hormone receptor 1	Agonist	Diagnostic agent	Corticorelin ovine triflutate
Follicle stimulating hormone receptor	Agonist	Fertility medicine	Follitropin alfa
Glucagon like peptide 1 receptor	Agonist	Type 2 diabetes	Lixisenalide, Exendin-4, Semaglutide, . . .
Glucagon like peptide 2 receptor	Agonist	Short bowel syndrome	Teduglutide
Glucagon receptor	Agonist	Severe hypoglycemia, diagnostic agent	Glucagon
Gonadotropin releasing hormone receptor	Antagonist	Hormone-sensitive cancers	Abarelix, Cetrorelix, Elagolix, . . .
Gonadotropin releasing hormone receptor	Agonist	Hormone-sensitive cancers	Leuprolide, Buserelin, Goserelin, . . .
Growth hormone releasing hormone receptor	Agonist	Diagnostic agent	Tesamorelin, Sermorelin
Hydroxycarboxylic acid receptor HCA2	Agonist	Hyperlipidemia	Acipimox, Nicotinic acid

(continued)

G-Protein-Coupled Receptors, Table 1 (continued)

Receptor	Mode of action	Clinical use (example)	Approved drug (example)
Luteinizing hormone/ Choriogonadotropin receptor	Agonist	Female infertility	Choriogonadotropin alfa
Melanocortin receptors 2	Agonist	Diagnostic agent	Corticotropin
Oxytocin receptor	Agonist	Labor induction	Oxytocin
Parathyroid hormone receptor 1	Agonist	Osteoporosis	Teriparatide
Secretin receptor	Agonist	Diagnostic agent	Secretin
Somatostatin receptors 1, 2, 3, 5	Agonist	Cushing's disease	Pasireotide
Somatostatin receptors 2,5	Agonist	Acromegaly, neuroendocrine tumors	Lanreotide
Gastrointestinal system			
Histamine H2 receptor	Antagonist	Peptic ulcer	Ranitidine, Cimetidine, Famotidine
Histamine H2 receptor	Agonist	Diagnostic agent	Betazole
Prostaglandin E receptor 3	Agonist	Gastrointestinal ulceration	Misoprostol
Immune system			
C-C motif chemokine receptor 5	Antagonist	HIV infection	Maraviroc
C-X-C motif chemokine receptor 4	Antagonist	Stem cell mobilization	Plerixafor
Histamine H1 receptor	Antagonist	Allergies	Cetirizine, Clemastine, Desloratadine, . . .
Sphingosine-1-phosphate receptor 1 + 5	Agonist	Multiple sclerosis	Fingolimod, Siponimod
Central nervous system			
5-Hydroxytryptamine (serotonin) receptor 1A	Agonist	Depressive disorder	Vilazodone
5-Hydroxytryptamine (serotonin) receptor 1B, 1D	Agonist	Migraine	Frovatriptan, Naratriptan, Elatriptan, . . .
5-Hydroxytryptamine (serotonin) receptor 2A	Antagonist	Schizophrenia, bipolar disorder	Asenapine*, Risperidone*, Sertindole*,. . ..
5-Hydroxytryptamine (serotonin) receptor 4	Agonist	Motility-related gastrointestinal disorders	Cisapride
5-Hydroxytryptamine (serotonin) receptors 2B, 2C	Agonist/ antagonist	Migraine, cluster headaches	Methysergide
Adrenoceptor alpha 2A, 2B, 2C	Agonist	Sedation in ICU setting	Dexmedetomidine
Cannabinoid receptors 1 + 2	Agonist	Antiemetic, analgesic	Nabilone
Dopamine receptor D2	Agonist	Parkinson's disease	Ropinirole*
Dopamine receptor D2	Antagonist	Schizophrenia	Risperidone*
Dopamine receptor D3	Agonist	Parkinson's disease	Ropinirole*
Gamma-Aminobutyric acid type B receptor 1 + 2	Agonist	Spasticity in multiple sclerosis	Baclofen
Histamine H3 receptor	Antagonist	Narcolepsy	Pitolisant
Hypocretin (Orexin) receptor 1 + 2	Antagonist	Insomnia	Suvorexant
Melatonin receptors 1A, 1B	Agonist	Insomnia	Ramelteon, Agomelatine, Tasimelteon
Muscarinic acetylcholine receptor M1	Antagonist	Parkinson's disease	Biperiden
Opioid receptor μ	Agonist	Pain management	Fentanyl, Alfentanil, Sufentanil
Opioid receptors μ, κ, δ	Antagonist	Opioid/alcohol dependence	Naltrexone

(continued)

G-Protein-Coupled Receptors, Table 1 (continued)

Receptor	Mode of action	Clinical use (example)	Approved drug (example)
Pulmonary system			
Adenosine A1 receptors A2A, A2B	Antagonist	Reversible airway obstruction	Theophylline
Adrenoceptor Beta 2	Agonist	Airway constriction, premature labor	Orciprenaline, Salbutamol, Salmeterol, . . .
Cysteinyl leukotriene receptor 1	Antagonist	Airway constriction	Zafirlukast, Montelukast
Muscarinic acetylcholine receptor M3	Antagonist	Chronic obstructive pulmonary disease	Tiotropium, Ipratropium
Other systems			
Adrenoceptor alpha 2A, 2C	Agonist	Elevated intraocular pressure	Apraclonidine
Adrenoceptor Beta 3	Agonist	Overactive bladder	Mirabegron
Muscarinic acetylchonine receptors M1, M2, M3	Antagonist	Gastrointestinal spasms, enuresis, hyperhidrosis	Propantheline
Prostaglandin E receptor 1	Agonist	Erectile dysfunction	Alprostadil
Prostaglandin E receptor 2	Agonist	Induction of labor/abortion	Dinoprostone
Prostaglandin F receptor	Agonist	Elevated intraocular pressure	Latanoprost
Smoothened, frizzled class receptor	Antagonist	Advanced basal cell carcinoma	Sonidegib

PAM Positive allosteric modulator. *Effects on other GPCRs contribute to therapeutic effect

obligatory heterodimers (Gurevich and Gurevich 2008; Milligan 2009). Dimerization of class C receptors is mediated by distinct structural features, for example, by the very large N-terminus containing a Venus flytrap motif (VFM), which mediates both ligand binding and dimerization of N termini. Additionally, mGluRs and CASR contain a cystein-rich domain (CRD) between VTF and heptahelical domain, which allows formation of disulfide bonds within the homodimer (Gurevich and Gurevich 2008).

Physiological Functions

GPCRs are involved in a wide variety of physiological processes. Some examples of their physiological roles include are shown in Fig. 4.

Pharmacological Modulation

In its simplest form, receptor theory proposes that a given GPCR can exist in two functionally distinct states, the inactive (R) and the active (R*) state. In the absence of ligands, most receptors are in the R state, but may spontaneously assume the R* conformation. This basal equilibrium between R and R* defines the basal (constitutive) activity of the receptor. GPCR ligands are classified according to their effect on basal activity of the receptor:

- Full agonists stabilize R* and elicit maximum signaling at the interrogated pathway.
- Partial agonists shift the balance towards R* without reaching maximum efficacy.
- Inverse agonists shift the balance towards R, i.e., they inhibit basal receptor activity.
- Neutral antagonists have no effect on receptor activity, but may block agonist effects.

Of note, a given GPCR may couple to different effector pathways, for example, different families of heterotrimeric G-proteins and/or arrestin. A specific ligand at this GPCR may exert the same effect, for example, full agonism, at all effector pathways, or it may have differential effects, for example, enhancing G-protein signaling while reducing β-arrestin signaling. Such ligand-dependent selectivity for certain signal transduction

pathways is referred to as "functional selectivity" or "biased signaling" (Smith et al. 2018).

Drugs

The unique combination of diversity and specificity within the GPCR family, together with the fact that they are readily targetable by exogenous agonists and antagonists, has made GPCRs a most successful group of drug targets (Wise et al. 2004): Approximately, 34% of all Food and Drug Administration (FDA) approved drugs target this family. Table 1 gives an overview of GPCRs targeted by approved drugs.

Cross-References

- ▶ Adenosine Receptors
- ▶ Ca^{2+}-Sensing Receptor (CaSR)
- ▶ Calcitonin Family Receptors
- ▶ Chemokine Receptors
- ▶ Free Fatty Acid Receptors
- ▶ Muscarinic Receptors
- ▶ Neurokinin/Tachykinin Receptors
- ▶ Proton-Sensing GPCRs
- ▶ Trace Amine-Associated Receptors

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Gram-Negative Bacteria

▶ Peptides and Peptidomimetics as Foundations for Drug Discovery

Granulocyte Colony-Stimulating Factor (G-CSF), Filgrastim, Pegfilgrastim and Lenograstim

▶ Hematopoietic Growth Factors

Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF), Molgramostim and Sargramostim

▶ Hematopoietic Growth Factors

Growth Factors

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Synonyms

Cytokines; Differentiation factors; Mitogens

Definition

Growth factors are relatively small and stable soluble polypeptides that mediate short-range cell-to-cell interactions. Growth factors and their cognate receptors, which often harbor enzymatic activity, function in the framework of modules regulating various cellular processes, such as proliferation, differentiation, and migration. These modules are evolutionarily conserved, and in many cases their primary developmental function is the determination of cell lineage through heterotypic cellular interactions. Many growth factors are versatile, promoting appropriate biological outcomes of several cell lineages, while others are more limited in scope. Growth factor expression is highly regulated, such that excessive growth factor activity is often associated with pathogenic hyper-proliferation (e.g., cancer, fibrosis, and psoriasis).

Historical Perspective

One of the first lines of evidence that led to the discovery of soluble growth factors (GFs) emerged from studies performed in the 1950s, in the laboratory of Victor Hamburger, by two fellows, Rita Levi-Montalcini and Stanley Cohen (Escudier et al. 2007). When studying mechanisms enabling limb innervation in chick embryos, they grafted a lump of a mouse sarcoma onto an embryo and observed a more extensive attraction of nerve fibers to the lump. They later found that snake venom and the murine submaxillary gland secrete a similarly active "nerve-stimulating factor," which instigated the isolation of the first two GFs: nerve growth factor (NGF) and epidermal growth factor (EGF). Two decades later, Cohen and Todaro reported that cells infected by the feline sarcoma virus lost their ability to bind EGF (Todaro et al. 1976). This observation led to the isolation from a murine sarcoma of two "transforming growth factors," TGF-α and TGF-β (Roberts et al. 1982). Subsequent studies revealed that not only virally transformed cells but also chemically transformed cells, as well as cells derived from human tumors, often secrete GFs, which are responsible for self-stimulation (autocrine) of growth. In support of autocrine theories, Waterfield and colleagues reported in 1983 that the transforming gene of the simian sarcoma virus is structurally related to the platelet-derived growth factor (PDGF) (Waterfield et al. 1983). In 1984 Waterfield provided yet another link between GFs and cancer: partial sequencing of the EGF-receptor (EGFR/ERBB1) uncovered homology to another oncogene, the *erbB* gene of the avian erythroblastosis virus (Downward et al. 1984).

Around the same time, recombinant DNA technology was about to transform biology. Scientists led by Paul Berg, Stanley Cohen, and Herbert Boyer devised a way to splice together different pieces of DNA using restriction enzymes. The benefits of gene technology were soon realized through the industrial production of recombinant GFs, such as human somatostatin and insulin, in the late 1970s. Human insulin (Humulin), made by a young biotechnology company, Genentech, was approved for clinical use in 1982. Subsequent molecular cloning of EGFR by Ullrich and colleagues (Ullrich et al. 1984) boosted the understanding of the intracellular mechanisms of GF action: like EGFR, the majority of GF receptors are single-pass transmembrane proteins harboring an intracellular tyrosine kinase domain (Yarden and Ullrich 1988) or a serine/threonine kinase in the case of TGF-β receptors. A similarly important development impacted our current understanding at the tissue level: similar to

their roles in embryogenesis, GFs are the short-range mediators of the interplay between tumors and both the extracellular matrix (Lee et al. 1984) and stromal, non-cancer cells, such as myofibroblasts, macrophages, and endothelial cells. This cross talk underlies processes fundamental to development and tumor progression, such as sprouting of blood vessels (Folkman 1971) and local inflammatory responses.

Concerted Actions of Growth Factors: Wound Healing as an Example

GFs are involved in progenitor cell migration and proliferation during embryogenesis, and they regulate various processes in adults, such as mammary gland development, inflammation, and wound healing (Werner and Antsiferova 2016). Skin injury and vascular damage instigate local blood clotting and inflammation. While the clot comprises mainly fibrin and fibronectin, it also acts as a pool of GFs essential for wound repair. Different cell types originating from the circulation and adjacent unaffected tissues are attracted to the wound. Like in wounds, malignant tumors often deposit an extracellular matrix and attract lymphoid and other cell types by means of GFs like EGF, which act as chemo-attractants. In addition, the GFs promote proliferation and migration of epithelial cells, thus allowing re-epithelialization of the injured body site. Importantly, re-epithelialization within the wound is tightly controlled, and the process is terminated when the new epidermis fully covers the wound. In contrast, unlimited epithelial cell growth characterizes carcinomas and other tumors, often due to the self-production of GFs or mutations preventing negative feedback of GF action. Wound healing involves loss of keratinocyte cell-to-cell contacts, enhanced migration, and secretion of matrix-degrading proteases. A spectrum of similar processes, collectively called epithelial-mesenchymal transition (EMT), repeatedly occurs during development and in tumor progression, such as in the course of metastasis (Terry et al. 2017). EMT, along with matrix deposition and degradation, is regulated by GFs, especially factors belonging to the TGF-beta family. The family includes activin, a crucial regulator of wound healing, scar formation, fibrosis, and the susceptibility to skin tumorigenesis (Antsiferova and Werner 2012).

Growth Factor Families

GFs are classified according to their structure and the family of receptors they activate. Whereas the definition of GFs may broadly apply also to lymphokines (e.g., interleukins; ILs) and cytokine-like members of the tumor necrosis factor (TNF) family, here we will consider only GFs that bind to receptors harboring kinase activities, either receptor tyrosine kinases (RTKs) or serine/threonine kinase receptors. GFs bind with such receptors with high affinity (in the low nanomolar range) and specificity, and they may be accordingly classified as ligands of a specific receptor subfamily (Blume-Jensen and Hunter 2001). For example, growth factors comprising an epidermal growth factor (EGF) motif bind to the ERBB subgroup of RTKs (also called type I RTKs). We will use this classification to elaborate on the shared mechanism of action of growth factors. Table 1 lists the major families of GFs acting by means of stimulating RTKs. In similarity to the EGF family, the fibroblast growth factor (FGF) family contains at least 22 distinct members and the TGFβ family comprises 35 known members, which fall into several subfamilies. Likewise, the neurotrophin family contains four members.

Biochemical Mechanisms Activated by Growth Factors

Cells of most if not all major tissue types are targets of GFs that mediate their effects by means of receptors with intrinsic tyrosine kinase activity. These receptors have an extracellular ligand-binding domain and an intracellular tyrosine-kinase domain responsible for transducing the mitogenic signal. Ligand binding induces the formation of receptor dimers or oligomers (Schlessinger and Ullrich 1992), and molecular interactions between adjacent cytoplasmic domains lead to activation of kinase function.

Growth Factors, Table 1 The major families of growth factors able to stimulate receptor tyrosine kinases. Listed are the major families of growth factors and the respective RTKs they bind with. The table also identifies the major biological functions of the respective ligands and receptor complexes

Growth factor family	Receptors	Major physiological functions
Epidermal growth factor (EGF) family and the neuregulin (NRG) family (altogether, 11 molecules: EGF, TGF-α, epiregulin, amphiregulin, HB-EGF, betacellulin, epigen), NRG1, NRG2, NRG3 and NRG4	ERBB family ERBB1 (EGFR), ERBB2 (Neu, HER2), ERBB3/HER3 and ERBB4/HER4	Morphogenesis, maintenance and proliferation of epithelial, mesenchymal and nerve tissues
Fibroblast growth factor (FGF) family (altogether, 22 members; FGF1 through FGF10 bind with RTKs) FGFs require heparan sulfate chains to activate their receptors	FGFRs Four members that form seven different receptors through alternative mRNA splicing	Proliferation of many cell types, embryo patterning and organogenesis, bone development, and angiogenesis
Platelets derived growth factors (PDGFs) Five different isoforms consisting of homo- and heterodimers of A- and B-polypeptide chains, and homodimers of C- and D-polypeptide chains	PDGFRs Comprising PDGFR-alpha and PDGFR-beta.	Embryonic development, particularly formation of the kidney, blood vessels, and various mesenchymal tissues. Proliferation of connective tissues, glial and smooth muscle cells
Hepatocyte growth factors (HGFs) The ligands are heterodimers of A and B subunits linked by a disulfide bond	MET family There are three receptors: MET, SEA, and RON	Motogenesis, morphogenesis, angiogenesis myogenesis, and embryonic development
Glial cell line derived neurotrophic factor (GDNF) family In addition to GDNF, the family includes neurturin (NRTN), artemin (ARTN), and persephin (PSPN)	RET RET is an RTK recruited by one of four co-receptors, called GFRs. Alternative mRNA splicing results in three isoforms of RET	Required for enteric neuron development, kidney development, and spermatogenesis
VEGF family There are four members in mammals (VEGF-A, -B, -C, and -D), and PGF (placenta growth factor). A viral gene encodes VEGF-E	VEGFR family There are three RTKs that bind with VEGFs (VEGFR-1, −2, and − 3). VEGFs also binds to receptor neuropilins	Formation and maintenance of the vasculature, including de novo establishment of the circulatory system in embryos and sprouting of existing blood vessels
Angiopoietin family There are four angiopoietin proteins (1 through 4).	TIE family Angiopoietin proteins 1 through 4 serve as ligands of Tie-2 receptors. TIE-1 heterodimerizes with TIE-2	Crucial for vessel stabilization
Stem cell factor (SCF or KIT-ligand) Due to mRNA splicing, there are soluble and membrane forms of SCF	c-KIT/SCFR	Hematopoiesis, gametogenesis, and melanogenesis
Nerve growth factor (NGF) family There are four members: NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and NT-4.	TRK family The family comprises three RTKs: TRK-A, -B, and -C NGF also binds the p75NTR which forms a heterodimer with TRK-A	The neurotrophins promote neurite outgrowth and neural cell survival. The family is involved in synaptogenesis and synaptic transmission
Ephrins There are five ephrin A and three ephrin B ligands, all are membrane bound (insoluble)	EPH family receptors There are 14 RTK members: nine EphAs and five EphBs	Patterning the developing hindbrain, axon pathfinding, angiogenesis, and long-term potentiation Rhombomeres, axon pathfinding, and guiding neural crest cell migration

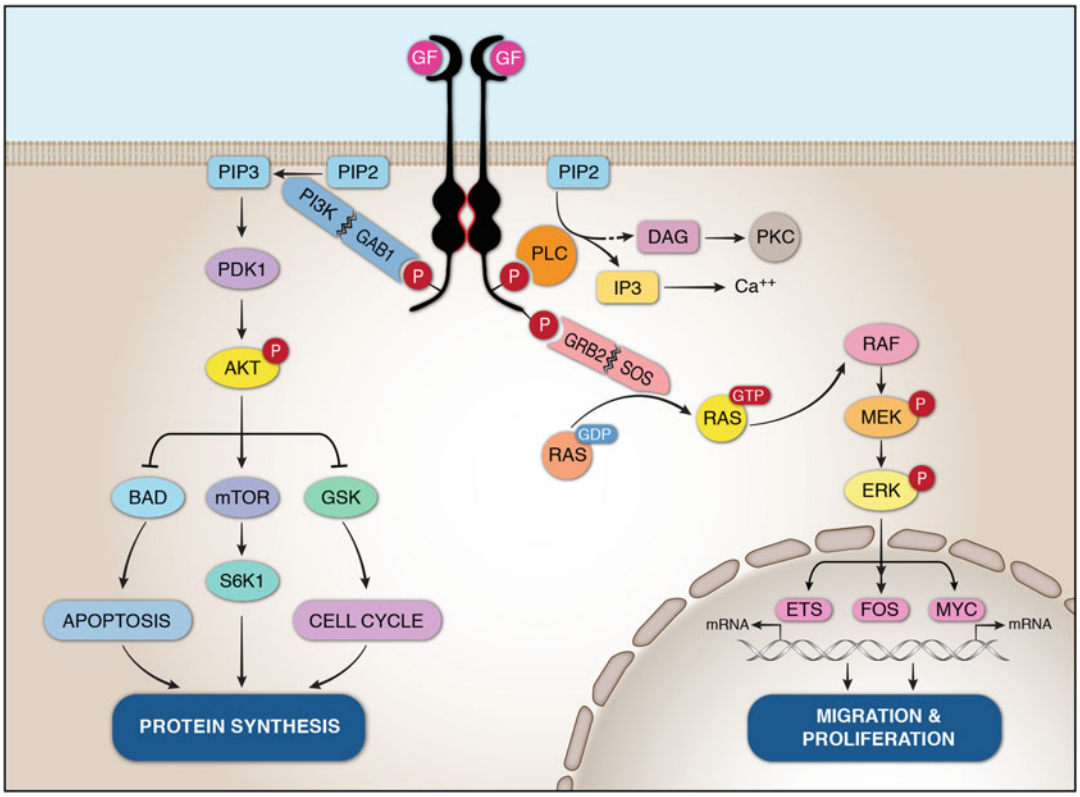
The tyrosine kinase domain is the most conserved and is absolutely required for receptor signaling. Mutation of a single lysine in the ATP (adenosine triphosphate) binding site, which destroys the ability of the receptor to phosphorylate tyrosine residues, completely inactivates biological function (Yarden and Ullrich 1988). The receptor itself is often the major tyrosine-phosphorylated species observed after stimulation with a ligand.

Formation of a signaling complex at the plasma membrane: Tyrosine phosphorylation modulates kinase activity and affects the ability of RTKs to interact with substrates. Conformational changes are relayed across the plasma membrane and orient the kinase domains of two receptors to promote auto- and transphosphorylation of key tyrosines within the kinase's activation loop (Kovacs et al. 2015). This shifts the loop in a manner that releases its autoinhibitory effect. Once activated, the kinase transfers phosphate groups to tyrosine residues of its own, as well as to nearby substrate proteins. Phosphorylated tyrosine residues of RTKs function as docking sites for a wide range of proteins. These proteins include phospholipase C (PLC-g), phosphatidylinositol 3′ kinase (PI3K), RAS guanosine triphosphatase (GTPase) activating protein (GAP), and SRC family tyrosine kinases (Pawson and Scott 1997). These molecules contain non-catalytic domains called SRC homology (SH) regions 2 and 3. SH2 domains bind preferentially to tyrosine-phosphorylated proteins while SH3 domains may promote binding to membranes or the cytoskeleton. Phosphatases like DEP1 and ubiquitin ligases (e.g., CBL) are recruited to the receptor either directly or through adaptor proteins (e.g., SHC and GRB2) with multiple docking sites. Thus, a large multi-protein signaling complex is assembled at the membrane (see Fig. 1).

Phosphoinositol signals and AKT activation: Though the outcome of signaling events initiated by receptors within a family is distinct, some common themes have emerged. Activation of the phospholipid PI3K pathway may be achieved by binding of the regulatory p85 subunit to an activated RTK or through the activation of the small GTP-binding protein RAS. On activation, PI3K induces the formation of phosphatidylinositol (3,4,5)-phosphate (PIP_3), which serves as a docking site for proteins containing phospholipid-binding domains (e.g., PH domains), including protein kinase B and AKT (Luo et al. 2003). Recruitment of AKT to the membrane enables its phosphorylation on two stimulatory residues. PTEN and INPP4B are phosphatases that abolish AKT activation and act as tumor suppressors. AKT has a large number of substrates that regulate mainly survival and metabolism. One example is the proapoptotic protein BAD, which is inhibited following phosphorylation by AKT. BAD induces permeabilization of the mitochondrial membrane to enable the release of cytochrome C. The latter is involved in the generation of a protein complex known as the apoptosome, which cleaves pro-caspases to generate the active form of caspase-9.

Mitogen-activated protein kinase (MAPK) and other pathways: The ERK pathway is a multiple layer kinase cascade, in which the most distal MAPK elements are activated following tyrosine/threonine phosphorylation (Seger and Krebs 1995). In the ERK1/2 kinase pathway, the binding of a ligand to an RTK enables autophosphorylation and recruitment of adaptors like GRB2 and SHC. The adaptors mediate recruitment of a GTP/GDP exchange protein, SOS, which loads GTP onto RAS. Active, GTP-loaded RAS molecules stimulate RAF kinases, which instigates the cascade leading to MEK (MAPK kinase) and then to ERK activation. Active ERK molecules translocate to the nucleus to stimulate multiple transcription programs essential for cell cycle progression and cell migration. The phospholipase C-gamma-protein kinase C (PKC) pathway is instigated on recruitment of PLC-gamma to ligand-bound receptors. The activated enzyme hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to form 2 second messengers, diacylglycerol (DAG), and inositol 1,4,5-triphosphate (IP_3). The binding of IP_3 to receptors on the membrane of the endoplasmic reticulum leads to Ca^{2+} release. Free cytosolic calcium ions, together with DAG, activate members of the protein kinase C (PKC) family, resulting in the phosphorylation of various effector proteins. In addition, cytosolic Ca^{2+} activates the calcium/calmodulin-dependent protein kinases and specific phosphatases.

Signal downregulation by means of endocytosis: Concomitant with the activation of



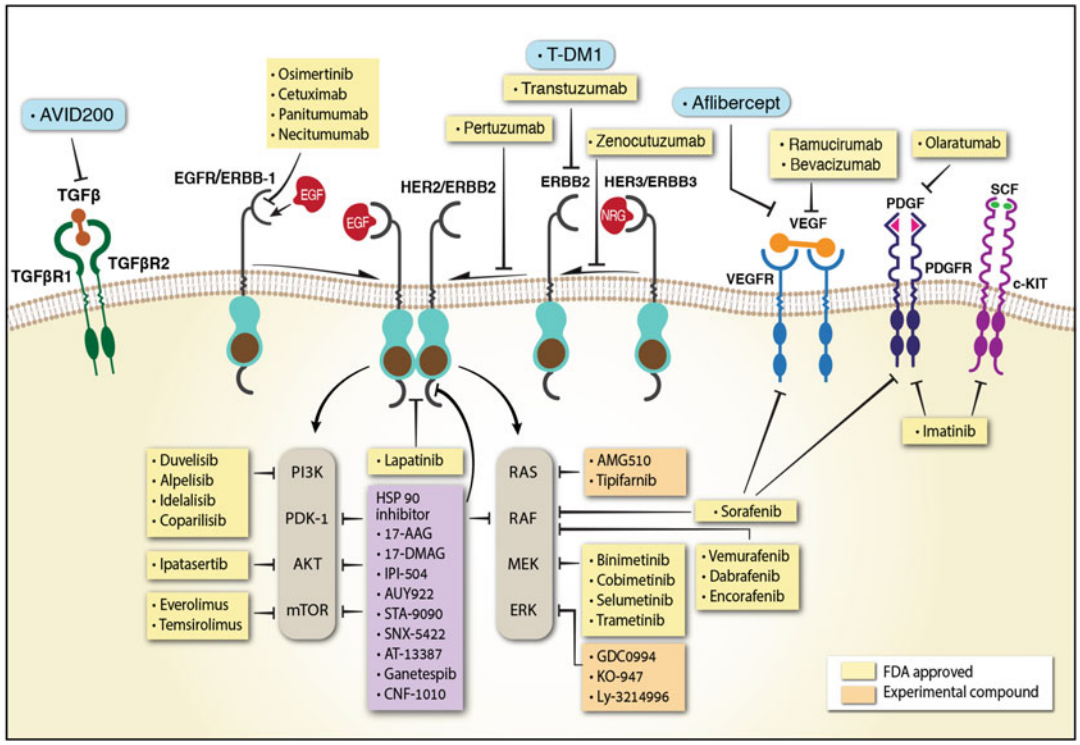
Growth Factors, Fig. 1 Growth factor-induced signal transduction pathways. The binding of growth factors (GFs) to their cognate receptors initiates the activation of intracellular signaling pathways, culminating in the nucleus. Demonstrated here are three of the best-understood pathways: (i) the pathway involving RAS and the MAPK cascade, which activates multiple transcription factors (e.g., ETS, FOS, and MYC) and regulates cell proliferation and migration; (ii) the PI3K/AKT pathway, which modulates the activity of enzymes involved in the control of apoptosis (e.g., BAD) and metabolism (e.g., mTOR, GSK); and (iii) PLC-gamma, through intrinsic SH2 domains, binds with specific phosphotyrosines located in the C-terminal tails of specific RTKs. This facilitates efficient tyrosine transphosphorylation by the RTK. Both tyrosine phosphorylation and membrane translocation of PLC-gamma, through binding of its PH domain to PtdIns (3,4,5)P3, are essential for complete activation of the catalytic activity. Note that several signaling pathways are activated by binding of non-catalytic adaptor molecules (e.g., GRB2 and GAB1), which bind specific phosphorylated tyrosine residues of the receptor, thereby stimulating the first enzyme in a linear cascade of enzymatic reactions

signaling pathways, multiple mechanisms are set in motion to regulate and eventually attenuate signaling by activated growth factor receptors. As in the case of EGFR and other RTKs, receptor internalization coupled to degradation is considered the most immediate and effective process that attenuates signaling by removing receptors from the cell surface and targeting them for degradation in lysosomes (Mosesson et al. 2008). Phosphorylated receptors are tagged by conjugation of a 76-amino-acid-long molecule called ubiquitin; tagging is performed by a three-step enzymatic pathway culminating in a ubiquitin ligase called CBL, which recognizes both the substrate receptor and the ubiquitin donor, an E2 ubiquitin-conjugating enzyme. A series of ubiquitin-binding endocytic adaptors subsequently recognize the ubiquitylated receptor and target it to regions of the plasma membrane called clathrin-coated pits. The latter invaginate to form a clathrin-coated neck that progressively moves inward, eventually generating a coated vesicle. This tiny vesicle shuttles to a vesicular compartment called the early endosome. Through acidification of the endosome's lumen, some ligand-receptor complexes are dissociated, leading to recycling to the plasma membrane,

while other complexes remain intact. The latter are handed to the subsequent sorting compartment, the multivesicular body, where hydrolases are accumulated. It is in this compartment and the subsequent one, the lysosome, where degradation of the receptor is completed. Receptor activation also causes the recruitment of a number of other attenuators, such as tyrosine phosphatases, like the density-enhanced phosphatase-1 (DEP1), which dephosphorylates EGFR, as well as other RTKs, GTPase-activating enzymes (e.g., GAP, which activates Ras' GTPase activity), and other negative regulators. These proteins inhibit the signaling cascade at multiple levels, thus realizing negative feedback loops, which help tuning and regulating the effect of GFs.

Drugs Targeting Growth Factors and Downstream Signaling Pathways

Several of the most formidable human diseases are characterized by aberrant signaling downstream of GFs and their cognate receptors, and thus these are constantly being pursued as targets for pharmacological intervention (Fig. 2). The ability of growth factors to stimulate cell proliferation and survival, for example, may help



Growth Factors, Fig. 2 Drugs blocking growth factor-induced pathways of signal transduction. The signaling cascades set in motion by growth factors can be blocked by pharmacological interventions at different levels. Monoclonal antibodies can specifically bind to growth factors (e.g., bevacizumab) or to the extracellular domain of their cognate receptors (e.g., cetuximab). Tyrosine kinase inhibitors, on the other hand, are small molecules that traverse the plasma membrane and block kinase domain activation. Some of them are specific to certain RTKs, like osimertinib and erlotinib, which are EGFR blockers, while others inhibit the activity of multiple kinases (e.g., lapatinib or sorafenib). Several drugs block enzymes downstream of the receptors, such as drugs targeting individual effectors of the MAPK pathway or the PI3K/AKT pathway. An additional site for pharmacological intervention is the chaperone protein HSP90, which is essential for the stabilization of many components of various signaling cascades (e.g., HER2, RAF1, PDK1, and AKT). Inhibition of HSP90 enhances degradation of its client proteins, leading to downregulation of the respective signals. See Table 2 for a complete list of drugs that have already been approved for clinical use

Growth Factors, Table 2 List of clinically approved antibodies and small-molecule kinase inhibitors targeting growth factor signaling. Note that names ending with "mab" refer to antibodies, whereas names ending with "nib" refer to kinase inhibitors. Drugs are arranged according to the timing of their first approval for clinical application. Also listed are respective major clinical indications. The abbreviations used are epidermal growth factor (EGF), transforming growth factor-b (TGF-b), neuregulin (NRG), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), nerve growth factor (NGF), rearranged during transfection (RET), tyrosine kinase receptor in endothelial cells (TIE), mammalian target of rapamycin (mTOR), anaplastic lymphoma kinase (ALK), breast tumor kinase (BRK), ephrins (EPH), breakpoint cluster region- Abelson (BCR-ABL), cyclin-dependent kinase (CDK), insulin-like growth factor (IGF), Janus kinase (JAK), mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinase (MEK/MAPK), tropomyosin receptor kinase (TRK), tyrosine kinase with immunoglobulin and EGF homology domains (Tie2), insulin receptor precursor (InsR), hepatocyte growth factor receptor (HGFR or c-MET)

Protein target	Name (trade name)	Year of first approval	Company	Condition	Remarks
PDGF	Becaplermin (Regranex)	1997	Ortho-McNeil Pharmaceutical	Treatment of lower extremity diabetic neuropathic ulcers	Available as a topical gel
HER2	Trastuzumab (Herceptin and Biosimilars)	1998	Genentech	Breast cancer, gastric and gastroesophageal junction cancer	Indicated in combination with chemotherapy for patients with tumors overexpressing HER2
PDGFRA, ABL, c-KIT	Imatinib mesylate (Gleevec)	2001	Novartis	Philadelphia chromosome positive chronic myelogenous leukemia (Ph+ CML), Ph+ acute lymphoblastic leukemia (Ph+ ALL), GIST, chronic eosinophilic leukemia (CEL)	
EGFR	Gefitinib (Iressa)	2003	AstraZeneca	Non-small cell lung cancer (NSCLC)	Resistance conferred by the T790M mutation and additional mechanisms
EGFR	Erlotinib hydrochloride (Tarceva)	2004	OSI	NSCLC and pancreatic cancer	Resistance conferred by the T790M mutation and additional mechanisms
EGFR	Cetuximab (Erbitux)	2004	ImClone	Colorectal cancer, squamous cell cancer of head and neck and small cell lung cancer (SCLC)	Applied in combination with chemotherapy. KRAS mutations confer resistance of colorectal tumors
VEGF-A	Bevacizumab (Avastin and biosimilars)	2004	Genentech	Multiple cancers: Ovarian and cervical cancer, colon, rectum, lung, glioblastoma, and renal cancer	Administered in combination with chemotherapy

(continued)

Growth Factors, Table 2 (continued)

Protein target	Name (trade name)	Year of first approval	Company	Condition	Remarks
VEGFR1/2/3, PDGFR, RAF, KIT, FLT3, and RET	Sorafenib (Nexavar)	2005	Bayer	Kidney cancer and certain type of thyroid cancer	EMT might confer resistance
EGFR	Panitumumab (Vectibix)	2006	Amgen	Colorectal cancer with wild-type KRAS	Applied in combination with chemotherapy. KRAS mutations confer resistance of colorectal tumors
PDGFR, VEGFR1/2/3, KIT, FLT3, CSF-1R, and RET	Sunitinib (Sutent)	2006	Pfizer	Renal cell cancer, gastrointestinal stromal tumors (GIST), pancreatic and neuroendocrine tumors	KIT mutations might confer resistance
VEGF-A	Lucentis (ranibizumab)	2006	Genentech	Macular degeneration	
EGFR and HER2	Lapatinib ditosylate (Tykerb)	2007	GSK	Canakinumab (Ilaris)	ER activation might confer resistance
VEGFR1/2/3, PDGFR, FGFR1/3, KIT, and FMS	Pazopanib (Votrient)	2009	GSK	Renal cell cancer, soft tissue sarcoma, gastrointestinal stromal tumors	
mTOR	Everolimus (Afinitor)	2009	Novartis	Some pancreatic cancers, kidney cancer, HER2+ breast cancer and neuroendocrine tumors	
ALK, MET, and ROS	Crizotinib (Xalkori)	2011	Pfizer	ALK-positive NSCLC	ALK mutations might confer resistance
VEGFRs, EGFR, RET, BRK, TIE2, EphRs, and SRC	Vandetanib (Caprelsa)	2011	IPR Pharms	Medullary thyroid cancer	
BRAF	Vemurafenib (Zelboraf)	2011	Daiichi-Sankyo and Genentech	Certain types of blood cancer and melanoma	
JAK	Ruxolitinib (Jakafi)	2011	Novartis	Myelofibrosis	
HER2	Pertuzumab (Perjeta)	2012	Genentech	Breast cancer	Inhibits heterodimer formation by HER2.Applied in combination with trastuzumab and chemotherapy
VEGF-A, VEGF-B, and PGF	Aflibercept (Zaltrap)	2012	Regeneron	Colorectal cancer	A recombinant decoy, combined with chemotherapy

(continued)

Growth Factors, Table 2 (continued)

Protein target	Name (trade name)	Year of first approval	Company	Condition	Remarks
VEGFR1/2/3	Axitinib (Inlyta)	2012	Pfizer	Renal cell and pancreatic cancer	Increased glucose metabolism
RET, MET, VEGFR1/2/3, KIT, TRKB, FLT3, AXL, and TIE2	Cabozantinib (Cometriq and Cabometyx)	2012	Exelixis	Metastatic medullary thyroid cancer, liver cancer and kidney cancer	MET activation might confer resistance
BCR-AAL, VEGFR, PDGFR, FGFR, EPH, SRC, KIT, RET, TIE2, and FLT3	Ponatinib (Iclusig)	2012	Ariad	CML, Ph chromosome positive ALL	BCR-ABL mutations confer resistance
VEGFR1/2/3, BCR-ABL, B-RAF, KIT, PDGFR, RET, FGFR1/2, TIE2, and Eph2A	Regorafenib (Stivarga)	2012	Bayer	Colorectal cancer, gastrointestinal stromal tumors (GIST) and liver cancer	An analogue of sorafenib
EGFR	Afatinib dimaleate (Gilotrif)	2013	Boehringer	NSCLC	Irreversible inhibitor of EGFR and HER2
HER2	Trastuzumab emtansine (Kadcyla)	2013	Genentech	Advanced breast cancer	An antibody-drug conjugate
BRAF	Dabrafenib (Tafinlar)	2013	GSK	Multiple cancers	
MEK1 and MEK2	Trametinib (Mekinist)	2013	GSK	Multiple cancers	
VEGFR-2	Ramucirumab (Cyramza)	2014	Eli Lilly	Certain types of CRC, gastric and esophageal junction cancer, metastatic NSCLC, hepatocellular carcinoma	Delivered as a single agent or combined with chemotherapy
VEGFR, FGFR, PDGFR	Nintedanib (Ofev)	2014	Boehringer Ingelheim	NSCLC and idiopathic pulmonary fibrosis	
ALK, IGF-1R, InsR, ROS1	Ceritinib (Zykadia)	2014	Novartis	ALK-positive NSCLC; applied post emergence of crizotinib resistance	ALK mutations might confer resistance
MEK	Cobimetinib (Cotellic and Zelboraf)	2014	Genentech	Certain forms of melanoma	Used in combination with vemurafenib (Zelboraf) to treat melanoma with BRAF mutations
EGFR	Dacomitinib (Vizimpro)	2014	Pfizer	Certain type of lung cancer	An irreversible inhibitor of EGFR
TGF-β	Pirfenidone (Esbriet)	2014	Shionogi	Idiopathic pulmonary fibrosis	

(continued)

G

Growth Factors, Table 2 (continued)

Protein target	Name (trade name)	Year of first approval	Company	Condition	Remarks
EGFR	Osimertinib mesylate (Tagrisso)	2015	AstraZeneca	NSCLC	Irreversible inhibitor of EGFR, specific to T790M-EGFR
EGFR	Necitumumab (Portrazza)	2015	Eli Lilly	Metastatic squamous non-small cell lung cancer	Applied in combination with chemotherapy drugs
VEGFRs, FGFRs, PDGFR, KIT, RET	Lenvatinib (Lenvima)	2015	Easai	Certain type of thyroid cancer, kidney and liver cancer	MET activation might confer resistance
ALK	Alectinib hydrochloride (Alecensa)	2015	Genentech	ALK-positive metastatic NSCLC	
CDK4 and CDK6	Abemaciclib (Verzenio)	2015	Eli Lilly	Certain type of breast cancer	
ALK and ROS1	Lorlatinib (Lobrena)	2015	Pfizer	ALK-positive metastatic non-small cell lung cancer (NSCLC)	
CDK4 and CDK6	Palbociclib (Ibrance)	2015	Pfizer	Hormone receptor positive and HER2-negative breast cancer	
PDGFRA	Olaratumab (Lartruvo)	2016	Eli Lilly	Soft tissue sarcoma	In combination with doxorubicin
ALK, EGFR	Brigatinib (Alunbrig)	2016	Ariad Pharmaceuticals	EGFR- or ALK-positive metastatic non-small cell lung cancer (NSCLC)	
HER2	Neratinib maleate (Nerlynx)	2017	Puma Biotechnology	Extended adjuvant treatment of adult patients with early-stage HER2-overexpressed breast cancer	
CDK4 and CDK6	Ribociclib (Kisqali)	2017	Novartis and Astex Pharmaceuticals	Certain types of breast cancer	
ALK	Lorlatinib (Lorbrena)	2018	Pfizer	ALK-positive metastatic non-small cell lung cancer (NSCLC) progressing while on other ALK inhibitors	
NTRK	Larotrectinib sulfate (Vitravki)	2018	Bayer	Cancers with NTRK gene abnormalities (fusions)	
FLT3	Gilteritinib fumarate (Xospata)	2018	Astellas Pharma	Relapsed or refractory acute myeloid leukemia (AML) with a FLT3 mutation	

(continued)

Growth Factors, Table 2 (continued)

Protein target	Name (trade name)	Year of first approval	Company	Condition	Remarks
NGF	Cenegermin (Oxervate)	2018	Dompé farmaceutici	Neurotrophic keratitis	
FGFRs	Erdafitinib (Balversa)	2019	Janssen-Cilag SpA	Locally advanced or metastatic urothelial carcinoma	
NTRK	Entrectinib (Rozlytrek)	2019	Genentech	NTRK gene fusion-positive solid tumors or ROS1-positive NSCLC	
CSF1R and FLT3	Pexidartinib hydrochloride (Turalio)	2019	Daiichi Sankyo	Tenosynovial Giant Cell Tumor	
HER2	Fam-trastuzumab deruxetecan-nxki (Enhertu)	2019	Daiichi Sankyo	Unresectable or metastatic HER2-positive breast cancer	HER2-directed antibody and topoisomerase inhibitor conjugate
HER2	Trastuzumab and hyaluronidase-oysk (Herceptin hylecta)	2019	Genentech	HER2-overexpressing breast cancer	A combination of trastuzumab and hyaluronidase, an endoglycosidase
PI3-kinase (PIK3CA)	Alpelisib (Piqray)	2019	Novartis	Certain type of breast cancer	
VEGF-A	Brolucizumab (Beovu)	2019	Novartis	Macular degeneration	
JAK-2	Fedratinib (Inrebic)	2019	Impact Biomedicines	Treatment of myelofibrosis	
KIT	Pexidartinib (Turalio)	2019	Daiichi Sankyo	Treatment of tenosynovial giant cell tumor	
PDGFR	Avapritinib (Ayvakit)	2020	Blueprint Medicines Corporation	Unresectable or metastatic gastrointestinal stromal tumors (GIST) harboring a platelet-derived growth factor receptor alpha (PDGFRA) mutations.	
HER2	Tucatinib (Tukysa)	2020	Seattle Genetics	Advanced unresectable or metastatic HER2-positive breast cancer, including patients with brain metastases, who have received one or more prior anti-HER2-based regimens in the metastatic setting	
MEK1/2	Selumetinib (Koselugo)	2020	AstraZeneca	Treatment of plexiform neurofibroma	In combination with trastuzumab and capecitabine

(continued)

G

Growth Factors, Table 2 (continued)

Protein target	Name (trade name)	Year of first approval	Company	Condition	Remarks
FGFR2	Pemigatinib (Pemazyre)	2020	Incyte Corporation	Metastatic bile duct cancer (cholangiocarcinoma)	
PDGFRα	Ripretinib (Qinlock)	2020	Deciphera Pharmaceuticals	Gastrointestinal stromal tumors	
IGF-1R	Teprotumumab (Tepezza)	2020	Genmab and Roche	Thyroid eye disease	
MET	Capmatinib (Tabrecta)	2020	Novartis	Non-small cell lung cancer (NSCLC)	

enhancing tissue healing and reduce organ damage in diseases of different origins. Recombinant forms of growth factors are currently being tested for potential usefulness in a variety of clinical settings, such as hepatitis, cirrhosis, renal fibrosis, multiple sclerosis, amyotrophic lateral sclerosis, and myocardial ischemia. Recombinant PDGF, for example, is currently employed as a drug (becaplermin) to facilitate the healing of diabetic ulcers (see Table 2). GFs may also be used for the production of artificial tissues, e.g., for skin replacements and bone and connective tissue reconstruction. In contrast, blocking the actions of specific GFs can help reduce uncontrolled cell proliferation, a characteristic of cancer (and also of diabetic retinopathy and vascular stenosis). Drugs in clinical use include small-molecule tyrosine kinase inhibitors (TKIs) and anti-receptor or anti-ligand monoclonal antibodies (mAbs). TKIs penetrate the plasma membrane and interact with the ATP-binding site of the receptor, thus preventing kinase activation. The EGFR's kinase inhibitors gefitinib and erlotinib, for instance, are both approved for a subset of non-small cell lung cancer (NSCLC) patients. Another example is the broad-spectrum inhibitor, sorafenib, targeting both VEGFR and PDGFR, as well as the RAF kinase, which has been approved for the treatment of renal cancer. In contrast to TKIs, anti-receptor monoclonal antibodies (mAbs) bind to the extracellular domain of the receptor and exert inhibition of cell proliferation by several mechanisms (Carvalho et al. 2016). Trastuzumab, which targets HER2/ERBB2, is approved for the treatment of metastasizing breast cancer and is thought to stimulate an immune response against the tumor and to induce receptor internalization. Examples of other mAbs include cetuximab (approved for colorectal and head and neck cancers) and panitumumab (approved for colorectal cancer), which are directed against EGFR and may act also by inhibiting ligand binding. Pertuzumab is another antibody targeting HER2/ERBB2 by inhibiting receptor dimerization. Some of the mAbs are directed against the ligands, rather than the receptors. For example, bevacizumab targets VEGF and inhibits angiogenesis. This mAb has been approved for the treatment of multiple cancers, as well as macular degeneration (Ferrara et al. 2004). Drugs that inhibit the signaling cascades downstream of the receptor at different levels (e.g., inhibitors of the MAPK or the PI3K/AKT pathways) have also been developed. Likewise, chaperone proteins like HSP90, which are essential for the stability and function of many receptors, effectors, and transcription factors, can also be targeted by specific inhibitors (e.g., 17-AAG, 17-DMGA), thus enhancing the degradation of their client proteins. The successful clinical application of RTK-based therapeutics in the last two decades has instigated further research aimed at generating the next generation drugs. Thus, antisense RNAs and aptamers and decoy receptors are being tested for their ability to reduce GF signaling.

Cross-References

▶ Cytokines

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G

Growth Hormone

▶ Ghrelin, Physiological Roles and Clinical Relevance of

Guanyl Cyclase

▶ Guanylyl Cyclase

Guanylate Cyclase

▶ Guanylyl Cyclase

Guanylyl Cyclase

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Synonyms

Guanyl cyclase; Guanylate cyclase

Definition

Guanylyl cyclases (GC) are a family of enzymes (EC 4.6.1.2) that catalyze the formation of the

second messenger cyclic GMP (cGMP) from guanosine triphosphate (GTP). GCs are subdivided into NO-sensitive GCs and transmembrane GCs stimulated by peptide hormones (Koesling et al. 2016; Kuhn 2016).

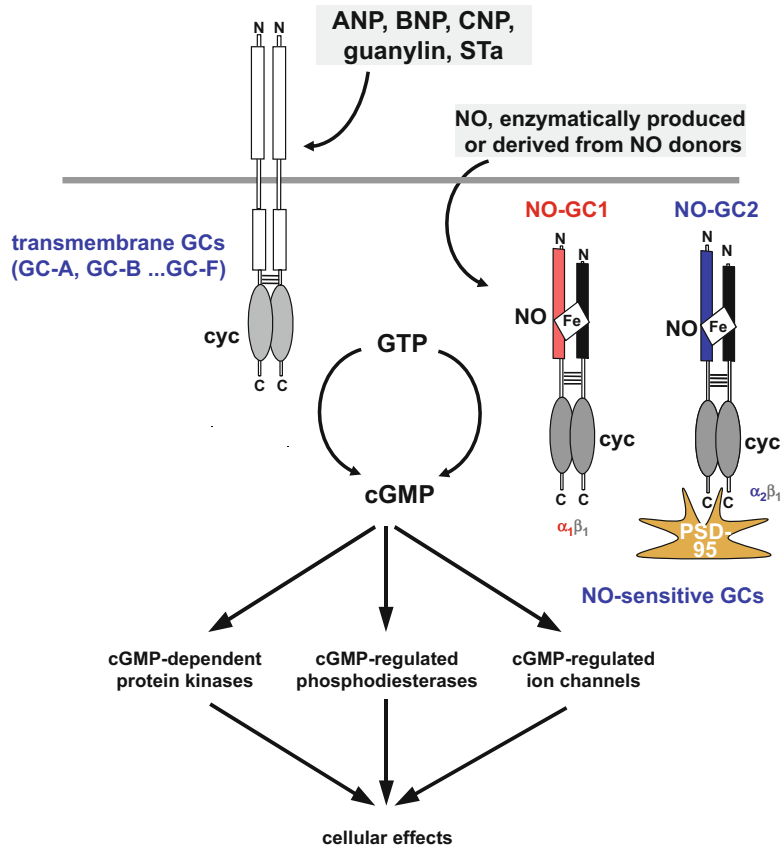
Basic Characteristics

Activation of GCs leads to an increase of the intracellular messenger molecule cGMP. Three different groups of cGMP effector molecules mediate the transduction of the cGMP-signal: cGMP-activated protein kinases, cGMP-regulated phosphodiesterases, and cGMP-gated ion channels, see Fig. 1. The cGMP increases are terminated by cGMP-degrading phosphodiesterases that reduce cGMP levels. cGMP executes established functions in smooth muscle relaxation, inhibition of platelet aggregation, retinal phototransduction, and alteration of synaptic transmission. Moreover, cGMP is involved in regulation of the water/electrolyte balance as well as in bone metabolism, exhibits antifibrotic properties, has been proposed to participate in cell growth/proliferation, and modifies the renin/angiotensin and the immune system.

According to their structural features and their regulation, GCs are grouped into NO-sensitive and transmembrane guanylyl cyclases.

NO-Sensitive GCs

NO-sensitive GCs (NO-GCs) represent the most important effectors for the signaling molecule NO, which is synthesized by NO synthases in a Ca^{2+}-dependent manner. NO-GCs contain a prosthetic heme group, acting as the binding site for NO. Formation of the NO–heme complex leads to a conformational change, resulting in an increase of up to 200-fold in catalytic activity of the enzymes (Koesling et al. 2016). The NO-releasing organic nitrates (see below)



Guanylyl Cyclase, Fig. 1 cGMP-signaling. Shown are the two groups of cGMP-forming guanylyl cyclases (transmembrane and NO-sensitive GCs) and the three effectors, which mediate the cGMP effects (see text for further explanation). *ANP* A-type natriuretic peptide, *BNP* B-type natriuretic peptide, *CNP* C-type natriuretic peptide, *Sta* heat-stable enterotoxin of *Escherichia coli, PSD-95* postsynaptic density protein 95

commonly used in the therapy of coronary heart disease exert their effects via the stimulation of these enzymes.

NO-GCs consist of two subunits, α and β, and two isoforms of the enzyme have been identified: the ubiquitous *NO-GC1* isoform ($\alpha_1\beta_1$) and the less broadly distributed *NO-GC2* isoform ($\alpha_2\beta_1$). Overall, the NO-GC subunits can be divided into four domains: the N-terminal domain, a Per/Arnt/Sim (PAS)-like domain, a coiled-coil domain, and a C-terminal catalytic domain (Koesling et al. 2016). In the N-terminal domain, the homology between the α_1 and α_2 subunit is quite low; the respective region in the β_1 subunit is responsible for heme binding and shares homology to the so-called HNOX (Heme Nitric Oxide/OXygen binding) domains. The cyclase catalytic domains are conserved among the subunits, in the transmembrane GCs as well as in the adenylyl cyclases (see below). The structure of NO-GC under stimulated and nonstimulated conditions has been elucidated and suggests a model of activation, which requires a bidirectional flow of information between the heme containing sensor domain and the catalytic module (Kang et al. 2019).

Both NO-GC isoforms show indistinguishable regulatory properties but may well differ in subcellular distribution as the α_2 subunit of *NO-GC2* is able to interact with PDZ domains and has been shown to be associated with the postsynaptic adapter protein PDS95.

NO-GCs occur in relatively high concentration in vascular smooth muscle cells and platelets as well as in lung, kidney, and brain. The NO-induced increase in cGMP causes smooth muscle relaxation and inhibition of platelet aggregation. Aside from the cardiovascular system, the NO/cGMP cascade has an important function in the nervous system, where it participates in synaptic plasticity, i.e., the use-dependent change in efficiency of synaptic transmission.

The phenotypes of knockout mice deficient in the β_1 or either one of the α subunits of NO-GC support and expand our current understanding of NO/cGMP signaling. The complete NO-GC knock out mice ($\beta_1^{-/-}$), which are completely devoid of NO-GC, show a greatly reduced life expectancy and die of fatal gastrointestinal symptoms. In these mice, NO-induced relaxation is totally abolished as is NO-induced inhibition of platelet aggregation. The mice show a pronounced increase in blood pressure underlining the important role of NO in blood pressure regulation.

The *NO-GC1* ($\alpha_1^{-/-}$)- or *NO-GC2* ($\alpha_2^{-/-}$)-deficient mice with a lack of the respective isoform allow to study the individual role of the GC isoforms (Koesling et al. 2016). Concerning distribution tissues, the isoforms occur in brain in similar amounts; in all other tissues tested, *NO-GC1* represents the major isoform. In aortic smooth muscle cells, *NO-GC2*, representing only 6% of the total GC content in WT, is sufficient to induce complete relaxation albeit with a reduced potency of NO. The results show that in smooth muscle cells, (I) *NO-GC2* can functionally substitute for *NO-GC1* and that (II) a minor cGMP increase induces relaxation. Studies on synaptic plasticity in the visual cortex and hippocampus revealed an involvement of both GC isoforms in long-term potentiation (LTP) suggesting the occurrence of two NO/cGMP-mediated signals that have to work in concert for the induction of LTP. Further studies with the isoform-specific KOs revealed a presynaptic and postsynaptic localization of *NO-GC1* and *NO-GC2,* respectively, in hippocampal glutamatergic neurons. Facilitation of the neurotransmitter release and an increase of postsynaptic responsiveness were assigned to *NO-GC1* and *NO-GC2*, respectively; in both cases, HCN channels were identified as cGMP targets.

Besides NO, other NO-GC-activating substances have been reported: Carbon monoxide (CO) known to bind to heme groups with high affinity activates the enzyme only marginally (three- to fivefold). YC-1 ([3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole]) represents the prototype of new GC-stimulating compounds, the NO-sensitizers (the so-called "GC stimulators"). YC-1 causes a tenfold activation of NO-GCs. Pharmacologically more interesting, YC-1 increases GC's sensitivity toward NO and CO suggesting potential beneficial effects of YC-1 by increasing the responsiveness of NO-GCs toward physiologically occurring NO. Thus,

YC-1 exemplifies a new class of drugs with potential use in the treatment of cardiovascular diseases and YC-1-related compounds with a higher affinity for NO-GCs have been developed (see below; "Drugs Acting on NO-Sensitive GCs"). In addition to these NO-sensitizers, a second class of compounds, the NO-heme-mimetics (the so-called "GC activators") that activate NO-GCs independently of NO by replacing and mimicking the conformation of the activated NO–heme group (cinaciguat, ataciguat) have been identified. The high potency of these NO-heme-mimetics in vivo led to the postulate of a highly responsive oxidized state of NO-GCs occurring under pathophysiological situations such as oxidative stress.

Transmembrane GCs

Transmembrane GCs belong to the group of receptor-linked enzymes with one membrane-spanning region that form homodimers (Kuhn 2016). All of these GCs share a characteristic structural arrangement of an extracellular ligand-binding domain coupled to a conserved intracellular region comprising a kinase homology domain and the cyclase catalytic domain at the C-terminus. As the extracellular ligand-binding domains differ among the isoforms, the transmembrane GCs are activated by different peptide hormones and accordingly, the GCs activated by natriuretic peptides are also referred to as natriuretic peptide receptors (NPRs). The guanylyl cyclase A (*GC-A, NPR-A*) isoform acts as the receptor for the natriuretic peptides ANP and BNP, two primarily cardiac hormones that are involved in the regulation of blood pressure as well as in water and electrolyte balance. ANP- and BNP-induced cGMP increases mediate physiological effects such as smooth muscle relaxation, modulation of endothelial permeability, inhibition of aldosterone secretion in the adrenal cortex, and salt and water excretion in the kidney. During chronic pressure or volume overload, the increased ANP and BNP levels exert local antiproliferative and antifibrotic effects in cardiac cells.

Another GC isoform, *GC-B* (or *NPR-B*), present in fibroblasts, chondrocytes, brain, lung, vascular smooth muscle, and uterus displays the highest affinity for the C-type natriuretic peptide (CNP). CNP is, e.g., produced by vascular endothelial cells and in an autocrine/paracrine fashion exhibits a fundamental role in the regulation of blood flow (Moyes and Hobbs 2019). CNP attenuates cardiac fibrosis and stimulates growth of long bones. Another form of NPRs, *NPR-C*, lacks the GC catalytic domain and contains only a very short intracellular C-terminal region. This receptor has been regarded as a clearance receptor involved in the internalization and degradation of the natriuretic peptides. However, a recently identified role for CNP in angiogenesis and vascular remodeling was found to proceed via *NPR-C*/Gi coupling (Bubb et al. 2019). It will be interesting to see whether CNP-induced *NPR-C* activation represents a general principle and acts as the underlying mechanism of other biological effects as well.

In contrast to other GC isoforms with a broader distribution, GC-C (guanylin receptor) is mainly restricted to the intestinal epithelium. GC-C is activated by the two endogenous peptides, guanylin and uroguanylin, secreted by the epithelium of the intestine, and the heat-stable enterotoxins of bacterial origin causing diarrhea (Rappaport and Waldman 2018). Activation of GC-C causes phosphorylation of the cystic fibrosis transmembrane regulator (CFTR) via the cGMP-activated protein kinases II (PKGII). This leads to the transepithelial secretion of chloride and bicarbonate ions, inhibition of sodium absorption by the sodium/hydrogen exchanger 3 and as a result to a net flux of ions and water into the lumen. In addition to the regulation of fluid secretion, recent research indicates participation of the GC-C/cGMP in various additional aspects of intestinal homeostasis and functions (epithelial proliferation, responses to DNA damage, intestinal barrier integrity, microbiome composition, epithelial-mesenchymal cross-talk, and colorectal cancer prevention) (Rappaport and Waldman 2018). Because of this wide variety of tasks, dysregulation of the signaling cascade has been implicated in the pathogenesis of intestinal motility disorders, in inflammatory bowel disease, and colorectal cancer. Accordingly, GC-C ligands have been developed to allow reconstitution or

enhancement of cGMP signals (see below; "GC-C Ligands").

Further, GC isoforms GC-D (pseudogene in humans), GC-E, and GC-F are restricted to sensory cells. GC-D is only expressed in a subset of olfactory neurons; GC-E and GC-F are exclusively found in the retina. Regulation of these GC isoforms by proteins that interact with the intracellular cGMP-forming domain has been demonstrated and the cGMP-forming activity does not appear to be controlled by the receptor domain.

Drugs

Drugs Acting on NO-Sensitive GCs

NO-Releasing Compounds

Clinically, the organic nitrates (glyceryl trinitrate, isosorbide dinitrate, isosorbide mononitrate, pentaerythrityltetranitrate) are typically used in the treatment of coronary heart disease. They exert their main therapeutic effect by activating NO-GC via NO (Münzel et al. 2014). Altogether, nitrate-induced vasodilation is more pronounced in veins than in arteries; the marked venorelaxation reduces central venous pressure, which, in turn, reduces pre- and cardiac workload thereby relieving symptoms of acute angina pectoris. In addition, the nitrates dilate the coronary arteries and arterioles.

In general, nitrates are nowadays preferentially used to treat or to prevent acute episodes of angina, for this purpose, sublingual and spray formulations of glyceryl trinitrate are preferred to avoid a pronounced first-pass metabolism. This way, a therapeutic blood level of glyceryl trinitrate is rapidly achieved and the antianginal effect occurs within minutes. However, the drug's duration is very short (15–30 min). Similarly, sublingual application of isosorbide dinitrate can be used that has a slightly delayed onset of activity but its duration ($\leq$1 h) is more sustained.

In the past, long-acting nitrates have been used chronically to provide long-term prophylaxis against episodes of angina in patients with frequent angina attacks. Due to lack of positive results on survival in clinical studies, the use of long-acting nitrates has dropped markedly. Long-acting nitrate therapy is only considered if initial therapy with a beta-blocker or a non-dihydropyridine calcium channel blocker is contraindicated, poorly tolerated, or insufficient in controlling angina symptoms (Knuuti et al. 2019). For a long-lasting drug effect, long-acting nitrate formulations (glyceryl trinitrate, isosorbide dinitrate, isosorbide mononitrate, or pentaerythrityltetranitrate) are applied orally at dosages sufficient to provide effective plasma levels after first-pass degradation. Another option to administer glyceryl trinitrate takes advantage of transdermal absorption from slow release preparations.

None of the nitrates releases NO spontaneously, instead they undergo a complex enzymatic bioactivation that either yields NO or bioactive S-nitrosothiols. Two main pathways of biotransformation have been postulated for glyceryl trinitrate/pentaerythrityltetranitrate and isosorbide dinitrate/isosorbide mononitrate, respectively (Münzel et al. 2014). An activity decline of the metabolizing enzymes contributes to the phenomena of nitrate tolerance (see below) but is without a major therapeutically relevant impact.

The major problem of long-term nitrate-based prophylaxis of angina is the loss of drug efficacy. The continuous application of nitrates for more than a few hours leads to the development of nitrate tolerance. Conceivably, tolerance is not caused by a single event but can be attributed to several factors, e.g., it occurs at the level of the drug-metabolizing enzymes and is partially due to an increase in the production of NO-scavenging superoxide ion. In addition, higher cGMP levels have been shown to be counteracted by cGMP-induced activation of phosphodiesterase 5 appearing as desensitization of the NO/cGMP signaling pathway. Moreover, counter-regulatory mechanisms like an increase of sympathetic tone and a rise in activity of the renin/angiotensin system are likely to contribute to the development of tolerance. However, since the marked attenuation of the nitrate effect is rapidly reversible upon discontinuation of the drug, development of tolerance can be controlled by allowing a "nitrate-free" period of about 10–14 h (usually at night) within 24 h.

Molsidomine represents another option for a long-term NO-releasing compound. The former notion that this compound does not induce tolerance and therefore should be used in the "nitrate-free" interval has been left. Molsidomine features a similar pharmacological profile as the organic nitrates. As a pro-drug, it is bioactivated in the liver and yields SIN-1 that decomposes spontaneously in a two-step reaction. In the first step, SIN-1 undergoes a base-catalyzed ring opening to form SIN-1A. This in turn yields NO and the stable metabolite SIN-1C. As the onset of action of molsidomine is comparatively slow, it is not used to acutely treat angina episodes.

The acute adverse effects of the organic nitrates as well as molsidomine are directly related to their therapeutic vasodilation as they can cause orthostatic hypotension, tachycardia, and throbbing headache.

NO-GC Sensitizers and NO-Heme-Mimetics (~ciguat)

The finding of new compounds that stimulate NO-GCs, the sensitizers and the NO-heme mimetics, led to the development of a variety of therapeutically applicable substances (Sandner 2018). After considerable testing of the compounds in animal disease models, they were subjected to clinical testing. In patients with pulmonary arterial hypertension and chronic thromboembolic pulmonary hypertension, the NO-sensitizer *Riociguat* improved hemodynamic parameters and exercise capacity in two phase III studies. Accordingly, *Riociguat* was the first NO-GC sensitizer to be approved for these two states of pulmonary hypertension. Several other NO-GC sensitizers are in preclinical and clinical testing for the treatment of various diseases like endothelial and vascular dysfunction, congestive heart failure, arterial hypertension, kidney diseases, fibrosis, inflammation, and others (Sandner 2018).

Compared to NO-GC sensitizers, the development of heme-mimetics yielded less compounds and their characterization is less advanced. In patients with acute decompensated heart failure, the heme-mimetic *Cinaciguat* improved cardiopulmonary hemodynamics. Yet, *Cinaciguat* caused hypotensive events and the study was terminated. So far, no NO-heme-mimentic is approved or in late stage of development (Sandner 2018).

Drugs Acting on Receptor-Linked GCs

GC-A Ligands

In theory, GC-A ligands, which increase the salt and water excretion, should reduce blood volume and lower blood pressure. *Nesiritide*, human recombinant BNP, was the first member of this new class of drugs approved for the intravenous treatment of acutely decompensated congestive heart failure. The outcome of the treatment with *Nesiritide* to relieve symptoms of decompensated heart failure was negative and the treatment is not recommended (O'Connor et al. 2011). In addition, the use of a synthetic analog of human ANP, *Carperitide*, in heart failure was associated with an increase of mortality (Matsue et al. 2015).

Elevating Natriuretic Peptides

Besides attempts to directly supply natriuretic hormones, e.g., the recombinant form of BNP, there are pharmacological approaches to elevate the concentration of natriuretic peptides by inhibiting their degradation by the neutral endopeptidase. Of special interest were dual-function inhibitors that do not only block the natriuretic peptide-degrading neutral endopeptidase to increase natriuretic peptides but also the angiotensin-converting enzyme (ACE), thereby causing an additional decrease of angiotensin II (Mangiafico et al. 2013). The best characterized of these also called "vasopeptidase inhibitors," omapatrilat, was shown to lower blood pressure in clinical trials; however, because of the occurrence of angioedema, omapatrilat has not been approved. By substituting the ACE inhibitory component (risk of angioedema) by an angiotensin receptor (AT_1) blocker lacking angioedema-inducing properties, the problem of a higher incidence of angioedema has been resolved. *Sacubitril* as inhibitor of the neutral endopeptidase (the so-called neprilysin) increases the concentration of natriuretic peptides, bradykinin and adrenomedullinin (McMurray et al.

2014). *Sacubitril* applied in a fixed combination with *Valsartan* (angiotensin receptor blocker) improved symptoms of heart failure when tested against (a sub-maximal dose of) *Enalapril* alone.

GC-C Ligands

Because of the importance of the GC-C/cGMP signaling cascade in various function of the gastrointestinal system, oral ligands for the receptor domain are of great interest (Rappaport and Waldman 2018). Available synthetic ligands are *Linaclotide,* an analog of the heat-stable enterotoxin, and *plecanatide*, an analog of the GC-C ligand uroguanylin. Both ligands are approved for the treatment of chronic idiopathic constipation and irritable bowel syndrome with constipation. Another uroguanylin analog, *Dolcanatide*, showed positive results when tested in rodent models of intestinal inflammation. If findings suggest that colorectal cancer arises in a microenvironment of GC-C ligand deficiency, oral GC-C ligands may emerge as a possibility to restore GC-C/cGMP signaling and prevent cancer.

Cross-References

- ▶ Adenylyl Cyclases
- ▶ Smooth Muscle Tone Regulation

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Hallucinogen

► Psychedelic Drugs

HCN Channels

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Definition

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are activated by membrane hyperpolarization. Cyclic nucleotides enhance HCN channel activity by affecting the voltage dependence of channel activation (Biel et al. 2009).

Basic Characteristics

A cation current that slowly activates upon membrane hyperpolarization was detected in a variety of excitable cells including neurons and cardiac pacemaker cells in the late 1970s and early 1980s. This current is inwardly directed at the physiological resting membrane potentials of most cells and is carried by Na^+ and K^+ ions and, hence, depolarizes the cell membrane. Additionally, cyclic adenosine monophosphate (cAMP) was found to facilitate current activation in a direct, PKA-independent manner. Because of these unique biophysical properties, this current was termed funny current (I_f) in the heart or hyperpolarization-activated current (I_h) in the brain.

HCN channels represent the molecular correlate of the I_h/I_f current. Structurally, these channels belong to the large superfamily of pore-loop cation channels (Yu et al. 2005). In mammals, the HCN channel family comprises four members (HCN1–4) that share approximately 60% sequence identity to each other. When expressed in heterologous systems, all four HCN channel subtypes generate currents displaying the typical features of native I_h/I_f: (1) activation by membrane hyperpolarization, (2) permeation of Na^+ and K^+ with a permeability ratio P_{Na}/P_K of approximately 0.2, (3) a positive shift in voltage dependence of channel activation by direct binding of cAMP, and (4) channel blockade by extracellular Cs^+ concurred with relative insensitivity to Ba^{2+}. HCN1–4 mainly differ from each other with regard to their speed of activation and deactivation and the extent in which they are modulated by cAMP. In HCN channels, the binding of cyclic nucleotides is not required for activation. However, cyclic nucleotides shift the voltage dependence of activation to a more positive membrane potential and thereby facilitate voltage-dependent channel activation. Unlike HCN2 and HCN4, whose activation curves are shifted by approximately +15 mV by cAMP,

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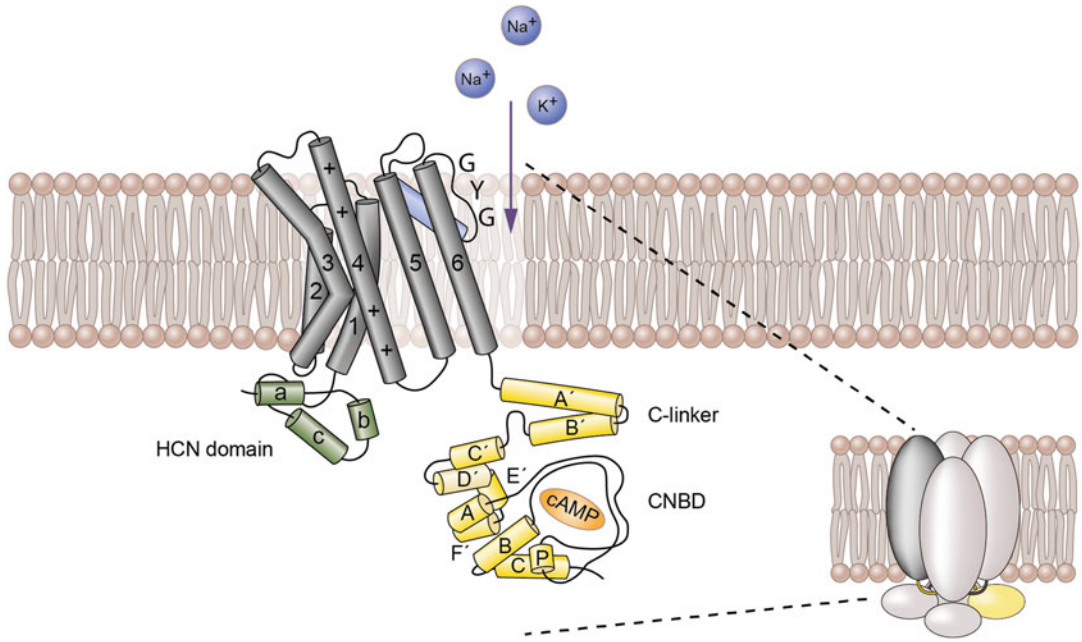
HCN1 and HCN3 are only weakly affected by cAMP.

HCN channels are protein complexes consisting of four subunits that are arranged around a centrally located pore. The proposed structure of a single subunit and a HCN channel tetramer is shown in Fig. 1. The ion channel core comprises six α-helical transmembrane segments (S1–S6) and the ion-conducting pore-loop between the S5 and S6 segment. The pore-loop contains a glycine-tyrosine-glycine (GYG) motif that is responsible for K^+ selectivity in several subtypes of potassium channels. Albeit in the presence of this motif, HCN channels also conduct Na^+. The voltage-sensing domain of HCN channels (S1–S4) contains a positively charged S4 helix containing nine regularly spaced arginine or lysine residues at every third position. Transition metal ion fluorescence resonance energy transfer (tmFRET) has shown that the S4 segment undergoes a substantial (~10 Å) downward movement in response to membrane hyperpolarization (Dai et al. 2019). The resulting conformational change in the channel protein is allosterically coupled by the channel domains to the opening of the ion-conducting pore. In the carboxy-terminus, HCN channels contain a cyclic nucleotide-binding domain (CNBD) that is highly conserved to CNBDs of CNG channels, cAMP-dependent protein kinases, cGMP-dependent protein kinases, and cAMP-guanine nucleotide exchange factors. The CNBD fulfills the role of an auto-inhibitory channel domain. In the absence of cAMP, the cytoplasmic carboxy-terminus inhibits HCN channel gating by interacting with the channel core and thereby shifts the activation curve to more hyperpolarizing

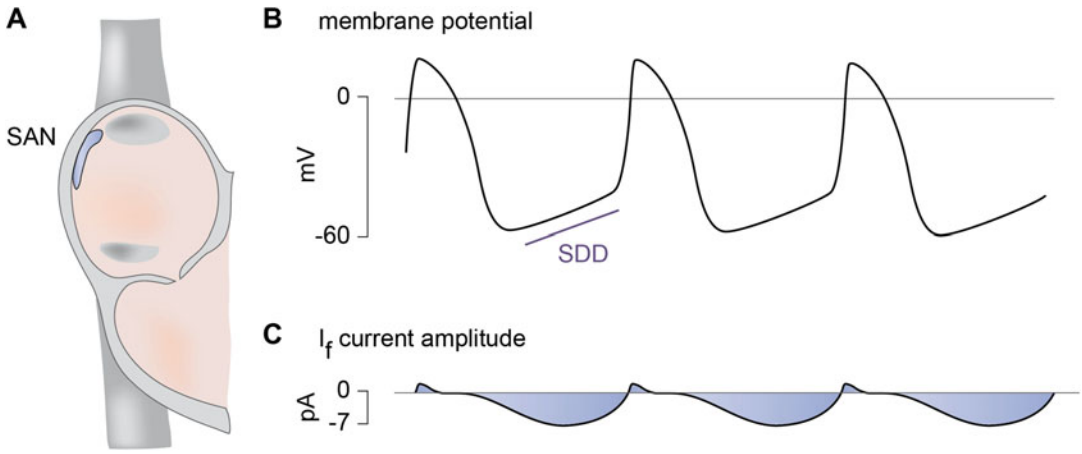


HCN Channels, Fig. 1 Topology of a single HCN channel subunit and an HCN channel tetramer (inset). The channel transmembrane domain (gray) consists of six α-helical segments (S1–S6). Helices S1–S4 serve as the voltage-sensing domain including the voltage sensor (S4) carrying charged arginine or lysine residues regularly spaced at every third position. Helices S5, S6, and the pore helix (purple) form the ion-conducting channel pore. The pore contains the glycine-tyrosine-glycine (GYG) motif that represents the selectivity filter. The C-terminus (yellow) contains the C-linker, composed of six α-helices (A′–F′), and the cyclic nucleotide-binding domain (CNBD), composed of three α-helices (A–C) and a β-roll between helix A and B. Cyclic adenosine monophosphate (cAMP) is bound to the CNBD. The amino-terminal HCN domain (green) consists of three α-helices (HCNa-HCNc). (Figure is courtesy of S. Fenske)

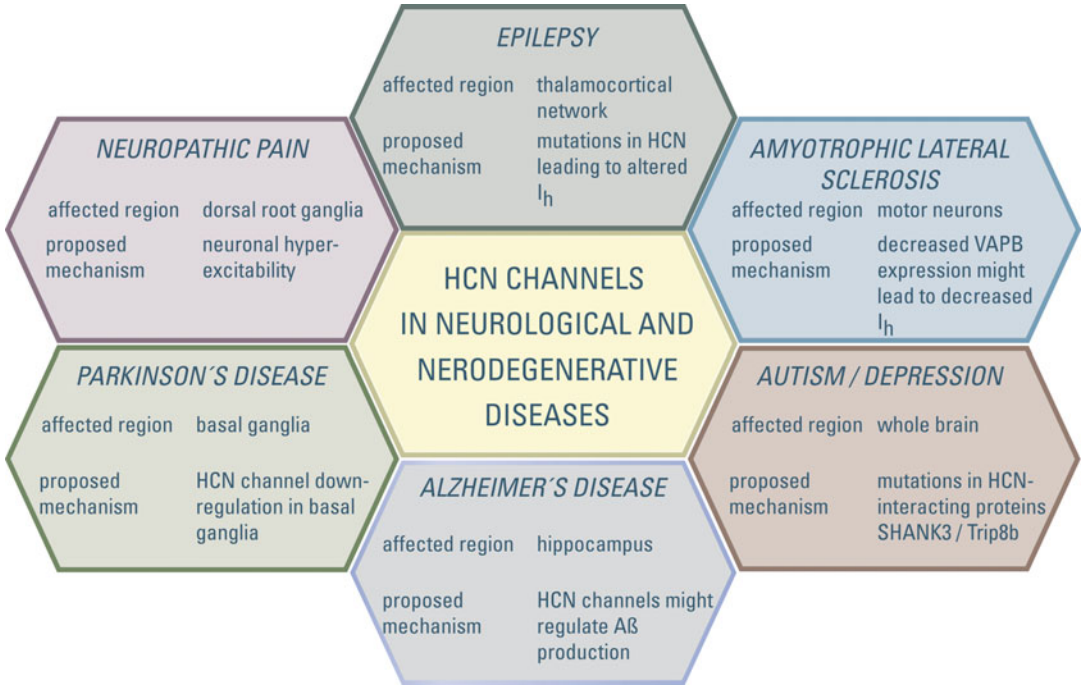
voltages. Binding of cAMP to the CNBD relieves this inhibition. Differences in the magnitude of the response to cAMP among the four HCN channel isoforms are largely due to differences in the extent to which the CNBD inhibits basal gating. Mutating two amino acids in the CNBD of the HCN channel (mHCN2: R591E, T592A) is sufficient to abolish cAMP-binding and cAMP-dependent modulation of I_h (Hammelmann et al. 2019). The amino-terminal HCN domain consists of three α-helices and is unique to HCN channels. Recent evidence suggests that this domain is involved in integration of ligand and voltage gating.

The best-characterized function of I_f is its contribution to the slow diastolic depolarization (SDD) phase in sinoatrial node (SAN) pacemaker cells of the heart (Fig. 2). I_f is activated during membrane hyperpolarization leading to an inward Na^+ current that slowly depolarizes the plasma membrane until it reaches the threshold to fire an action potential. The rhythmic action potentials generated in this way propagate through the conducting systems of the heart and trigger myocardial contraction. The autonomic regulation of I_f and its specific impact on the SDD are still a matter of debate. I_f has been suggested to play a pivotal role in heart rate (HR) acceleration mediated by the sympathetic nervous system, as I_f increases upon binding of cAMP to HCN channels. However, there is strong evidence from human channelopathies and genetically engineered mouse models that suggest HCN channels are not needed for principal heart rate acceleration by the sympathetic nervous system. HCN channels rather set the intrinsic heart rate, blunt the parasympathetic response of the sinoatrial node, and thereby protect the heart from destabilizing influences of the parasympathetic nervous system.

In the brain, I_h fulfills diverse functions: it modulates neuronal excitability as well as the rhythmic activity of spontaneously spiking neurons ("neuronal pacemaking"). Additionally, I_h is one of the critical quantities keeping the resting membrane potential stable and tuned as well as modulating dendritic integration and synaptic transmission. In the brain, HCN channels are tightly regulated by the cytoplasmic auxiliary subunit tetratricopeptide repeat-containing Rab8b-interacting protein (TRIP8b) that interacts with the CNBD and the last three amino acids of the C-terminus. Binding of TRIP8b to the HCN



HCN Channels, Fig. 2 **I_f contributes to the slow diastolic depolarization phase in sinoatrial node pacemaker cells.** (**a**) Scheme of the heart depicting the location of the sinoatrial node (SAN) in the right atrium. (**b**) Specialized pacemaker cells within the SAN generate spontaneous action potentials due to their ability to slowly depolarize (*SDD* slow diastolic depolarization) until the threshold to fire an AP is reached. (**c**) The I_f inward cation current, carried mainly by HCN4 channels, contributes to the SDD and, hence, is important for cardiac pacemaking. (Figure is courtesy of S. Fenske)



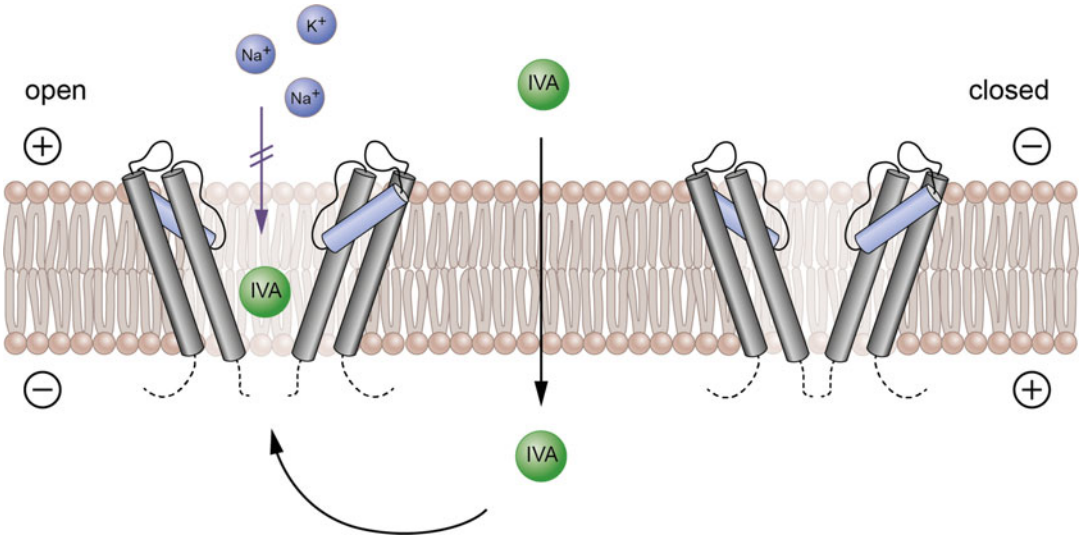
HCN Channels, Fig. 3 Potential use of HCN channel modulators in neurological diseases. HCN channels play a major role in diverse neurological diseases. The neurological disease, the corresponding affected region, and the proposed mechanisms are listed

channel increases cell surface protein expression and thereby the density of I_h. Recent studies showed that deletion of TRIP8b promotes antidepressant behavior in mice. I_h plays an important role in shaping firing properties of neuronal circuits that control motor function, sleep and wakefulness, learning and memory, and somatic sensation. In recent years, HCN channel dysfunction has become associated with pathophysiological states in a variety of neuronal pathways such as epilepsy, neuropathic pain, autism, and neurodegenerative disorders including Parkinson's disease, Alzheimer's disease, or amyotrophic lateral sclerosis (Fig. 3).

Drugs

The only blocker of HCN channel, which is currently used in clinics, is the drug ivabradine. It is approved for the treatment of stable, symptomatic chronic heart failure and stable angina pectoris in patients who are on maximally tolerated doses of beta-blockers or have a contraindication to beta-blocker use (e.g. asthma or obstructive pulmonary disease) (Prescribing Information CORLANOR® 2019).

Studies with heterologously expressed HCN channels indicate that ivabradine preferentially blocks the HCN4 channel, which is highly enriched in pacemaker cells of the SA node (Fig. 4). Ivabradine is able to diffuse into SAN cells where it has free access to the binding site in the inner vestibule of the HCN channel only when channels are in the open state. Hence, the inhibition is use-dependent, and the heart rate reduction with ivabradine is greater when the initial heart rate is high and lower when the initial heart rate is low. In clinical terms, this



HCN Channels, Fig. 4 Use-dependent block of HCN channels by ivabradine. Left, HCN channel in the open conformation at hyperpolarized membrane potential. The S5, S6, and pore helix of two HCN subunits are depicted representing the ion-conducting pore. For clarity transmembrane segments S1–S4, the C-terminus, and HCN domain were omitted. Ivabradine passively diffuses through the cell membrane, enters the open channel, binds within the inner cavity of the channel, and thereby blocks the ion-conducting pore. Right, HCN channel in the closed conformation at depolarized membrane potential. Ivabradine is not able to bind to the closed channel. Hence, it blocks HCN channels in a use-dependent way being more effective at higher heart rates, while its action declines during bradycardia. (Figure is courtesy of S. Fenske)

means that the risk of pronounced bradycardia with ivabradine is lower compared to beta-blockers and non-dihydropyridine calcium channel blockers. At recommended doses, heart rate reduction is approximately ten beats per minute.

Electrophysiological studies in SAN cells isolated from several species have demonstrated that, at therapeutic doses, ivabradine does not act on any other cardiac ion currents (I_K, $I_{Ca,L}$, or $I_{Ca,T}$). Therefore, ivabradine has no effect on cardiac repolarization, on heart muscle function (contraction and relaxation), and on heart and coronary artery adaptation to exercise. Since HCN channels are not expressed in vascular smooth muscle, there are no side effects on the peripheral resistance and blood pressure.

Following oral administration, peak plasma concentrations are reached in approximately 1 h under fasting conditions. Food intake extends absorption by approximately 1 h. Because of pronounced first-pass elimination in the gut and liver, the oral bioavailability is approximately 40%. Ivabradine is metabolized in the liver and intestines by CYP3A4-mediated oxidation. Hence, coadministration of drugs and substances that affect CYP3A4 metabolism (e.g. verapamil, diltiazem, amiodarone, grapefruit juice) should be avoided.

The most common adverse reactions of ivabradine are bradycardia and atrial fibrillation. In addition, ivabradine also blocks HCN channels in photoreceptors and other neurons in the retina. There, the I_h current is involved in the processing of retinal response to bright light stimuli. Via this mechanism of action ivabradine is thought to cause medication-induced perception of phosphenes. Phosphenes are luminous phenomena described as a transiently enhanced brightness in a limited area of the visual field, image

decomposition (stroboscopic or kaleidoscopic effects), or multiple images.

Over the last years, several attempts have been made to develop subtype-specific HCN channel blockers. For example, HCN4-specific blockers would be beneficial for cardiological indications, while HCN1/2-specific blockers would be useful to target neurological or psychiatric disorders. However, a major unsolved problem in the development of such blockers is the very high structural similarity of the four HCN channel types. Recent progress made in determining high-resolution structures of HCN channels may facilitate in the future the specific design of novel classes of compounds tailored to specific HCN channel types.

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Head Injury

► S100 Proteins

Heart Attack

► Myocardial Infarction

Heart Failure

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Synonyms

Cardiomyopathy (see "Definition")

Definition

Chronic heart failure is defined by the presence of typical symptoms and clinical signs, including dyspnea, edema, and fatigue, which are related to reduced cardiac output or elevated filling pressure and caused by structural or functional alterations of the heart (Ponikowski et al. 2016). The diagnosis of heart failure relies on clinical signs and symptoms, echocardiography, and the detection of the biomarker NT-proBNP (see "Natriuretic Peptides"). Normal levels of NT-proBNP largely exclude heart failure. For the pathophysiological understanding and therapy of heart failure it is necessary to distinguish whether systolic or diastolic function is predominantly disturbed. According to left ventricular ejection fraction (LVEF) which is an echocardiographic measure of systolic function, heart failure is classified as heart failure with reduced ejection fraction (HFrEF, LVEF $<$ 40%), mid-range ejection fraction (HFmEF, LVEF 40–49%) or preserved ejection fraction (HFpEF, LVEF $\geq$ 50%) (Table 1) (Ponikowski et al. 2016). Today, HFpEF is considered to account for approximately 50% of heart failure cases (Gladden et al. 2018).

Heart Failure, Table 1 Classification of heart failure

	HFrEF	HFmEF	HFpEF
Criteria	Symptoms and signs		
	LVEF < 40%	LVEF 40–49%	LVEF ≥ 50%
	Elevated natriuretic peptides[a]	1. Elevated natriuretic peptides 2. Relevant structural heart disease or diastolic dysfunction	

HFrEF heart failure with reduced ejection fraction, *HFmEF* heart failure with mid-range ejection fraction, *HFpEF* heart failure with preserved ejection fraction, *LVEF* left ventricular ejection fraction

[a]Elevated natriuretic peptides are frequently observed but not required to diagnose HFrEF according to ESC guidelines (Ponikowski et al. 2016)

In addition, heart failure can be categorized by the New York Heart Association (NYHA) functional classification, ranging from patients showing structural changes without symptoms (NYHA class I) to patients with mild (II), moderate (III), or severe symptoms (IV).

Cases in which the myocardium is directly affected, for example, in ischemia or by gene mutations, are also referred to as cardiomyopathy. However, valve defects, arrhythmia, pericardial effusion and others may cause heart failure as well.

Basic Mechanisms

Heart Failure with Reduced Ejection Fraction

Arterial hypertension and coronary artery disease are the most common reasons for HFrEF (Burchfield et al. 2013). In hypertension, cardiac myocytes respond to increased afterload with hypertrophic growth. While this mechanism is in short term adaptive to cope with elevated wall stress, in long term myocyte growth leads to disproportional wall thickness, capillary rarefaction, and chronic ischemia (Oka et al. 2014). In coronary artery disease, acute or chronic ischemia due to hypoperfusion induces cardiac myocyte necrosis and scarring of the ventricular wall while the remaining cardiac myocytes have to compensate workload. In cases of persistently elevated wall stress, adaptive remodeling may turn into a decompensated state. Ischemia impairs contractile function, drives cardiac myocyte loss, and ends up in chamber dilatation. Increased chamber diameters in turn increase wall stress which again fuels cardiac myocyte hypertrophy and myocardial ischemia (Burchfield et al. 2013).

Cardiac myocyte hypertrophic growth is a complex process involving sarcomere reassembly, protein synthesis and degradation, electrical and metabolic remodeling (Schiattarella and Hill 2015; Burchfield et al. 2013). As part of this process cardiac myocytes undergo profound transcriptional reprogramming including re-expression of the fetal myosin heavy chain isoform and natriuretic peptides (Schiattarella and Hill 2015; Gilsbach et al. 2018). Altered expression and phosphorylation of calcium handling proteins impairs contractility and causes pro-arrhythmogenic action potential prolongation and calcium leakage (Burchfield et al. 2013).

Crosstalk between cardiac myocytes and non-myocytes is crucial for the cardiac remodeling process. Macrophages accumulate in failing heart tissue. Though resident macrophages mostly perish after acute myocardial infarction the pool is rapidly expanding by invasion of spleen-derived monocytes and local macrophage proliferation (Honold and Nahrendorf 2018). In the early phase after myocardial injury, macrophages promote infarct healing by pursuing phagocytosis and secretion of factors that stimulate fibroblast proliferation and activity and thereby facilitate stable scar development. However, sustained activation of fibroblasts in the chronic phase by profibrotic factors derived from macrophages, for example, tumor necrosis factor, leads to excessive collagen production, interstitial fibrosis, and myocardial stiffening (Honold and Nahrendorf 2018; Frangogiannis 2017).

Heart Failure with Preserved or Mid-Range Ejection Fraction

More than HFrEF, heart failure with preserved ejection fraction is considered as a syndrome including multiple other organ systems. HFpEF is frequently accompanied by other disorders including arterial hypertension, diabetes, renal dysfunction, pulmonary disease, sleep disorders, or anemia. Female, aged, and obese individuals are at higher risk to develop HFpEF (Mentz et al.

2014). Due to its multifaceted nature there is no standard experimental model for HFpEF (Valero-Munoz et al. 2017). However, it is a widely accepted concept that cardiac and non-cardiac comorbidities bidirectionally impact each other (Mentz et al. 2014; Gladden et al. 2018). Mechanistically, it has been proposed that multimorbidity promotes a systemic inflammatory state with high levels of pro-inflammatory cytokines, endothelial cell activation, and attraction of circulating immune cells to the heart tissue (Paulus and Tschope 2013). Endothelial cell activation is associated with increased reactive oxygen species production and reduced nitric oxide (NO) bioavailability. Lack of endothelial cell-derived NO impairs the production of cyclic guanosine monophosphate (cGMP) in cardiac myocytes and thereby promotes cardiac myocyte hypertrophy and diastolic dysfunction (Paulus and Tschope 2013).

Heart failure with mid-range ejection fraction has been newly introduced to the classification in the 2016 revision of the European Society of Cardiology guidelines to acknowledge the gap of evidence for patients with left ventricular ejection fraction of 40–49% (Ponikowski et al. 2016). It remains unclear whether these patients represent a separate collective or a transition area between HFpEF and HFrEF and what is the underlying pathophysiology.

Neuroendocrine Activation

Chronic heart failure is accompanied by an activation of the sympathetic nervous system and the renin-angiotensin-aldosterone system (RAAS). In the initial phase, this is a compensatory mechanism to increase cardiac output and sustain organ perfusion. However, in long term sympathetic drive and RAAS activation promote adverse cardiac remodeling and failure.

Adrenergic activation increases vascular tone via activation of α_1-adrenergic receptors (Lymperopoulos et al. 2013). In the heart, adrenaline acts predominantly on β_1-adrenergic receptors to improve cardiac inotropy and relaxation through phosphorylation of L-type calcium channels, ryanodine receptors, and phospholamban. As a consequence, adrenergic stimulation increases energy and oxygen demand. Sustained adrenergic activation drives the risk for ventricular arrhythmia and leads to cardiac myocyte hypertrophy and interstitial fibrosis (Engelhardt et al. 2004; Lymperopoulos et al. 2013). Adrenergic stimulation, hypotension, and low sodium levels are key drivers for the release of renin from the kidney. Renin mediates the cleavage of circulating angiotensinogen to angiotensin I, which is then converted to angiotensin II by the angiotensin-converting enzyme (Lother et al. 2015). Angiotensin II mediates its effect on the heart and the vasculature through activation of angiotensin II type 1 receptors (AT_1). Angiotensin II is vasoconstrictory and increases blood pressure but independently promotes cardiac myocyte hypertrophy and fibroblast activation (Karnik et al. 2015; Hein et al. 1997). In addition to its direct effects of the heart, angiotensin II increases the production of aldosterone in the adrenal gland (Lother et al. 2015). While the physiological role of aldosterone is to regulate salt and water homeostasis in the kidney, it acts detrimentally on the cardiovascular system by activating the mineralocorticoid receptor in different cell types (Lother and Hein 2016; Cole and Young 2017).

Natriuretic Peptides

In response to increased wall stress the expression of natriuretic peptides type A and B (ANP, BNP) is increased in heart failure. Natriuretic peptide precursor B is secreted and the N-terminal fragment (NT-proBNP) is cleaved. While NT-proBNP can be used as a biomarker, active BNP binds to the extracellular domain of the natriuretic peptide receptor A, a membrane-bound guanylate cyclase, that facilitates the production of intracellular cGMP (Kuhn 2016). cGMP is vasodilatory and thereby increases renal blood flow and diuresis and lowering blood pressure. In the heart, cGMP signaling has beneficial effects by inhibiting hypertrophy, inflammation, and fibrosis (Kuhn 2016; Braunwald 2015). Together with other

peptides, including angiotensin II, natriuretic peptides are degraded by the endopeptidase neprilysin (Braunwald 2015).

Pharmacological Intervention

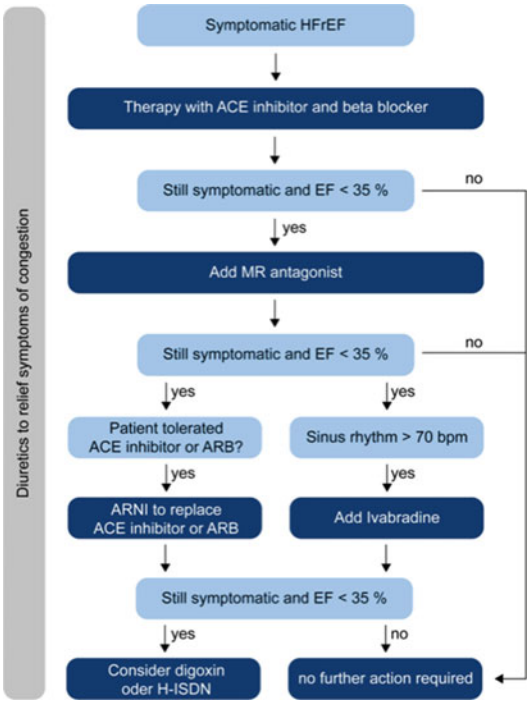
The current guideline recommends initial treatment of HFrEF patients to receive a diuretic (to relieve congestion), an ACE inhibitor (or angiotensin receptor blocker, ARB) plus a beta-blocker (BB). RAAS inhibitors, beta-blockers, and MRAs have been shown to improve survival of patients with HFrEF (Ponikowski et al. 2016). For HFpEF, no pharmacological treatment has shown to lower mortality of patients yet (Ponikowski et al. 2016) (Fig. 1).

Diuretics

In order to reduce congestive symptoms, diuretics are recommended in HFrEF. Diuresis lowers body fluid retention and thus reduces edema, breathlessness, and other related symptoms. The effects of diuretics of mortality of heart failure have not been studied in randomized clinical trials. If renal function is normal or medium impaired (eGFR > 30 mL/min/1.73 m^2), thiazide diuretics, for example, hydrochlorothiazide or chlorthalidone, may be used. With stronger impairment of renal function, loop diuretics (e.g., furosemide, torasemide) are recommended. Typical side effects are hypokalemia, hyponatremia, hypovolemia, renal dysfunction, hypotension, or hyperuricemia.

Beta Blockers (BB)

In HFrEF, the sympathetic system is activated to compensate for the loss of contractile function and elevated catecholamine levels are associated with increased mortality (Cohn et al. 1984). However, it took three decades until the protective effect of BB in HFrEF were shown in clinical trials in 1999 (CIBIS-II and MERIT-HF trials) (Ponikowski et al. 2016). Bisoprolol, carvedilol, metoprolol, and nebivolol were shown to reduce mortality and morbidity in HFrEF patients. This effect was even observed in the presence of an ACEI. Thus, ACEI and BB act synergistically and should be given together. It is essential that BB therapy starts with a very low dose to prevent acute decompensation of heart failure, and BB doses should be gradually up-titrated to the maximum recommended or tolerated dose. Contraindications for BB (second- or third-degree AV block, critical limb ischemia, asthma) should be observed. Typical side effects are bardykardia, hypotension, AV block, and bronchoconstriction.



Heart Failure, Fig. 1 Pharmacological treatment of heart failure with reduced ejection fraction. Treatment algorithm for patients with symptomatic heart failure with reduced ejection fraction according to present ESC guidelines. (Modified from Ponikowski et al. 2016). ACE, angiotensin converting enzyme; ARB, angiotensin receptor blocker; ARNI, angiotensin receptor-neprilysin inhibitor; EF, ejection fraction; HFrEF, heart failure with reduced ejection fraction; H-ISDN, hydralazine-isosorbide dinitrate; MR, mineralocorticoid receptor

ACE Inhibitors

ACEI are the first drugs which have been proven in clinical trials to increase survival of HFrEF patients (enalapril; CONSENUS trial, 1987 (Ponikowski et al. 2016)). They improve heart

failure symptoms, increase exercise capacity, and lower the risk of hospitalization. ACEI prevent the generation of angiotensin II and thus reduce systemic effects of RAAS activation in HFrEF, lowering afterload, reducing blood pressure, and inhibiting cardiovascular remodeling and hypertrophy (Lother and Hein 2016). Several ACEI can be prescribed to treat HFrEF. Frequently used ACEI are ramipril, enalapril, and lisinopril. Contraindications which prevent use of ACEI are angioedema, bilateral renal artery stenosis, or pregnancy. Typical side effects are hyperkalemia, renal dysfunction, hypotension, and cough. If cough does not resolve, the patient may be switched to an ARB.

Angiotensin Receptor Blockers (ARB)

Several blockers of the G-protein-coupled angiotensin II type 1 (AT_1) receptor (ARB) are available, including candesartan, valsartan, and losartan. They inhibit the vasoconstrictory action of angiotensin II in the vasculature and in cells of the heart to attenuate cardiac remodeling due to RAAS activation. The evidence of reduction of mortality of HFrEF patients is stronger for ACEI than for ARBs (Ponikowski et al. 2016). Thus, ARBs are recommended for patients who do not tolerate an ACEI. Contraindications and side effects are similar to ACEI with the exception of cough which is typically associated with ACEI-induced accumulation of bradykinin. Due to increased risk for hyperkalemia, the combination of ACEI with ARBs is no longer recommended (Ponikowski et al. 2016).

Angiotensin and Neprilysin Inhibitors

Inhibition of neprilysin prevents the degradation of natriuretic peptides and thereby enhances their beneficial effects in heart failure. However, neprilysin inhibition may increase the levels of other vasoactive peptides, for example, angiotensin II, endothelin-1, and bradykinin, which partially counteracts the beneficial effect (Braunwald 2015). As a consequence, a combination drug consisting of the angiotensin receptor blocker valsartan and the neprilysin inhibitor sacubitril has been developed. In the PARADIGM-HF trial, sacubitril-valsartan significantly reduced the rate of death from cardiovascular causes or first hospitalization for heart failure in HFrEF patients when compared to an ACE inhibitor (McMurray et al. 2014). Subsequently, sacubitril-valsartan was evaluated in patients with HFpEF, but did not reduce the rate of hospitalizations for heart failure or cardiovascular mortality (Solomon et al. 2019). Interestingly, subgroup analysis revealed a potential benefit of sacubitril-valsartan in women with HFpEF (Solomon et al. 2019**;** O'Connor and deFilippi 2019). Various differences in the pathophysiology of HFpEF between males and females have been described (Beale et al. 2018), but at present the underlying reasons for that finding remain speculative.

Mineralocorticoid Receptor Antagonists

Mineralocorticoid receptors (MR) belong to the family of ligand-activated nuclear transcription factors. MR is expressed in different cell types of the cardiovascular system, including cardiac myocytes, endothelial cells, and immune cells (Lother and Hein 2016; Lother et al. 2015; Cole and Young 2017). MR activation in heart failure drives cardiac myocyte hypertrophy, inflammation, and extracellular matrix deposition. In addition, MR takes a pivotal role in the translation of cardiovascular risk factors into cardiac disease (Lother and Hein 2016).

The use of the steroid-based competitive MR antagonists spironolactone or eplerenone improved mortality and reduced mortality of patients with mild to severe HFrEF in large randomized trials (Pitt et al. 1999, 2003; Zannad et al. 2011). In HFpEF, the TOPCAT trial spironolactone did not significantly reduce cardiovascular mortality (Pitt et al. 2014). However, significant discrepancies in event rates, responses to spironolactone, and in the reported use versus metabolite concentrations of spironolactone severely compromise the validity of the study (de Denus et al. 2017). Another phase III trial on spironolactone in HFpEF is ongoing (SPIRIT-HF, EudraCT 2017-000697-11).

Ivabradine

Ivabradine is a HCN channel inhibitor. Reduction of the pacemaker current (I_f) delays f spontaneous depolarization in sinoatrial node myocytes and

thereby decreases heart rate in sinus rhythm (Bois et al. 1996). High heart rate is considered to drive the pathophysiology of heart failure, potentially due to altered calcium cycling and a mismatch of increased oxygen consumption but decreased coronary perfusion time (Grande et al. 2018). In a phase III clinical trial, ivabradine improved the outcome of patients with reduced ejection fraction, mostly driven by a reduced hospitalization rate (Swedberg et al. 2012).

Additional Drugs

In case the HFrEF patients are still symptomatic with the combination of these drugs, hydralazine and isosorbide dinitrate or digitalis glycosides are additional options. Recently, inhibitors of the sodium/glucose transporter 2 (SGLT2) have shown beneficial effects in patients with HFrEF. SGLT2 is the main glucose transporter mediating glucose reabsorption from kidney tubules. SGLT2 inhibitors, for example, dapagliflozin and empagliflozin, are approved for treatment of type II diabetes. By preventing renal glucose reabsorption, they reduce blood glucose levels. In a recent trial, dapagliflozin significantly reduced cardiovascular mortality in HFrEF patients with NYHA II-IV even independent of diabetes (McMurray et al. 2019). The mechanism of action has not been uncovered (Packer 2020) and SGLT2 inhibitors have not been included in clinical treatment guidelines for HFrEF yet.

Conclusion

Pharmacological inhibition of neuroendocrine activation of the adrenergic and renin-angiotensin-aldosterone systems is well established to reduce morbidity and mortality of patients with HFrEF. The underlying molecular mechanisms of HFrEF involve multiple signaling events at the cardiac myocyte cell surface, intracellular pathways (Schmid et al. 2015) as well as long-term transcriptional adaptations (Gilsbach et al. 2018). In contrast, further studies are required to uncover the molecular pathophysiology of HFpEF and to develop clinical treatments which improve prognosis of HFpEF.

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Heat Shock Proteins (Hsp)

► Chaperones

Hedonic Appetite

► Ghrelin, Physiological Roles and Clinical Relevance of

Hematopoietic Growth Factors

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Synonyms

Granulocyte colony-stimulating factor (G-CSF), filgrastim, pegfilgrastim and lenograstim; Granulocyte macrophage colony-stimulating factor (GM-CSF), molgramostim and sargramostim; HGFs; EPO, recombinant erythropoietin, epoetin and darbepoetin alfa; Interleukin 11 (IL-11)

Definition

Hematopoietic growth factors (HGFs) are a specific type of cytokines that regulate hematopoiesis, which is a process related to the production of blood cells in the bone marrow. More specifically, HGFs directly stimulate the proliferation, differentiation, and maturation of bone marrow cells, originating functional blood cells (Silva et al. 2019; Pérez-Ruixo 2019; Walsh 2013; Groopman et al. 1989).

HGFs molecules are proteins and peptides produced through DNA recombinant techniques and used in the management of diseases related to blood and bone marrow. Food and Drug

Administration (FDA) and European Medicines Agency (EMA) approved several HGFs for clinical use (Table 1), which include recombinant erythropoietin or epoietin (EPO) and darbepoetin alfa, granulocyte colony-stimulating factor (G-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF), and interleukin 11 (IL-11) (Silva et al. 2019; Pérez-Ruixo 2019; Walsh 2013; Kaushansky 2006).

Erythropoietin (EPO)

EPO is mainly synthetized in the kidneys, being released in response to tissue hypoxia, that is, when the blood oxygen level is low. Nonetheless, it was detected that the liver and brain also produce EPO (Silva et al. 2019; Pérez-Ruixo 2019; Walsh 2013; Elliott et al. 2008).

Mechanism of Action

EPO stimulates and regulates erythropoiesis, which is the body's mechanism of producing red blood cells. The result of this process is an increase of differentiated red blood cells in the bloodstream (Silva et al. 2019; Pérez-Ruixo 2019; Walsh 2013). Briefly, EPO binds directly to the red blood cells surface receptors, originating their conformational change, which brings the Janus family tyrosine protein kinase 2 (JAK2) to

Hematopoietic Growth Factors, Table 1 Examples of recombinant hematopoietic growth factors (HGFs) and respective clinical use

HGFs	Clinical use	References
Epoetin alfa and epoetin zeta	Anemia related to different conditions	Silva et al. 2019; Walsh 2013; (FDA), FDA 2017a
Epoetin beta and epoetin theta	Anemia related to chronic renal failure or nonmyeloid malignancies originated by chemotherapy	Silva et al. 2019
Darbepoetin alfa	Anemia related to renal failure or myelosupressive chemotherapy	Silva et al. 2019
G-CSF (granulocyte colony-stimulating factor) or filgrastim, pegfilgrastim, and lipegfilgrastim	Neutropenia in patients undergoing myelosupressive chemotherapy and myeloablative chemotherapy, patients with idiopathic, congenital, or cyclic neutropenia, and patients with hematopoietic syndrome of acute radiation syndrome	Silva et al. 2019; Walsh 2013
	Amyotrophic lateral sclerosis	(EMA), E.M.A. 2015a
	Spinal cord injury	(EMA), E.M.A. 2015b
GM-CSF (granulocyte macrophage colony-stimulating factor) or molgramostim	Acute respiratory distress syndrome	(EMA), E.M.A. 2016
GM-CSF (granulocyte macrophage colony-stimulating factor) or sargramostim	Neutropenia recovery after: Induction chemotherapy in acute myelogenous leukemia, autologous transplantation of peripheral blood progenitor cells, autologous and allogenic bone marrow transplantation for myeloid reconstitution, bone marrow transplantation failure or engraftment delay Decrease the mortality of patients with hematopoietic syndrome of acute radiation syndrome	(FDA), FDA 2017c, 2018b
IL-11 (interleukin-11) or oprelvekin	Avoidance of thrombocytopenia and need of platelet transfusions after chemotherapy treatments	Silva et al. 2019; Walsh 2013; Lipiäinen et al. 2015

the vicinities and originates its phosphorylation, promotes the phosphorylation of tyrosine residues in the EPO receptor cytoplasm, and activates signaling molecules. This mechanism induces the proliferation and differentiation of red blood cells precursors while avoids their apoptosis (Elliott et al. 2008).

Clinical Use

Epoetin, which is the recombinant form of human erythropoietin, is used to manage anemia caused by different disorders, reducing or avoiding the patient's need for blood transfusions. For example, to treat patients with chronic renal failure, who had myelosuppressive chemotherapy, undergoing bone marrow transplantation, and on antiretroviral therapy or hepatitis C infection. There are five different subtypes of epoetin clinically available, which include the epoetins alfa, beta, theta and zeta, and darbepoetin alfa. Among these, epoetin alfa was first approved, although this subtype of epoetin show several side effects, such as hypertension and other cardiovascular events, thromboembolism, seizures, stroke, tumor return or development, and anemia (Silva et al. 2019; Walsh 2013; (FDA), F.a.D.A. 2017a). Darbepoetin alfa was also approved for the same clinical applications, showing the same side effects ((FDA), F.a.D.A. 2017b). Epoetin beta and epoetin theta were further approved to treat similar conditions. The most common side effects observed for epoetin beta are hypertension, headache, and thromboembolic events (Silva et al. 2019; (EMA), E.M.A. 2015c, 2012; (FDA), F.a.D.A. 2018a), while epoetin theta can also cause skin reactions, joint pain, and influenza-like symptoms in addition to these effects ((EMA), E.M.A. 2009a, b). Latter, epoetin biosimilars that include the subtypes alfa and zeta were approved for the same therapeutic indications as the reference epoetin alfa. In addition, these biosimilars have also been used to support surgeries in anemic patients, avoiding the need for blood transfusions. Regarding side effects, epoetin biosimilars show the same as the reference epoetin. Although the use of these biosimilars is more cost-effective than the reference medicines, some clinicians are uncertain about its real effectiveness and prefer to prescribe the reference epoietin (Santos et al. 2019).

Granulocyte Colony-Stimulating Factor (G-CSF) and Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF)

G-CSF and GM-CSF are blood cell growth factors responsible for the proliferation, differentiation, and maturation of white blood cells from hematopoietic stem cells (Silva et al. 2019; Walsh 2013).

Mechanism of Action

Bone marrow stromal cells, macrophages, and fibroblasts generate G-CSF, which regulates the proliferation and maturation of neutrophils, induces the proliferation and migration of endothelial cells, and participates in the differentiation of other hematopoietic cells (Silva et al. 2019; Walsh 2013). G-CSF stimulates the neutrophils production, upon binding to the receptors expressed on the surface of myeloid progenitor cells, changing their conformation, and triggering several signaling pathways, such as JAK/STAT, PI3K/AKT, and MAPK/ERK. Besides, G-CSF improves the phagocytic activity of neutrophils, avoiding infections, and the neutrophils recovery after chemotherapy-induced neutropenia (Dwivedi and Greis 2017).

Endothelial cells, fibroblasts, macrophages, and T-lymphocytes produce GM-CSF, which binds to the respective cells receptors and stimulates the JAK2, STAT1, and STAT3 signaling transduction pathways that promote the proliferation of neutrophils, and other hematopoietic cells, such as macrophages, eosinophils, erythrocytes, and megakaryocytes (Silva et al. 2019; Walsh 2013; Chen et al. 2006).

Clinical Use

G-CSF and GM-CSF have been widely used in the treatment of neutropenic patients, although they also show effectiveness against some infectious diseases, cancer, and in the recovery of patients undergoing bone marrow transplants. The recombinant forms of G-CSF under clinical use are called filgrastim and lenograstim, while the recombinant forms of GM-CSF are called molgramostim and sargramostim (Silva et al.

2019; Pérez-Ruixo 2019; Walsh 2013). Among these, filgrastim was the first approved to reduce neutropenia related to different conditions, including in patients undergoing myelosupressive chemotherapy (except in chronic myeloid leukemia and myelodysplastic syndrome) and myeloablative chemotherapy; patients with idiopathic, congenital, or cyclic neutropenia; and patients with hematopoietic syndrome of acute radiation syndrome (i.e., patients acutely exposed to myelosupressive radiation). To improve the pharmacokinetics of filgrastim, pegfilgrastim and lipegfilgrastim were approved for the same therapeutic indications. These medicines contain as active substance a filgrastim molecule linked to a polyethylene glycol (PEG) molecule, which improves its half-life, reducing the number of administrations per patient. The most common side effects associated to filgrastim and derivatives are bone and muscle pain, nausea, fever, rash, headache, cough, difficulty breathing, and nose bleeding. Due to the expiration of patents, several biosimilars were approved for the same therapeutic indications as the references filgrastim and pegfilgrastim. Nonetheless, some of these biosimilars were withdrawn (Silva et al. 2019; Walsh 2013). Furthermore, filgrastim has been granted with two orphan designations for the management of amyotrophic lateral sclerosis ((EMA), E.M.A. 2015a) and spinal cord injury ((EMA), E.M.A. 2015b).

Regarding GM-CSF, molgramostim was granted with the orphan designation to treat the acute respiratory distress syndrome, which is a lung disorder that originates insufficient oxygen supply from the lungs into the blood ((EMA), E.M.A. 2016). In contrast, sargramostim is indicated to improve neutropenia recovery and avoid infections after induction chemotherapy in acute myelogenous leukemia, autologous transplantation of peripheral blood progenitor cells, autologous and allogenic bone marrow transplantation for myeloid reconstitution, bone marrow transplantation failure or engraftment delay, and to decrease the death of patients with hematopoietic syndrome of acute radiation syndrome. Sargramostim is well tolerated by most patients. Some of the reported side effects include pain, thrombosis, hepatic dysfunction, hypotension, arrhythmia, syncope, and eosinophilia ((FDA), F.a.D.A. 2017c, 2018b).

Interleukin-11 (IL-11)

IL-11 is a cytokine that belongs to the interleukins group, which are cytokines that regulate cells growth, and the inflammatory and immune responses within the body (Silva et al. 2019; Walsh 2013).

Mechanism of Action

IL-11 is a thrombopoietic growth factor that stimulates thrombopoiesis by inducing the proliferation of hematopoietic stem cells and megakaryocyte progenitor cells. This process results in an increase in platelets production (Silva et al. 2019; Walsh 2013).

Clinical Use

Oprelvekin, which is the recombinant IL-11, is used to avoid severe thrombocytopenia and the need of platelets transfusion in patients submitted to chemotherapy treatments. Oprelvekin is well-tolerated by patients, although some side effects were reported, including arrhythmia, edema, dyspnea, and oral yeast infections (e.g., moniliasis) (Silva et al. 2019; Lipiäinen et al. 2015).

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Heparin and Related Drugs

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Synonyms

Dalteparin; Enoxaparin; Fondaparinux; Low molecular weight heparin (LMWH); Tinzaparin

Definition

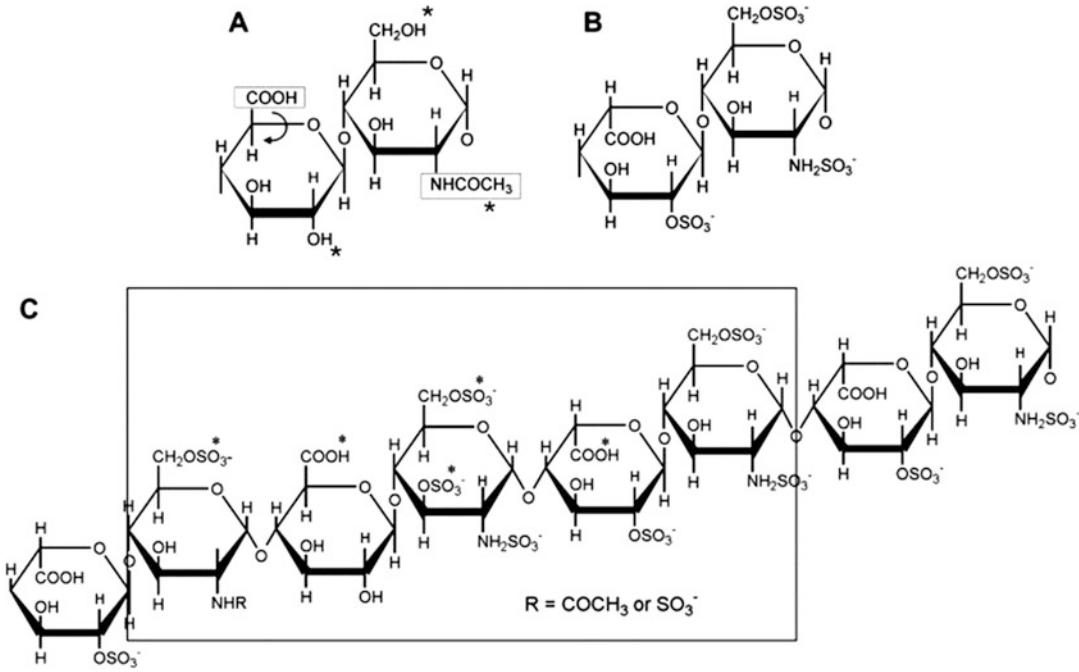
Heparin is a naturally occurring sulfated polysaccharide, and as such it is composed of many sugar units or saccharides. It has a long history being first marketed as a pharmaceutical product in the USA in 1939. It is still used in the prevention and treatment of coagulation disorders. It is an anticoagulant, inhibiting the clotting of blood collected into a tube, and an antithrombotic, inhibiting the formation of clots in blood vessels in the body (Mulloy et al. 2016).

Structure and Origin

The structure of heparin is complex. It is a completely linear molecule resembling a long chain (unlike many other polysaccharides which

may be branched). It is made up of alternating glucosamine and uronic acid monosaccharide units, heavily substituted with sulfates attached to the nitrogen in the glucosamine, and oxygen in the glucosamines and the uronic acids (N- and O-sulfates, respectively). A minor proportion of the glucosamines carry an acetyl group on their nitrogen, and these are N-acetylglucosamines (Fig. 1a, b). The biosynthetic process by which the heparin polysaccharide is elaborated is summarized in Fig. 1a. This process does not yield an absolutely regular polysaccharide but introduces sequence heterogeneity. This means that most heparin chains are different from each other in their structural details, including in the unusual but important modifications that give rise to the pentasaccharide sequence in heparin that has high affinity for antithrombin (see below). This sequence is very important for heparin's anticoagulant activity.

Heparin is a member of the glycosaminoglycan (GAG) family of polysaccharides, all of which contain an amino sugar (a glucosamine, or a galactosamine) alternating with either a uronic acid or galactose. Like other GAGs, heparin is synthesized in the cell attached to a protein core to form a proteoglycan: the heparin proteoglycan is called serglycin (Fig. 2). Unlike other GAGs that are found on the cell surface or outside of cells in their surrounding extracellular matrix of proteins and polysaccharides, heparin occurs inside the cell, in the granules of mast cells. Mast cells are one of the many cell types that make up the body's immune system. When first synthesized

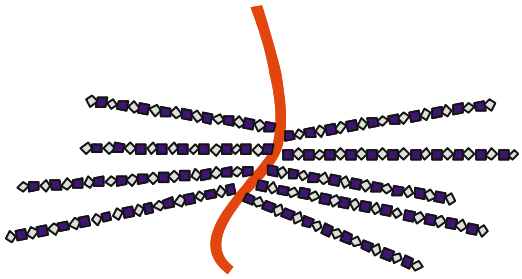


Heparin and Related Drugs, Fig. 1 Heparin substructures and biosynthesis. (**a**) The second most common repeat disaccharide unit in heparin: β-D-glucuronic acid and N-acetyl α-D-glucosamine, often abbreviated to GlcA-GlcNAc. In the biosynthesis of heparin, the GlcA-GlcNAc disaccharide is polymerized from a protein linked oligosaccharide, after which post-polymerization enzymes remove most *N*-acetylation, add sulfates (where indicated by asterisks), and epimerize the glucuronic acid to iduronic acid. (**b**) The main repeating unit of heparin is a disaccharide: α-L-iduronic acid *O*-sulfated at the 2-position and α-D-glucosamine *N*-sulfated at the 2-position and *O*-sulfated at the 6-position: often abbreviated to IdoA2S-GlcNS6S. This highly sulfated disaccharide sequence makes up more than 70% of heparin. All the monosaccharide residues in heparin are 1–4 linked. (**c**) The octasaccharide sequence in heparin with high affinity for antithrombin. The core pentasaccharide is outlined. Asterisks indicate some essential structural features for the interaction with antithrombin. (Reproduced with permission from Mulloy et al. (2010))

the heparin chains on serglycin range in size from 60 to 100 kilo Daltons (kDa). However, the heparin chains are subject to partial enzymatic digestion in the mast cells of the tissue of origin, resulting in free heparin chains that range in molecular weights from 5 to 20 kDa. The enzyme performing this function is called heparanase (Gong et al. 2003).

Heparin is extracted from tissues such as porcine (pig) or bovine (cow) intestinal mucosa that are rich in mast cells. The manufacturing process involves digestion of mucosa with proteolytic enzymes to remove protein; the heparin chains are then captured onto an ion exchange resin. Purification and bleaching and then final finishing and drying follow (van der Meer et al. 2017). The API product is either heparin sodium or heparin calcium and has an average molecular weight of around 16,000 g/mole.

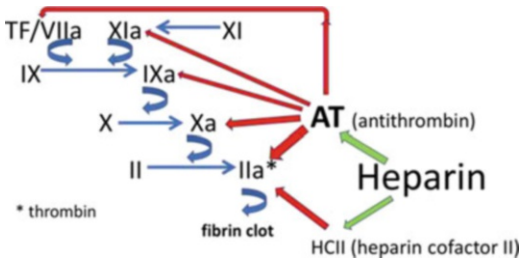
In some parts of the world, such as Europe and the USA, heparin for clinical use comes only from porcine intestinal mucosa (at the time of writing). Heparin from bovine intestinal mucosa is available in some other areas, and in the past bovine lung heparin was used. Heparins from these different sources vary systematically in their composition. They contain the same disaccharide units as are described above but in different proportions, which vary according to both the species and the tissue of origin. Other animals, such as sheep, have also been explored as sources of heparin.



Heparin and Related Drugs, Fig. 2 A cartoon representation of the intracellular PG serglycin, bearing closely packed heparin chains, on a peptide core. The protein chain is shown as a red ribbon, and GAG chains are depicted as symbols: blue squares represent GlcNS6S (or GlcNAc) and open lozenges represent uronic acid, usually IdoA2S but with minor proportions of IdoA and GlcA. (Reproduced from Pomin and Mulloy (2018))

Mechanism of Action

The coagulation cascade (for a simplified diagram, see Fig. 3) depends on a stacked set of serine proteases, each of which cleaves the inactive pro-enzyme which is next in the cascade into an active form. This culminates in the cleavage of fibrinogen to fibrin by thrombin (also known as factor IIa) to form the clot itself. This enzyme cascade acts as an amplification mechanism so that a small initial stimulus can lead to a substantial clotting response. Control of the coagulation system is complex, and the naturally occurring plasma protein antithrombin (a serpin, or *ser*ine *p*rotease *in*hibitor) can inhibit the process at several stages (Fig. 3). When heparin binds to antithrombin, through its high-affinity pentasaccharide sequence (Fig. 1c), the conformation of antithrombin is altered so that its affinity for its targets, principally factors IIa and Xa, is increased several thousand fold. Antithrombin interacts with the target enzyme to form a covalent, inactive complex. While antithrombin is consumed by this process, heparin dissociates from the complex and is free to bind to another antithrombin molecule. It is this catalytic action that makes heparin such a



Heparin and Related Drugs, Fig. 3 A much simplified representation of the coagulation cascade, in which serine proteases cleave pro-enzymes to give active serine protease coagulation factors. Factor IX is converted to active factor IXa in two ways, by the complex of tissue factor (TF) and factor VIIa and by factor XIa. Factor IXa cleaves factor X to Xa, and Xa cleaves factor II (prothrombin) to IIa (thrombin). IIa goes on to cleave fibrinogen to fibrin, which is incorporated into the blood clot along with platelets. Heparin potentiates the inhibitory activity of AT (which inhibits active coagulation factors throughout the cascade) and HCII (which inhibits only thrombin). Blue arrows signify activation by cleavage; red arrows signify inhibition; green arrows signify potentiation of inhibition. Figure reproduced by kind courtesy of Dr. Elaine Gray

powerful anticoagulant, as long as the antithrombin concentration is not depleted.

In order to potentiate the inhibition of factor Xa by antithrombin, a heparin molecule must include a copy of the high-affinity pentasaccharide (Fig. 1c); this is the only structural requirement. This means very small heparin fragments can be active against factor Xa, and indeed a synthetic heparin mimetic, fondaparinux, closely modelled on the pentasaccharide has been developed and is currently in clinical use (Zhang et al. 2019). Inhibition of factor IIa, however, needs not only a copy of the pentasaccharide but an additional length of heparin of at least 13 monosaccharide residues attached at the non-reducing side of the pentasaccharide (the left side in Fig. 1c). This adds up to a minimum molecular weight of 5400 g/mole, assuming a molecular weight of 600 g/mole per repeating disaccharide.

Additional Anticoagulant Mechanisms of Action

A second serpin present in the circulation in lower concentrations than antithrombin is called heparin cofactor II (HCII). Heparin can potentiate the action of HCII against factor IIa (HCII is not active against factor Xa). There is no specific sequence in heparin that has high affinity for HCII, and many other sulfated polysaccharides can also enhance its activity. Dermatan sulfate (DS), another GAG, can activate HCII; as DS is a component of the network of proteins and polysaccharides comprising the extracellular matrix that underlies the endothelial cells of the blood vessel wall, it may be exposed when a blood vessel wall is injured. When this occurs DS can act like heparin and potentiate the inhibition of factor IIa by HCII.

Low Molecular Weight Heparins: Mechanisms of Action

It is possible, by a process of controlled, partial depolymerization, to make lower molecular weight heparin that has reduced anti-IIa activity and a high ratio of anti-Xa to anti-IIa activity. This is because the anti-IIa activity of heparin is restricted to molecules with a molecular weight of above about 5000 g/mole. Several proprietary versions of low molecular weight heparin (LMWH) are in clinical use (e.g., tinzaparin, enoxaparin, dalteparin), each with its characteristic molecular weight distribution and activity profile. The average molecular weights of LMWHs are less than half that of unfractionated heparin (UFH). These products have improved absorption and bioavailability as compared with UFH; they are removed less quickly from the body's circulation. They also have fewer adverse side effects as their smaller size reduces nonspecific reactions with plasma proteins. As a result, depending on the medical condition being treated, LMWHs may be preferred over UFH, for example, to prevent thrombosis during surgical procedures.

Units of Activity of Heparin

Heparin is a polydisperse, heterogeneous polysaccharide in which not every molecule contains a copy of the high-affinity pentasaccharide motif necessary for antithrombin binding. This means some heparin samples are more effective as anticoagulants than others. As a consequence, heparin is administered to patients in doses defined by units of activity rather than by weight. Activity may be measured by several methods, including assays that depend on the measurement of plasma clotting times, but the values that appear on the labels of heparin products are derived from assays in which purified antithrombin acts on purified factor Xa or IIa as appropriate. Activity is measured relative to a heparin standard reference material. The International Standard for Unfractionated Heparin and the International Standard for Low Molecular Weight Heparin, established by the World Health Organization, are the primary global standards, and activity values determined against these reference materials are expressed in International Units (IU) (Mulloy et al. 2010).

Clinical Uses of Heparin

Heparin has been approved by the drug-regulating authorities for use in the prevention and treatment of venous thromboembolism, which is a blood clot that begins in a vein. There are two types of

venous thromboembolism: deep vein thrombosis (DVT) and pulmonary embolism. The first is a clot deep in a vein, usually in a leg but can occur in an arm or other veins, whereas the latter occurs when a clot detaches from the vein wall and travels to the lung where it stops part of, or all of, the blood supply. Commonly surgery, immobilization, or cancer triggers this condition, and it can lead to stroke and other cardiovascular disorders, both of which may be life threatening. Where a risk of thrombosis can be foreseen, for example, when surgery is planned, heparin (often LMWH) can be administered in advance to prevent clotting. Patients are generally treated with an anticoagulant, commonly heparin, very soon after DVT diagnosis is confirmed with the objectives of preventing an extension of the clot and pulmonary embolism (Gresele et al. 2012).

Heparin is also used to treat cardiac conditions like heart attacks (acute myocardial infarction) or unstable angina, where the heart, due to poor blood flow and narrowing of the coronary arteries, does not receive sufficient oxygen. Heparin is given following diagnosis, and during and after surgery to widen an artery by inserting a stent. The aim being to reduce the possibility of the stent triggering the formation of a thrombus or clot. Other agents like aspirin are used in combination with heparin to limit thrombosis in such settings.

Heparin is often administered to patients in hospital via a drip into a vein, the solution in the bag being pumped into the patient's vein at a controlled rate. This is called intravenous infusion. It can also (especially for LMWH) be given by an injection under the skin (subcutaneous injection), but then the anticoagulant effect is not seen until 1 or 2 h after administration. In contrast, when given intravenously an immediate anticoagulant effect is seen. Heparin cannot be given orally.

Some forms of open heart surgery require the circulation and oxygenation of blood outside of the body; this cardiopulmonary bypass process involves exposing blood to foreign surfaces in the heart-lung machine and then recirculating it throughout the body. When a patient's blood is exposed to surfaces that differ from the walls of blood vessels, this triggers an inflammatory response comprising the initiation of protein cascades of the immune system and the activation of platelets and leukocytes (white blood cells) with their release of inflammatory mediators. The result is a state of heightened coagulation. The management of patients on extracorporeal life support has only been possible because of the use of anticoagulants to prevent thrombus formation within the circuitry and in the patient's vasculature. Heparin is the usual anticoagulant. Managing the balance between anticoagulation and clotting is particularly challenging for patients on extracorporeal life support, and heparin has the advantages of a short half-life and the possibility of reversing, or neutralizing, its effects by the addition of a peptide called protamine.

These days extracorporeal circuitry coated in heparin is increasingly being used during cardiopulmonary bypass surgery, with circuitry in which the heparin is covalently bonded to the surface being preferred. The discovery of the critical pentasaccharide sequence for antithrombin binding prompted the covalent immobilization of partially depolymerized heparin chains by their reducing termini as a way of retaining heparin's bioactivity. This technology has now been used to covalently coat heparin onto not only cardiopulmonary bypass equipment but also vascular stents, vascular grafts, and hemodialysis catheters. In this way the possibility of thrombosis and inflammation triggered by the device is reduced (Biran and Pond 2017).

Heparin Clearance from the Circulation

Heparin is cleared from the circulation by a combination of saturable and non-saturable mechanisms. The saturable mechanism is due to the binding of heparin to cell surfaces, while the non-saturable mechanism is due to renal excretion. Both UFH and LMWH are primarily cleared from the circulation by the liver, through attachment to the HARE (Hyaluronic Acid Receptor for Endocytosis) receptor. UFH has a half-life of about 1 h in the circulation, but LMWH has a longer circulatory half-life of 2–6 h, having lower affinity than UFH for the receptor (Harris et al. 2009). The renal route of clearance is more

significant for LMWH than for UFH (Johansen and Balchen 2013).

Heparin Neutralizing Agents

The risk of bleeding is a potential side effect of heparin treatment. Heparin treatment is a balance between the prevention of thrombotic events and the triggering of excessive bleeding. Generally elderly patients and patients with poor renal function have a higher risk of bleeding following anticoagulant therapy. Patients undergoing cardiac surgery and interventions that require the use of extracorporeal circuitry need to have any excess heparin in their bloodstreams neutralized to prevent postoperative bleeding. Linear positively charged molecules, such as the arginine-rich protamine peptide, can bind to heparin and neutralize its bioactivity. This property is used in the clinic to remove excess heparin. Protamine neutralizes heparin very rapidly acting within 5 min following its administration, which in a cardiac operating room is usually by an intravenous 10–15 min infusion. Protamine is less effective at neutralizing LMWH, with an efficacy of around 60%, and protamine does not neutralize fondaparinux, the synthetic analogue of heparin modelled on the pentasaccharide sequence of heparin that binds antithrombin. Excessive bleeding following fondaparinux use may be partially corrected by injections of factor VIIa, but there is no specific antidote yet.

In order to optimize protamine doses for each patient, the quantity of anticoagulant active heparin in a patient's blood must be measured. This is usually done using the clotting-time assays, activated partial thromboplastin time (aPTT), and anti-factor Xa assays, with the latter being particularly important for monitoring the amounts of LMWH in plasma. The reliability of these assays for estimating the anticoagulant response is greater for LMWH than for UFH. The reasons being heparin can more readily bind to the surfaces of endothelial cells effectively taking it out of plasma, and it can also readily bind certain proteins that may be present in plasma that have heparin-neutralizing properties, including platelet factor 4 (PF4), histones, histidine-rich glycoprotein, and others. The limitations of the clotting-time assays are why research is being undertaken to find alternative assays that can reliably estimate the quantities of heparin and LMWH in plasma and blood (Ourri and Vial 2019).

Non-bleeding Side Effects of Heparin Treatment

Non-bleeding side effects of heparin treatment arise because heparin binds to proteins other than antithrombin and factors involved in coagulation. Possibly the best known side effect is immune heparin-induced thrombocytopenia (HIT). It is relatively uncommon, with a reported incidence of 0.2% to 5% of patients who are exposed to UFH developing this complication. There are two types of HIT. Type I HIT is a transient fall in platelet numbers which is probably caused by heparin-activating platelets; it generally resolves without complications. Type II HIT can be life threatening, and it is caused when heparin forms complexes with PF4, a protein released from activated platelets. These complexes can trigger the body's immune system to produce antibodies that recognize and bind to the heparin-PF4 complexes, further increasing their size. These large immune complexes bind to platelets causing platelet activation and a cascade of events that can lead to the massive generation of thrombin, the formation of blood clots, and thromboembolism. Associated with this is a fall in platelet numbers. The likelihood of HIT occurring following the use of LMWH is much lower than when UFH is used. This is because the size of the initial UFH-PF4 complexes is generally larger than complexes of LMWH and PF4, and larger complexes more effectively trigger the immune response and antibody production. If HIT is suspected, heparin treatment must be stopped and an alternative anticoagulant used to prevent clot formation in the capillaries (Alban 2012; Gupta et al. 2015).

The most common side effect of long-term heparin use is osteoporosis, but if heparin is used on a short-term basis to treat acute thromboembolism, osteoporosis does not occur. Osteoporosis is a bone disease associated with a low bone mass, fragile bones, and an increased risk of fractures. The mechanisms contributing to heparin's effects

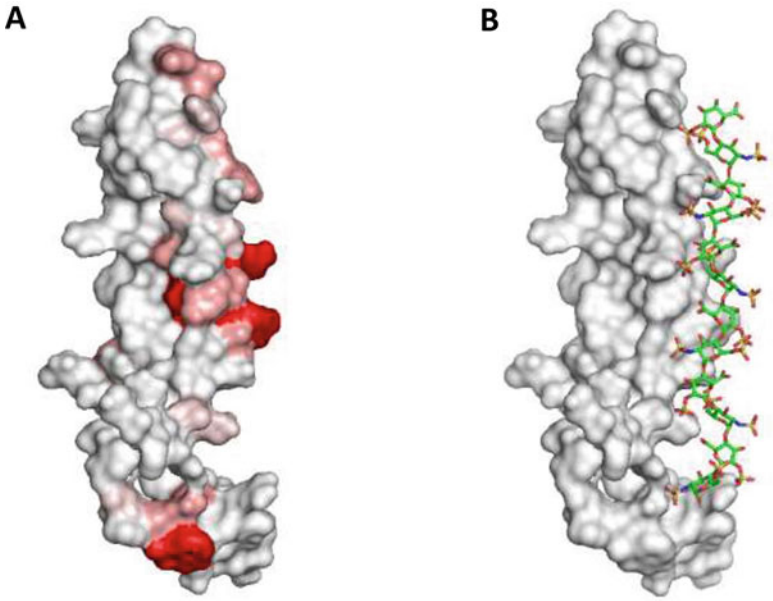
on bone density are not well understood, but it is believed that the binding of heparin to a protein (called osteoprotegerin) that regulates bone density is a key contributing factor. Heparin binding prevents this protein from inhibiting bone resorption, and this results in increased rates of bone resorption compared to bone formation, which leads to osteoporosis. LMWHs do not bind osteoprotegerin so well, and so their use has less of an adverse effect on bone densities than that of UFH (Signorelli et al. 2019).

Heparin Contamination

In 2007–2008, some patients suffered a severe adverse reaction to heparin involving loss of blood pressure, nausea, and shortness of breath; there were some fatalities. These events were linked with specific heparin batches that were contaminated with an unusual compound, oversulfated chondroitin sulfate (OSCS). Though chondroitin sulfate itself is a naturally occurring and harmless GAG, OSCS is made by a chemical treatment to increase its sulfate content and its ability to inhibit plasma clotting. Pharmacopoeial specifications for heparin have been improved worldwide to ensure that events of this type do not happen again (see, e.g., Szajek et al. 2016).

Other Heparin-Protein Interactions

The number of proteins that bind heparin is unknown but amounts to over 400 (Ori et al. 2011). Heparin, as the most highly negatively charged biopolymer found in nature, can be expected to interact with positively charged molecules or to an area of positive charge on the surface of a protein. As heparin has a regular repeating structure with a linear conformation, the spacing and orientation of charges on the protein surface can affect the affinity of any interaction. Structurally, heparin resembles heparan sulfate, a GAG found on the surface of cells that modulates the actions of soluble protein mediators like growth factors, morphogens, cytokines, and chemokines. Heparin similarly binds to proteins in all these categories in addition to the coagulation factors and their inhibitors. The size and the nature of the heparin binding site on proteins varies according to the protein's structure. Some of these binding sites are extended and comprise amino acids that are widely separated in the protein's amino acid sequence (Fig. 4), while others are compact. Extended binding sites interact with longer heparin chains, while compact sites may require only a pentasaccharide or hexasaccharide. The biological consequences of heparin binding



Heparin and Related Drugs, Fig. 4 (**a**) A surface view of the protein sclerostin, in which residues are colored according to the perturbation of nuclear magnetic resonance (NMR) signals induced by heparin binding. (**b**) A representative view of a heparin fragment docked by molecular modelling techniques onto its binding site on the surface of sclerostin. Heparin is shown in a stick representation colored by atom type: carbon is green, oxygen red, nitrogen blue, and hydrogen white. (Reproduced from Veverka et al. (2009))

these proteins are many, and they vary depending on the protein bound and its function. Despite being a topic of current research, it is known these consequences include blocking an enzyme's activity, regulating the binding of growth factors to their cell surface receptors, regulating white blood cell migration, and inhibiting some viral infections. Heparin binding to sclerostin as shown in Fig. 4 was found to release sclerostin into the culture medium from the cell layer, presumably by blocking it from associating with the heparan sulfate chains of cell surface proteoglycans (Veverka et al. 2009). Sclerostin is an inhibitor of bone formation that plays a role in skeletal development; sclerostin inhibitors are being developed for use in the treatment of postmenopausal osteoporosis.

The diversity of heparin's activities is the stimulus for current research to make molecules by synthetic chemistry that resemble aspects of heparin's structure and which display particular subsets of heparin's activities. The goal being to develop synthetic heparin-like molecules as drugs for diseases like cancer or inflammation (Mohamed and Coombe 2017). Although we have known about heparin for over a century, there remains more to be understood about how this complex molecule can be used to benefit our lives.

Cross-References

- ▶ Anticoagulants
- ▶ Antiplatelet Drugs

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Hepatocyte Stimulating Factor

▶ Interleukin-6

Heterodimerization

▶ Ghrelin, Physiological Roles and Clinical Relevance of

HGFs

▶ Hematopoietic Growth Factors

High Content Screening

▶ High-Throughput Screening (HTS) Technology

High Voltage-Activated (HVA) Calcium Channels

▶ Voltage-Dependent Ca^{2+} Channels

High-Throughput Screening

▶ High-Throughput Screening (HTS) Technology

High-Throughput Screening (HTS)

▶ Small-Molecule Screens

High-Throughput Screening (HTS) Technology

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Synonyms

Assay development; Chemical probes; Early drug discovery; High content screening; High-throughput screening

Definition

HTS is a translational research technology used for identifying effectors (compounds, peptides, and biologics) that modulate target-specific biochemical or cell-based assays. In the context of small molecule drug discovery, HTS involves performing assays in the presence of large collections of compounds. The compounds identified from HTS are referred to as "hits" once the actives are reconfirmed for activity and specificity in downstream assays. The method uses automation for dispensing compounds and assay reagents and for reading detection signals. Advances in automation, liquid handing, and signal detection, as well as large data computing and analysis, have greatly facilitated screening of mid- to large (10^3 – $>10^6$) compound collections in HTS. Depending upon the type of robotic instrumentation available, high-throughput screening can yield up to 40,000 data points per day, whereas ultrahigh-throughput screens (uHTS) can generate 10^5 to 10^6 data points daily. High content screening (HCS) uses automated microscopy to capture images and quantify changes in cells exposed to compound collections. In contrast to the wet bench HTS- or HCS-based approaches to identifying hits, theoretical in silico methods are also used for screening virtual compound databases to identify compounds that dock into computational models of protein binding or interaction sites based on theoretical models or on actual X-ray

diffraction-based models. The compounds identified from HTS/HCS or virtual screenings are tested for activity (potency and efficacy) and selectivity using counterscreens and orthogonal assays. Compounds that show sub-micromolar potency against a target and exhibit reasonable selectivity serve as probes or chemical tools for mechanism of action studies. The probes can also serve as starting points for optimization of hits to chemical leads that have the potential of being developed into marketed drugs.

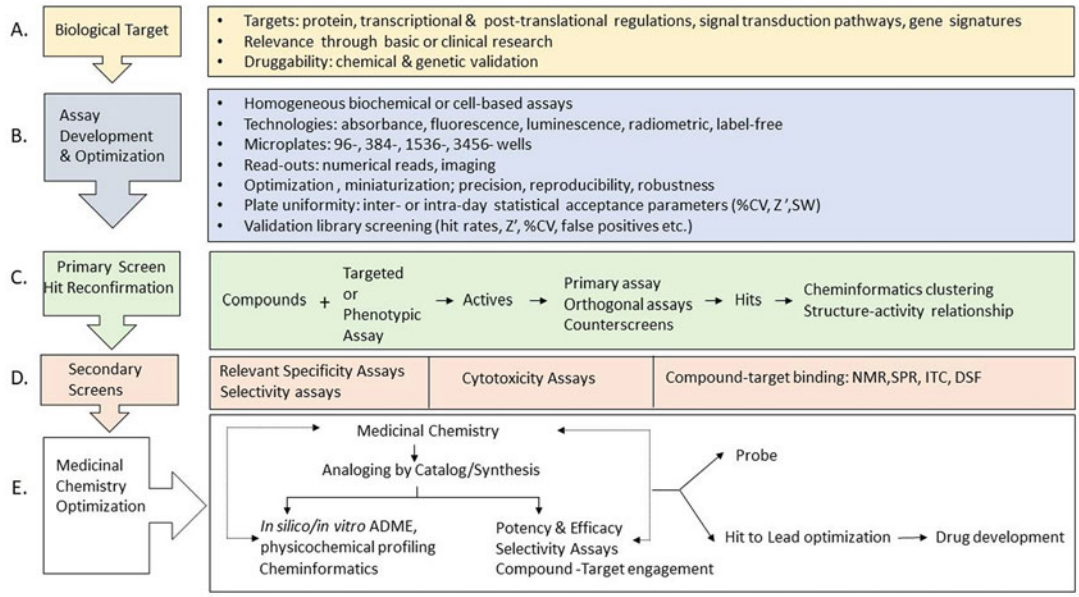
Description

Any screening campaign, medium-, high-, ultrahigh-throughput starts with identification of a biological target and a quantifiable assay. The optimized assay is first validated against a small test set of compounds which may be a focused or bioactive collection or a small diversity compound set representative of most of the scaffolds in larger library sets. Data from the validation screen is useful to evaluate statistical parameters in the presence of compounds, determine hit rates, and validate the assay workflow. The following steps are involved to execute the screening campaign (Fig. 1).

Target Identification and Validation. The process of translational research starts with identification of a biological target such as an enzyme, protein, receptor, regulatory DNA or RNA sequence, a signaling pathway effector, or modulator/adaptor macromolecules through basic research or via clinical observations. The unequivocal role of target in disease or a phenotype is first validated either (a) chemically (Wyatt et al. 2011), using available peptides, antisense oligonucleotides, aptamers, compounds, and antibodies, and/or (b) genetically, via knockouts, knockins, or knockdown experiments using gene-editing tools (e.g., clustered regularly interspaced short palindromic repeats, CRISPR-Cas9 editing, CRISPR interference) and RNA interference or microRNAs. A target is of therapeutic value or "druggable" once a dose-dependent relationship between target function and biological phenotype in homeostasis or pathogenesis is established. Alternatively, a genetic variant that directly correlates to a clinical condition can also serve as a validated target for developing therapeutics. Data integration and extraction from high-throughput genomics, transcriptomics, proteomics, and other such "omics" databases can also support target identification and validation efforts (Roy and Chaguturu 2017).

Targeted and Phenotypic Assays. Identifying an assay that best represents target biology and function is a critical success factor for HTS (Roy 2018). Assays are conceptualized and executed exploiting some known aspect of target function, like enzymatic activity or interaction with another protein or DNA or RNA sequences or post-transcriptional regulation. When target function is unclear, biophysical assays are set up to screen for compounds that directly bind to the target. Several of the target functions can be set up using purified components in biochemical assays, where reaction components are well defined. Targeted biochemical screens, or the reverse chemical genetic screens, help to identify compounds that modulate function of the target either by binding directly to the protein or nucleic acid or to interaction interfaces between the reactants. The hits from targeted screens require cellular validation.

Several of the target functions can also be analyzed in the context of their cellular environments. In such cell-based assays, the target activity can be tracked using engineered cells, stably or transiently expressing reporters like luciferase, green fluorescent proteins (GFP) or its variants under the nucleic acid regulatory elements or as protein fusions or via in-cell enzyme assays (e.g., caspases), or target-dependent toxicity assays. Assays using whole organisms, primary cells, or cancer cell lines are utilized for executing phenotypic screens. In phenotypic screens or the classical forward chemical genetic screens, whole organisms or cells are treated with chemical collections in microplate formats. Deconvolution of compound mechanism of action in phenotypic screens is more complex, as screening will identify cell-permeable compounds that through various specific or non-specific interactions within



High-Throughput Screening (HTS) Technology, Fig. 1 Schematic showing high-throughput screening workflow. (**a**) The relevance of biological target in therapeutics is validated chemically and/or genetically. (**b**) Biochemical and cell-based assays are established for primary screening of large compound libraries. The assays are optimized and miniaturized for screening in low-volume high-density microplates. Once the assay meets statistical acceptance parameters, it is validated in a small screen using test set of compounds (up to 10,000 compounds). (**c**) The assay is used to screen large compound libraries at single concentration, and primary screen actives are reconfirmed for dose-depending activity. The actives are also tested in parallel against assay platform-dependent counterscreens to eliminate assay interference compounds. Various orthogonal assays are used to confirm target-specific activity. Cheminformatics analysis is performed to flag promiscuous actives, chemical liabilities, and structure-activity-based clustering. A few synthetically tractable chemical series are selected by medicinal chemists. The compounds and their analogs are purchased as fresh powders from commercial vendors. (**d**) The purity and structural integrity of fresh powders is evaluated, and compounds that are >95% pure are tested in a series of assays that profile the compounds for potency, efficacy, selectivity, and direct binding. (**e**) The physicochemical properties of hits as well as their in vitro and in silico ADME (adsorption, metabolism, and excretion) are evaluated along with compound potency, selectivity and toxicity. The ultimate goal is to identify compounds that can serve as probes or chemical tools or serve as starting points in hit to lead drug development programs

cells will cause changes in cell morphology, spatial regulation, and viability. Phenotypic screens also can help in the identification of prodrugs or compounds that show activity after chemical transformations within the cells. Signaling pathways are queried in cells expressing reporter coding sequences under the control of target promoter or regulatory sequences. The reporter proteins include firefly and renilla luciferase, β-galactosidase, secreted alkaline phosphatase, green fluorescent proteins, and β-lactamase. Unmodified whole cells and organisms that are commonly used for screening range from bacteria, algae, fungi or mammalian cells, multicellular vertebrate organisms like Zebrafish, *Xenopus laevis*, drosophila, and *Caenorhabditis elegans*.

A large number of cell-based screens have used two-dimensional (2D) cultures of immortalized primary or cancer cell lines or induced pluripotent stem cell (iPSC)-derived normal or patient cell lines. While the throughput of screening large compound libraries in cell lines in 2D monolayer formats is high and convenient, more relevant preclinical information (Vincent et al. 2015) is obtained from testing compound activity in three-dimensional (3D) single-cell or multicellular co-cultures or organoids. Organoid cultures capture structural and functional interactions

between relevant cell populations and with extracellular matrices in solid tumors. While in some organoids some cellular subtypes do not exactly recapitulate normal levels and patterns of gene expression profiles, migration, and layering, the use of the 3D models is a significant technological advancement in drug discovery. Organs-on-a-chip microfluidic models derived from human origin cells are available for the livers, pancreas and prostate glands, brains, and kidneys and are predicted to serve as more relevant alternatives to rodent models. Advances in organoids (Rossi et al. 2018) that completely recapitulate human organ cellular complexity and function will be invaluable for allowing more direct translation of compound behavior in clinical trials.

Homogeneous versus heterogeneous assay formats. Whenever possible, a primary homogeneous assay that is simple (mix and read, up to two steps), precise, robust, and amenable to automated handling is used to execute large compound screens. Depending upon target complexity, biology, and availability of reagents, heterogeneous formats like ELISA (enzyme-linked immunosorbent assay) or radiofiltration assays involving multiple steps of reagent additions or removals or washing cycles can also be optimized for primary screening. Heterogeneous formats limit batch sizes of screening plates and are generally more prone to plate-to-plate and screening batch-wise variability, which ultimately complicates global data analysis and hit identification of primary screen. While a homogeneous assay is desirable for screening large compound libraries, secondary assays for activity reconfirmation, specificity, and selectivity for small numbers of hits can be both homogeneous and heterogeneous formats.

Assay Detection Technologies

Assays for HTS are developed using platform technologies based on optical properties, like absorbance, fluorescence, and luminescence (Inglese et al. 2007). Absorbance-based or colorimetric assays exhibit small signal dynamic range but are easy to set up. Fluorescence assays exploit the intrinsic light absorption and emission characteristics of proteins or fluorophores (fluorescein, Texas Red, Pacific Blue, Rhodamine, etc.) at two distinct wavelengths. All fluorescence measurements require external light source for activation. Upon absorption of light at specific wavelength, the assays can use fluorescence intensity, for direct emission measurements, or measure emissions from energy transfer between donor and short-lived fluorophore acceptors or to quenchers (Förster resonance energy transfer (FRET)). The more specialized FRET assays like time-resolved FRET (TR-FRET) or HTRF ((homogeneous time-resolved fluorescence) assays are based on the use of long emission fluorophores as donor and acceptors as well an integrated time delay between initial excitation and final fluorescence measurement. The fluorescence polarization/anisotropy (FP/FA) assays are based on selective excitation of fluorophore absorption vectors that are parallel to the electric vector of incident linearly polarized light. Low polarization values are obtained when fluorophores on small rapidly rotating probes (small molecules or peptides or nucleic acids) orient randomly. When the fluorophore-tagged probes bind to a large protein, high polarization values are obtained due to slower rotation in the plane of polarized light. All fluorescence formats are very sensitive and show high dynamic range. Majority of fluorescence formats are applicable to both biochemical and cell-based assays.

Luminescence-based assays are based on quantification of photons emitted after chemical (chemiluminescence) or enzymatic (bioluminescence) reactions between the luciferase enzymes and photon-emitting substrates (Fan and Wood 2007). Luminescence detections do not require a light source for excitation and use a photomultiplier tube that proportionately converts the photons to electrons. HTS assays are based on luciferase enzyme from *Photinus pyralis* (firefly luciferase) which catalyzes luciferin substrate in the presence of ATP. Bioluminescence assays also use *Renilla* (*R. reniformis*) luciferase or secreted *Gaussia* luciferase, both of which use coelenterazine as substrate. The activity of aequorin enzyme from *Aequorea victoria* is

calcium sensitive and is used extensively in calcium channel assays. Luciferase assays for biochemical and cell-based assays are configurable in various ways to evaluate target-related activity. Some of the formats used include genetic reporter constructs expressing luciferase under target promoters or regulatory untranslated regions of mRNA, or protein-luciferase fusions, adding luciferase enzyme and substrate to quantify ATP levels of kinase reactions and within cells. A wide variety of luciferin tagged substrates for protease, assays are available to set up biochemical assays that are sensitive to ATP concentrations in purified luciferase enzymatic reactions. A number of novel formats integrate both luminescence and fluorescence for developing protein-protein interactions or for multiplexing different readouts. These include BRET or bioluminescence resonance energy transfer assays (Machleidt et al. 2015) for protein-protein interactions in which energy transfers occur from bioluminescence donor to a fluorescence acceptor.

Many assay formats are used in more restricted niche due to requirement for specialized detection systems or due to expense-related and biohazard issues. For example, radiometric assays, despite their high sensitivity, are not used extensively for primary screening due to the biohazard nature of radioactivity-based assays. Screening for various targets is executed based on Scintillation Proximity Assays (SPA) in which radioactive decay of β-particles released from ^{3}H. ^{14}C, ^{33}P, and ^{125}I isotopes is used to generate light via scintillant material in microbeads or coated on the wells of flash plates. Label-free assays measure changes in native proteins or cells based on biophysical principles and do not require modified proteins, fluorescent tags, or reporter expression constructs. The label-free detection systems require specialized instrumentation and assay plates. Label-free systems based on optics are employed for primary screening and for orthogonal hit confirmation, e.g., Optical/resonant waveguide Grating (OWG) technologies (Corning Epic® EnSpire label-free module and SRU Biosystems BIND®) and surface plasmon resonance (SPR). In another form of label-free technology, measuring changes in electrical impedance across the interdigitated microelectrodes is used for quantifying compound modulated changes in cell growth, migrations, and invasion. In recent years high-throughput screening is also being performed using advanced Mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy technologies (Dahlin et al. 2018).

All platform technologies have their pros and cons and can contribute toward artifactual activities resulting in false positives and negatives. Compound interference arises from intrinsic absorbance, fluorescence, aggregation, redox activity, or direct binding of the compound to reporter or to the recombinant protein tags (Nickel chelate-Histidine (His), glutathione S-transferase (GST), maltose-binding protein (MBP) tags used for affinity purification). To minimize assay false positives, the HTS workflow also incorporates additional downstream assays during hit reconfirmation. These include (1) orthogonal assays, which use a platform technology distinct from that used in the primary screen to confirm compound activity against a target, and (2) counterscreen assays that help deprioritize compounds interfering with primary assay signal detection. Sometimes, compounds that exhibit assay interference can also be true positives if they show activity in a physiologically relevant secondary assay.

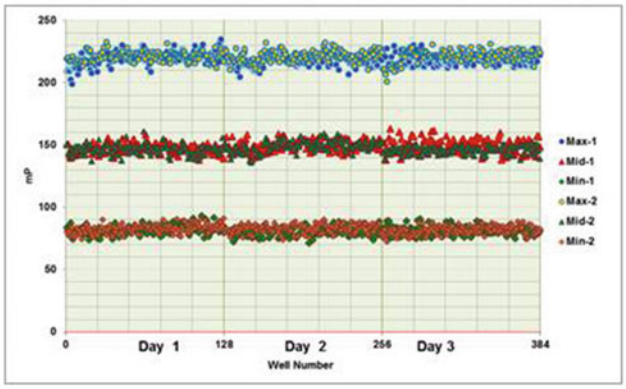
Assay Microplates. The assays are performed in polystyrene multiwell microplates that vary in the total number of wells (96-, 384-, 1536-, or 3456-wells) and in their well volumes (5ul–100ul). The footprint and tolerances of standard microplates manufactured by commercial vendors conform to microplate dimension standards established by the Society for Biomolecular Screening (SBS, American National Standards Institute (ANSI 1-2004)). Primary screening of large compound libraries in high-density plates using small reagent volumes is both cost- and time-effective. Assay performance is significantly affected by microplate color as microplates can contribute to inner filter effects by absorbing light. UV transparent plates are used for signal detection at wavelengths <320 nm, while standard clear microplates are used for absorbance assays in visual spectrum; black plates reduce background

signals for fluorescence intensity and polarization, and the white plates are used for TR-FRET and luminescence-based assays. AlphaScreen™ technology (Perkin Elmer Inc.) utilizes gray plates for reducing cross-talk and background. The clear bottom plates used for HCS permit image capture from bottom of the plate. Based on their ability to bind nucleic acids and proteins, microplate surfaces can be non-binding, low to high binding to facilitate setting up of different assay formats. Cell-based assays require lidded plates that are untreated for suspension cell growth or treated with agents that enhance attachment of adherent cells (cell culture-treated plates, poly-D lysine plates, etc.). A large number of ultralow attachment hydrophilic surfaces and geometries are currently available to promote formation of spheroids in three-dimensional assay systems. More specialized assays incorporate use of modified 96-well or 384-well plates. In radiometric scintillation proximity assays, flash plates are coated with scintillant material. Label-free assays like xCELLigence (Roche Applied Science) may have microelectrodes integrated on the bottom of cell culture plates.

Assay Optimization. In routine HTS campaigns, as an example, a screening campaign of 1000 plates may be divided into batches of 50–100 plates/day each, depending upon the assay. The datasets from all 1000 plates must show high degree of uniformity from plate to plate, and on day to day basis, as signal variability creates issues in hit identification. Both biochemical and cell-based assays are optimized for signal and uniformity, signal dynamic range, signal to background ratios, and batch consistency. Various parameters like reagent stability, reagent concentrations, buffer components (salts, reducing agents, and detergents), temperature, order of addition, preincubation time, dispense volumes, incubation times, read times, positive and negative controls, and DMSO sensitivity are defined for all types of assays. The cell lines used for assay development are authenticated via short tandem repeat (STR) PCR analysis, routinely screened for mycoplasma, preferably grown in the absence of general antibiotics (penicillin/streptomycin) in media and tested for the assay response in relation to DMSO concentration, passage number in culture, number of cells per well, and growth kinetics in multiwall plates. Cell-based assays that require transient transfections or transductions to express reporter constructs have to be validated for batch-wise transfection efficiencies and baseline drifts. Assay variability using cell lines expressing recombinant reporter constructs or proteins is greatly reduced by using well-characterized stable cell lines derived from a single high-expressing cell clone.

Once the assay is optimized on a small scale, all steps in assay setup are transitioned to robotic handling for improving efficiency and throughput of screening. For any new assay, experiments addressing plate uniformity are performed using reagent amounts or cell numbers or inhibitor/activators that give low, medium, and high signals with any platform technology. In its most extensive format, raw values and distribution of signals across three plates run within a day and over 3 days are evaluated for signal drifts, for variability, and for row-wise/column-wise edge effects. When assays are transferred from one lab to another, two-plate, 2-day formats for plate uniformity assessment are used. Assay conditions are defined for generating three types of signals on a plate: (1) "Max," highest value of a read out, using high enzyme concentration or higher cell numbers per well; (2) "Mid," ~50% of Max signal; and (3) "Min" (<80% of Max signal) which are performed at concentrations of enzyme or cell numbers giving moderate and low signals. The Mid and Min signals can also be obtained by adding different inhibitor or substrate concentrations to the reactions or to cells ((Roy and Mir 2017) Fig. 2). The assay robustness and variability are defined by a number of statistical parameters: the absolute value of the signal, coefficient of variability (CV<20%), signal windows (SW>2), midpoint signal shifts (>2), and Z' ratios (between 0.5 and 1). The Z' factor ($Z' = 1 - (3SD_{Max} + 3SD_{Min})/|Mean_{Max-} Mean_{Min}|$) is key parameter that measures the signal spread (standard deviation, SD) around the positive and negative controls as well as the window of separation between the two controls. In screening plates, the Z' score is defined in terms of positive

A.



B.

Day	Signal	Plate	n	Mean	SD	%CV CV<20
1	Max	1	128	220.9	3.7	1.69
1	Max	2	128	219.7	6.1	2.76
2	Max	1	128	220.7	4.5	2.02
2	Max	2	128	217.4	4.5	2.05
3	Max	1	128	220.8	5.0	2.28
3	Max	2	128	218.3	4.4	2.02
1	Mid	1	128	145.4	4.1	2.84
1	Mid	2	128	146.2	3.8	2.63
2	Mid	1	128	148.2	4.5	3.05
2	Mid	2	128	148.7	4.1	2.74
3	Mid	1	128	144.8	3.1	2.12
3	Mid	2	128	148.7	5.4	3.60
1	Min	1	128	82	3.6	4.37
1	Min	2	128	81	3.9	4.78
2	Min	1	128	82	3.4	4.09
2	Min	2	128	80	3.9	4.85
3	Min	1	128	81	3.4	4.18
3	Min	2	128	80	3.7	4.59
					SD<20	
1	%Mid	1	128	54.1	3.0	5.49
1	%Mid	2	128	52.3	2.8	5.33
2	%Mid	1	128	52.1	3.2	6.23
2	%Mid	2	128	50.5	3.0	5.85
3	%Mid	1	128	54.6	2.2	4.04
3	%Mid	2	128	50.5	3.9	7.68

C.

Day	Z'	SW	
1	0.996	37.0	SW>=2 and/or Z'>0.4
1	0.953	21.7	
2	0.976	30.3	
2	0.987	30.5	
3	0.965	26.9	
3	0.984	30.8	

High-Throughput Screening (HTS) Technology, Fig. 2 Representative example of assay optimization parameters. (**a**) A two-plate assay was set up over 3 days, and inter-plate and intra-plate reads were evaluated statistically to study signal drifts and variability. The millipolarization counts from a protein-RNA fluorescence polarization assay show tight separation of counts at max, mid, and min signals in all plates across all 3 days. (**b**) The table shows assay robustness and variability data from the plate uniformity assay including signal values, means, standard deviations, and CVs. (**c**) The Z' scores and signal window calculations are shown. The assay meets the statistical acceptance parameters for optimized assay: coefficient of variability (CV<20%), signal windows (SW>2), midpoint signal shifts (>2), and Z' ratios (between 0.5 and 1)

and negative controls on each assay plate. The Z' score of >0.5 is acceptable for cell-based assays; the acceptable scores generally lie between 0.7 and 0.9 for biochemical screens. Z' values are evaluated not only during assay development and optimization but are used throughout the HTS campaign. Genomic screens with siRNAs are noisier, and additional statistical evaluation parameters are used to define assay readiness.

An assay that meets all statistical acceptance criterions is ready for screening compounds (small molecule/natural products/fragment screens) or siRNAs (genomic screens) or antibodies (biologics screens) or peptides (peptide screens). Screening large collections efficiently with high precision and fidelity requires use of reliable, well-calibrated robotic/automated instrumentation.

HTS Laboratory Instrumentation. In addition to basic research equipment (balances, incubators, shakers, pH meters, centrifuges, biosafety cabinets), the resources of a typical HTS laboratory include (a) screening collections, compound management and handling (b) automated liquid handling robotics, and (c) multimodal plate readers (Jones et al. 2004). In addition to this, HTS programs require large data storage and processing abilities. The HTS labs also have dedicated spaces and equipment for aseptic mammalian cell culture and/or for handling infectious disease organisms. Screenings with more infectious BSL3 organisms require more specialized enclosed spaces that harbor all screening equipment.

Screening Collections. Compound libraries are an important resource for screening centers, all of which maintain medium to large compound collections. Majority of the compounds are purchased from commercial vendors, e.g., ChemDiv, Inc. (San Diego, CA), ChemBridge Corp (San

Diego, CA), Life Chemicals Inc. (CT, US), and Prestwick (Illkirch, France), but each center may have varying numbers of proprietary or legacy compounds, which may include natural products, metabolites, synthesis intermediates, focused compound collections, etc. Compound collections include small organic molecules containing diverse chemical scaffolds that range in size from <300 Da (fragments), small molecules (300–500 Da) or macrocycles, and natural products (>1000 Da). Small or high molecular weight natural products and metabolites isolated from plants, microbes (soil, marine), and other living organisms are also attractive candidates for drug discovery and often utilized in screenings. Peptides, siRNAs, biologics, and CRISPR libraries are also employed in identifying mechanism or genomic sequences that will modulate a target identified through basic or clinical research. Library collections are characterized via computational selection tools or filters. These include Lipinski's rule of five (Ro5) which selects drug-like and potentially bioavailable compounds that are <500 Da, <5 hydrogen (H) bonds donors (NH &OH groups), <10 H-bond acceptors (N + O), with octanol-water partition coefficient, ClogP<5. The rule of three (Ro3) for fragment library selection identifies compounds <300 Da, <3 H-bond donors, <3 H-bond acceptors, ClogP≤3. At least 275 rules have evolved over the last two decades that identify compound liabilities from screening datasets (Bruns and Watson 2012), and several filters like rapid elimination of swill (REOS), Lilly Rules, and PAINS filters are employed to flag or exclude compounds that show promiscuous chemical reactivity, oxidizing/reducing agents, intercalators, large lipophilic molecules, heavy metals, or synthetically challenging scaffolds. Compound libraries also include a drug-repurposing collection, which includes FDA-approved drugs, used for screens directed toward identifying novel indications for marketed drugs. Since safety and human clinical trial data is already available, the FDA-approved drugs can be developed quickly if shown to be efficacious against a novel target both in vitro and in vivo. Over the last decade, the use of compounds tagged with DNA barcodes (DNA-encoded chemical libraries, DECLs) has evolved for use in screens that can be conducted in single tubes (Franzini et al. 2014). The DECLs are still not readily accessible, and their success in hit identification will become evident over the next few years.

Compound Storage and Management. The compounds in HTS collections are stored as liquid stocks or as solids (powders). For primary screens, compounds are solvated in 100% dimethyl sulfoxide (DMSO) as vast majority of small organic molecules are soluble in DMSO at 10 mM. Some labs also store pre-weighed amounts of solid powders in barcoded vials at various temperatures (room temperature, refrigerated, or −20 °C, depending upon the collections). The solids are generally used for verification of hit structures, for reconfirmation of activity, or for secondary assays. The solvated compounds are stored, sealed, and frozen in polypropylene (PP) or cycloolefin microplates at −20 °C, flushed with nitrogen to reduce humidity and minimize DMSO hydration. The automated plate sealing systems (e.g., Agilent PlateLoc TM Thermal microplate sealer) can also flush nitrogen prior to heat sealing the microplates containing compounds. Various automated compound storage and retrieval systems are available commercially with software that also tracks frequency of freeze-thaws. DMSO is hygroscopic, and compound-processing protocols that include repeated freeze-thaws, plate unsealing, and compound transfers facilitate slow hydration of DMSO, which, in turn, reduces solubility of some compounds causing them to eventually crash out of solution. HTS labs are also equipped with multi-wall plate sonicators like Covaris L8 for homogeneous compound plate preparation. Covaris L8 uses high-frequency, ultrasonic acoustic energy to resuspend compounds prior to transfer. In addition to using DMSO as a solvent, occasionally, ethanol, methanol, acetonitrile, or other solvents are also used for solvating natural products. Peptides tend to require different solvents based on

their net charge and size. All compound libraries are managed through inventory databases containing information on barcodes, plate ID, unique compound identification numbers, batch information, molecular weight, chemical formula, structure SMILES (simplified molecular-input line-entry system), salt form, and vendor catalog number (Vendor ID) or internal synthesis notebook ID, enantiomer forms, etc. While primary screens are performed using HTS library stocks that undergo repeated freeze-thaws, the hits from HTS are further reconfirmed from less utilized HTS stocks or freshly dissolved compound powders that are first analyzed for structural integrity and purity.

Compound Dispensing. Daughter plates that contain compound stocks are stored in 384-well or 1536-well plate formats in DMSO. Compound transfers are performed via either contact tip-based methods or non-contact transfers acoustically. Robotic platforms with mechanical arms and tip-heads are utilized for transfer of small volumes of compounds to the assay plates. Tip-based transfers are made via air displacement disposable tips (250 nl or higher) or via fixed positive displacement tips (25 nl or higher) or stainless steel pre-calibrated pin tools (2–5 nl). Depending upon the stock volumes and the concentration of compound screened in the final assay volume, intermediate concentration plates may be required for compound transfers. Reusing plastic tips or pin tools requires washes between transfer to prevent cross-contamination or carryover of compounds. Non-contact acoustic transfer systems (Labcyte Inc., EDC Biosystems™) use sound energy to transfer volumes as low as 1 to 2.5 nanoliter directly from stock plates to assay plates without any cross-contamination, or any need for intermediate plate dilutions or tips. Acoustic transfers are especially convenient for setting up combination screens where two drugs of interest can be transferred from single-source well to a single destination test well with ease. Some screening labs also use piezo actuator-based technologies that allow electrical to mechanical energy conversions which can create 0.1 nl to 1 nl transfers.

Screening Formats. In vast majority of screens, a single compound is screened per well of the microplate, which requires screens of hundreds of microplates to screen large compound/peptide collections. Appropriate vehicle controls are always included in each assay plate during screenings. Generally, large compound collections are screened at a single concentration (e.g., 1 uM or 10 uM) for time- and cost-effectiveness. In quantitative HTS (qHTS) mode, compound libraries are screened at six to eight concentrations to enable hit selection based on their dose-responsiveness, shapes of curves, and approximate IC50 values. In drug repurposing-focused screens, the FDA-approved collection of drugs or known bioactives are screened alone at very low concentrations (1uM–2.5 uM) or may be screened in the presence of a known standard of care in drug combination screens to identify synergistic combinations. Synthetic lethality screens (Huang et al. 2020) can be set up using an FDA-approved or chemotoxic compound that at low concentrations is non-lethal to cells but, in combination with a hit from siRNA screen, identifies a protein product and eventually another drug, which sensitizes a tumor to the drug, thus identifying a novel chemotherapy combination.

Bulk Reagent Dispensing. The reagents are dispensed into assay plates before or after the compound transfers, depending upon the assay design. Generally for biochemical screens, the compounds and vehicle controls or known positive controls are first transferred from HTS stock plates ("daughter plates") into the assay plates. For cell-based screens, the cells are first plated and allowed to attach to the plates before the compounds are dispensed. For most assays, the dispensing volumes of cells, media, proteins, substrates, and buffer into the wells of 96-, 384- or 1536-well plates in a screening batch range between 2.5 ul and 120 ul. Bulk volume dispensing is conducted with either automated pipetting platforms that use plastic or stainless steel tips attached to 384- or 1536-well heads that move in

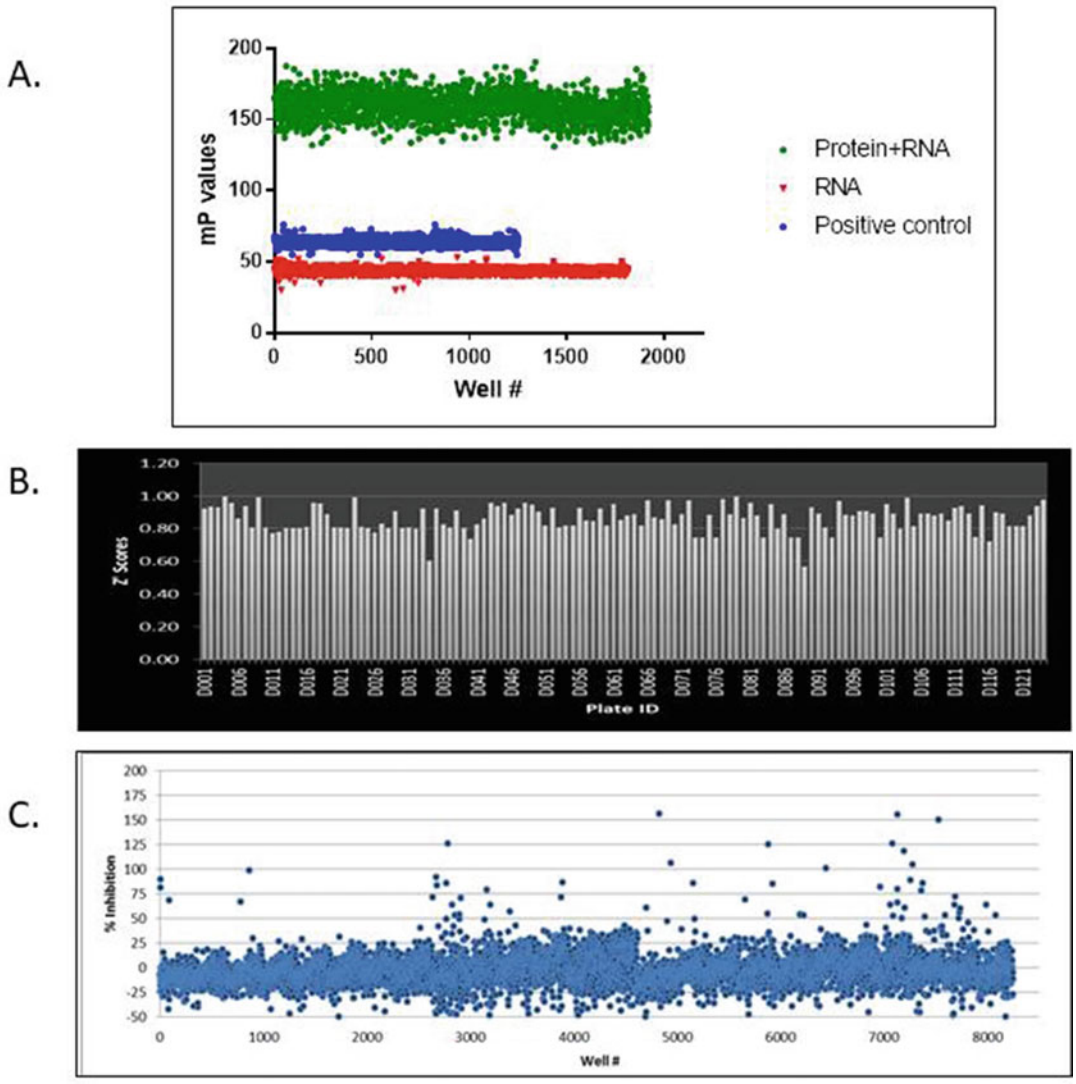
x-, y-, and z-axes with a robotic arm across all pipetting positions on instrument deck. Reagent dispensing is also performed with more affordable peristaltic pump-based dispensers containing multichannel tubing cassette assembly, which are calibrated for dispensing variable volumes. In a typical plate map for screening, each 384-well assay plate may contain 16 wells each of negative and positive controls in the first two columns of assay plate. Compounds are transferred to the remaining wells of the plate (columns 2–24). The control wells are also backfilled with 100% DMSO to ensure the same final percent of DMSO in all the wells of the plate.

Plate Readers. The plates containing compounds and assay components are incubated under defined optimal conditions, and the signals are read on multimodal plate readers that are capable of reading all available assay technologies. The microplate readers are equipped with plate stackers that can harbor up to 100 microplates that are inserted in defined sequence inside the plate reader to quantify absorbance-, fluorescence-, or luminescence-based detection technologies through use of monochromators and/or filter sets of specific wavelengths. The plate readers also capture the assay plate barcodes and store raw data of all the plates for quality control management. Rapid decaying signals are captured one well at a time using reagent injectors in plate readers that inject the detection reagent just before a well is read. Specialized modules in plate readers facilitate reading of AlphaScreen counts or TR-FRET or HTRF-based assays. Specialized drug discovery systems like Functional Drug Screening System (FDSS, Hamamatsu) allow fast kinetic measurement and analysis of fluorescent and luminescence signals in all wells of 384- or 1536-well microplate in millisecond intervals without time lags using highly sensitive camera. The RT-PCR systems are utilized in capturing qRT-PCR profiles of selected primers to evaluate changes in gene expression induced by compound exposure or to capture protein unfolding in presence of compounds for direct compound binding screens using differential scanning fluorimetry. Although not widespread in practice, a small number of HTS laboratories also use highly specialized screening formats like automated flow cytometry, mass spectrometry, and NMR for primary screening.

The readouts of phenotypic screens include direct reads from absorbance, fluorescence, or luminescence or imaging-based high content screening (HCS). The images of all the wells in clear bottom plates containing cells and compounds are captured with automated microscopy either directly or after fixing and immunostaining. The automated confocal microscopic platforms provide resolution at subcellular level and can quantify spatial and temporal signals from fluorescent reporter proteins, fluorescently labeled antibodies, and stains. Imaging readouts include quantification of cell morphology, cell number, nuclei, cell membrane changes, apoptosis, translocations within cells, neuronal or vascular outgrowths, cell migrations, cell differentiation, etc.

Data Analysis

The raw data from 10^3 to 10^6 wells is transferred from plate readers or imagers to assay and compound databases like IDBS ActivityBase or in-house databases. Large compound screens are performed at $n = 1$ and are therefore not statistically reliable. The data is susceptible to compound purity, stability, solubility, and intrinsic spectral properties. Issues with physicochemical properties of compounds can result in identification of false positives due to aggregation or assay interference. Despite extensive optimization, assays in 1536-well or 384-well plates can sometimes show edge effects, row- or column-dependent patterns in HTS workflows. The data analysis for entire screening campaign is analyzed using a variety of excel or informatics tools. The readouts from the positive and negative controls per plate are utilized for evaluating the quality control of the screen (Fig. 3). The plates that show unacceptable Z' scores or show row- or column-wise issues can be rejected and rescreened if time and cost permit. As a general guideline, hits are identified as compounds that inhibit or activate an assay at plate median plus/minus 3 standard deviations. Depending upon the hit rates, the hit selection



High-Throughput Screening (HTS) Technology, Fig. 3 Data analysis from a representative primary screen. (**a**) Scattergram showing uniform distribution of high (protein + RNA) and low (RNA only) control signals across all the plates. (**b**) Distribution of Z' scores across assay plates. An average of 0.8 ± 0.02 was obtained showing acceptable separation of controls. (**c**) Scattergram of percent inhibition across the primary screen. Primary screen actives are identified as compounds that inhibit the assay at plate median plus three standard deviations

criterion can also be changed accordingly. Most targeted biochemical screens show very low hit rates (<1%), whereas cell-based or phenotypic screens can show anywhere between 0.5 and 4% hit rates. Cheminformatics analysis is used again to flag compounds with PAINS (Pan Assay Interference Compounds) or reactive groups, chelators, and covalent binders. The hits from a screen are also evaluated for their promiscuous activity against other targets or specific targets using internal screening databases or using the publicly available PubChem database (Wang et al. 2017). Depending on the number of hits from a screen, hits can be clustered based on substructure similarity at this stage or after the hits from $n = 1$ screen is reconfirmed for

concentration-dependent activity experiment. Imaging data management for phenotypic screens requires large data storage clusters and development of analysis algorithms (CellProfiler https://cellprofiler.org) to analyze multiparametric high content information.

Hit Reconfirmation. Compound attrition is an essential step in viable hit identification in both academic and pharmaceutical settings. The hits from primary screen are reconfirmed in dose-response studies using compound aliquots cherry-picked from in-house library DMSO or powder stocks. The reconfirmation assay is the primary screening assay that is run in parallel with counterscreen to remove assay interference compounds or cytotoxicity assays in relevant cell lines to eliminate non-specific toxics or with an orthogonal assay to confirm compound activity against the target of interest. The hits that are dose-responsive and show higher potency in target-specific screen are analyzed further for structure-activity relationships by cheminformatics analysis (Shun et al. 2011). The clusters are analyzed by medicinal chemists for exploring SAR using minimal scaffold analysis, and some clusters are prioritized for further analysis by medicinal chemists based on their amenability to chemical optimization. At this stage, the hits are also examined for their promiscuity or activity against other targets using internal databases or public databases like PubChem (https://pubchem.ncbi.nlm.nih.gov/). The compounds of interest are repurchased in larger amounts as fresh powders of high purity (>95% purity) for reconfirmation and further profiling. A limited number of compounds may be synthesized by medicinal chemists if they look interesting but are not available from commercial vendor sources.

Hit Profiling. A number of biochemical and cell-based assays are used to define biological potency and selectivity and physicochemical properties of compounds (Arrowsmith et al. 2015). In addition to activity and potency in the primary assay, the hits are evaluated for toxicity in relevant cell lines and activity in 3D models, quantifying target-relevant signaling pathway proteins. Other orthogonal assay schemes also include several biophysical approaches to show direct binding of the compound to the target of interest. Depending upon availability of resources, the direct binding of hits to targets is performed using Surface Plasmon Resonance (SPR), Differential Scanning Fluorimetry (DSF), Isothermal Titration Calorimetry (ITC), or Nuclear Magnetic Resonance (NMR) assays. Identification of targets of hits from phenotypic assays is complex and utilizes various techniques to establish target engagement. The binding of the hit compounds to cellular targets in intact cell or in cell lysates is performed via Cellular Thermal Shift Assay (CETSA). The proteins stabilized by compound binding at increasingly high temperatures are detected by immunodetection or via activity assay. The more complete profile of targets stabilized by compounds in CETSA is obtained via mass spectrometry. Alternative methodologies that are used in combination with mass spectrometry to establish hit-target interactions include affinity elution of proteins from compounds immobilized on solid-phase resins pull-down experiments, or use of protein arrays. Compound profiling also includes in silico or in vitro profiling of compounds for their physicochemical properties like structure, aqueous solubility, protein binding, microsome stability, and other ADME/pharmacokinetic profiling in vitro or in silico screens. The selectivity, specificity, and ADME profiles form the basis of identifying probes that can be used for chemical genetics or provide starting points for a more involved and cost-intensive drug development programs. The hits that bind the target non-covalently show high potency (<0.1 uM biochemical and ~up to 1 uM cell based); selective (>50–100-fold) and quantitative modulation of a specific biological target are very useful in defining the mechanism of action.

Pharmacological Relevance

HTS as a standardized translational research exploratory platform evolved largely in pharmaceutical sector as an approach toward identifying

starting compounds for drug discovery programs. The National Institutes of Health (NIH) funded initiative Molecular Libraries Probe Production Centers Network (MLPCN), and the NIH Molecular Libraries Program (MLP) brought the technology to academic screening centers by providing protocols, guidelines, novel compound collections, screening methods, as well as funding mechanisms to expand the scope of basic research. The MLP successfully identified ~375 high-quality probes against diverse therapeutic targets (Schreiber et al. 2015). Several of these probes are currently being advanced for late-stage drug discovery programs. There are several notable examples of hits from HTS that were developed ultimately to marketed drugs (Macarron et al. 2011). High-throughput targeted or phenotypic screening has also resulted in the discovery of several first-in-class drugs (Eder et al. 2014). HTS, hit identification and probe, and lead development are a constantly evolving technology that serves to impact our understanding of basic biology and therapeutic development to treat various conditions in pathology and progression of human diseases.

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Hippo Pathway

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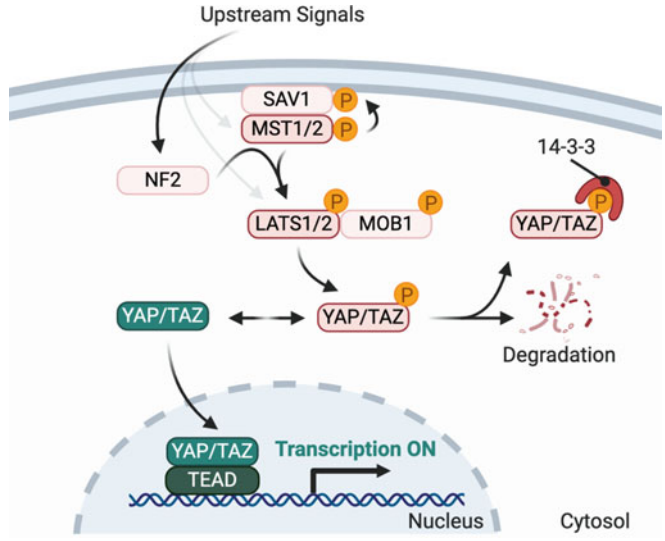
Definition

The Hippo pathway is a highly conserved signaling pathway that regulates multiple cellular and developmental processes within metazoans. It is composed of a kinase cassette, Yes-associated protein (YAP), and WW-domain-containing transcription regulator 1 (WWTR1; or TAZ). YAP and TAZ are paralogs that function, separately, as transcriptional co-activators upon binding to the transcriptional enhanced associate domain (TEAD) family of proteins and TEAD's cognate DNA binding sites. Formation of a YAP/TAZ-TEAD complex in the nucleus can activate transcription of genes involved in cell fate, growth, proliferation, and survival. The kinase cassette functions as a switch to regulate YAP/TAZ activity. When the kinase cassette is active, YAP/TAZ activity is suppressed via phosphorylation. In contrast, when the kinase cassette is inactive, YAP/TAZ can translocate into the nucleus, bind TEAD, and induce target gene transcription. This pathway gets its atypical name from the *Drosophila melanogaster* kinase Hippo as initial deletion studies of Hippo in *D. melanogaster* led to an overgrowth phenotype. Aberrant Hippo signaling has been linked to a range of different cancers, therefore tight control of the Hippo pathway is critical due to the important role of the Hippo pathway in development and tumorigenesis.

Basic Characteristics

The Hippo pathway regulates the activity and cellular localization of transcriptional co-activators YAP and TAZ to control organ size, tissue homeostasis, and cell growth. Unlike most conventional cell signaling pathways, Hippo activity is not controlled by a dedicated ligand-receptor pairing but is instead regulated by multiple mechanisms including – but not limited to – the cell microenvironment, cell polarity, metabolic pathways, and extracellular growth factors. When the Hippo kinase cassette is activated, transcriptional co-activators YAP and TAZ are localized to the cytosol, where they bind to 14-3-3 proteins or are degraded by the proteasome (Fig. 1) (Ma et al.



Hippo Pathway, Fig. 1 Key components of the Hippo pathway in mammalian cells

2019). In contrast, when the kinase cassette is inactive, YAP/TAZ can translocate into the cell nucleus, bind TEAD, and induce a transcriptional response (Fig. 1). Genes activated for transcription by YAP/TAZ are involved in cell survival, migration, and proliferation, such as *BIRC5*, *CTGF*, and *Cyr61*.

Key Hippo pathway components were first identified in *Drosophila melanogaster* via genetic mosaic screens (Pocaterra et al. 2020). Deletion of the kinase genes for Hippo and Warts resulted in an overgrowth phenotype (Wu et al. 2003) thought to be visually reminiscent of a hippopotamus, hence the colorful pathway name. While the gene names of Hippo pathway components between *D. melanogaster* and mammals are quite different, both the domain composition and functional roles of each protein is conserved (Table 1). *D. melanogaster* has thus served as a useful model system for identifying and characterizing components of the Hippo pathway.

Hippo Pathway, Table 1 Hippo pathway components and functional homology shared between mammals and *D. melanogaster*

Mammalian gene	Fly gene (*D. melanogaster*)	Role in Hippo pathway
NF2	Merlin	Binds and localizes LATS1/2 to the plasma membrane
MST1/2	Hippo	Phosphorylate LATS1/2
Sav1/WW45	Salvador	Scaffold for MST1/2
LATS1/2	Warts	Phosphorylate YAP/TAZ
MOBKL1A/B or Mob1	Mats	Adaptor protein for LATS1/2
YAP, TAZ	Yorkie	Nuclear localization activates transcription in presence of TEAD
TEAD1, TEAD2, TEAD3, TEAD4	Scalloped	Co-transcriptional activator for YAP/TAZ

Hippo Pathway Activation-Inactivation

The upstream signals that regulate the Hippo pathway are diverse and not fully characterized. Activity of the Hippo kinase cassette can be affected by hormonal signaling through GPCRs, cellular stress, phosphatases such as the STRIPAK complex, and cell biological properties like cell-cell contact, cell polarity, and sheer stress (Ma et al. 2019). As an inverse pathway, it is able to integrate and convert multiple biochemical, cellular, and mechanical cues into a transcriptional response. This requires the kinase cassette to be activated in order to turn off transcription, and the kinase cassette to be inactivated for transcription to be turned on.

Hippo Kinase Cassette

The Hippo kinase cassette is composed of tumor suppressor kinases MST1/2 and LATS1/2, along with their respective accessory proteins SAV1 and MOB1. Activation of the kinase cassette is initiated by NF2, which functions as a scaffold by binding the LATS1/2-MOB1 complex and delivering it to the membrane bound MST1/2-SAV1 complex (Manning et al. 2020). Following autophosphorylation of MST1/2, the LATS1/2–MOB1 can be phosphorylated by MST1/2. Other kinases, such as MAP 4K and TAO kinases, can also activate LATS1/2 via phosphorylation (Meng et al. 2015). Importantly, LATS1/2 phosphorylation enables it to function as a kinase for YAP/TAZ; whose kinase activity is promoted through interactions with the angiomotin family of proteins (Mana-Capelli and McCollum 2018). Both YAP and TAZ contain WW domains that bind the PPxY motif of LATS1/2, where P is a proline, Y is a tyrosine, and x is any residue. The YAP WW and LATS1/2 PPxY interaction is essential for YAP/TAZ phosphorylation. Based on gene deletion studies, LATS1/2 and MOB1 are also indispensable for YAP/TAZ phosphorylation (Bae et al. 2016; Plouffe et al. 2016). Therefore, maintaining a functional kinase cassette and accessory proteins is imperative for controlling YAP/TAZ activity.

YAP/TAZ

YAP and TAZ serve as the terminal effectors of the Hippo pathway. These are paralogs that share 46% sequence identity and activate similar pro-growth transcription profiles (Pocaterra et al. 2020). While YAP and TAZ are essential for development, they lack a DNA binding domain and require the transcriptional enhanced associate domain (TEAD) family of proteins to activate transcription.

Transcriptional activation requires YAP/TAZ to translocate into the nucleus and form a transcriptional complex with TEAD. Under normal physiological conditions, TEAD is exclusively located within the nucleus, whereas YAP/TAZ cellular localization is regulated by the Hippo kinase cassette. LATS1/2 mediated phosphorylation of YAP/TAZ occurs at the consensus HxRxxS motifs on YAP/TAZ where H is histidine, R is arginine, S is serine, and x is any amino acid. Phosphorylation of YAP at S127 or TAZ at S89 enables binding to 14-3-3 (Callus et al. 2019). Phosphorylation of YAP also triggers subsequent phosphorylation by casein kinase 1δ/ε at the YAP-encoded phosphodegron, which in turn serves as a substrate for $SCF^{\beta\text{-}TrCP}E3$ ligase-induced degradation.

At the molecular level, regulation of YAP/TAZ activity is accomplished through modulating gene expression, ubiquitin directed proteasome degradation, increased nuclear YAP/TAZ export rates, YAP/TAZ phosphorylation, and binding to 14-3-3 in the cytosol (Pocaterra et al. 2020). Together, these mechanisms serve to regulate YAP/TAZ nuclear levels, retain YAP/TAZ to the cytosol, and limit the pro-growth transcription profile induced by nuclear YAP/TAZ-TEAD complex formation. Although the full range of YAP/TAZ binding partners has not yet been elucidated, it appears their roles are likely both context- and tissue-dependent (Callus et al. 2019).

TEAD

The TEAD family protein Scalloped was first characterized in *Drosophila* to promote wing development and control organ size (Zhang et al. 2008). Unlike *Drosophila* whose genome encodes for one TEAD protein, mammals have four distinct TEAD genes whose translated protein sequences (TEAD1, TEAD2, TEAD3, and TEAD4) share a high sequence identity. Each TEAD protein is composed of an N-terminal DNA binding domain, a C-terminal YAP binding domain, and a proline-rich sequence between the two domains (Holden and Cunningham 2018). The TEAD DNA binding domain is highly conserved across all TEADs and is composed of a helix-turn-helix homeodomain fold that directs TEAD towards the muscle-specific cytidine-adenosine-thymidine (MCAT) consensus sequence (Holden and Cunningham 2018). Despite high sequence homology shared between human TEAD1–4, the individual TEAD proteins are differentially expressed in a tissue- and development-dependent manner. A clear understanding of how TEAD1–4 interact and complement one another in a physiological setting remains unknown.

Crystal structures and binding studies have enabled a detailed mapping of critical interactions between YAP/TAZ-TEAD (Holden and Cunningham 2018; Bokhovchuk et al. 2020). In short, YAP/TAZ are able to wrap around a highly conserved surface on TEAD to form a heterodimer complex. The YAP binding domain of TEAD is posttranslationally modified by a palmitate group with the lipid tail extending into a conserved hydrophobic pocket. This palmitoylation is required for TEAD activity and stability. While YAP/TAZ are the best-known coactivators and regulators of TEAD transcriptional activity, the vestigial-like protein family (VGLL) and p160s also interact with TEAD to function – in part – as competitive YAP/TAZ antagonists (Callus et al. 2019). On a similar note, YAP has also been shown to have a TEAD-independent role in intestinal Wnt inhibition (Li et al. 2020).

Genetic Dysregulation of the Hippo Pathway

Gene fusions that promote tumorigenesis have also been identified within the Hippo pathway. Notably, epithelioid hemangioendotheliomas

(EHEs) result from a TAZ-CAMTA1 gene fusion that is constitutively expressed and can no longer be regulated by the Hippo pathway. YAP can also be fused to transcription factor E3 resulting in EHE. In lung cancer, gene fusions to TAZ, NF2, and LATS1/2 have also been identified and correlated to poor overall survival (Meng et al. 2016). A missense mutation that ablates the TEAD-YAP interaction and results in the human disease Sveinsson's chorioretinal atrophy – also referred to as helicoid peripapillary chorioretinal degeneration, atropia areata, or circumpapillary dysgenesis – a progressive degenerative disease of the eye has also been identified on TEAD1 at the TEAD-YAP interface.

Pathophysiology and Therapeutic Potential

There is increasing interest in harnessing the therapeutic potential of compounds that modulate the Hippo pathway to affect YAP/TAZ mediated transcription. Modulation of the pathway's activity is envisioned for two disparate approaches: (1) limiting cell growth in various cancers with aberrant YAP/TAZ induced transcription and (2) inactivating the Hippo kinase cassette to promote cell growth for tissue repair and wound healing.

Suppressing YAP/TAZ transcriptional activation in cancers represents a significant therapeutic opportunity. While both proteins are critical in early development and tumorigenesis, their activity is expendable in most adult tissues. This indicates that YAP/TAZ antagonists could be tolerated and a viable therapeutic target. Although there are multiple hypothesized benefits of deactivating YAP/TAZ activity in the context of cancer, directly inhibiting the transcription profile induced by YAP/TAZ is challenging. Viable inhibition of YAP/TAZ activity will require a therapeutic that can either (1) prevent YAP/TAZ from entering the nucleus or (2) modulate the YAP/TAZ interaction with TEAD. These two general strategies represent potential therapeutic avenues for modulating YAP/TAZ activity and the discovery of new anticancer therapeutics.

Due to the pro-growth and pro-survival transcription profile induced by YAP/TAZ, it might also be possible to leverage this to accelerate wound healing and tissue repair mechanisms. In wounded *in vitro* cultured cells, YAP is activated and thus able to translocate into the nucleus and induce transcription of its target genes (Meng et al. 2016), enabling cell migration and proliferation at the site of the wound. The cellular response to injury also requires the Hippo pathway to be suppressed for the repair mechanisms to be enabled. In short, therapeutic control of YAP/TAZ localization represents an exciting opportunity to advance healing of damaged tissues.

Genetic disruption of core Hippo pathway components and subsequent YAP translocation into the nucleus leads to sustained liver growth and cancer, such pathway inhibition enhances regenerative capacity *in vivo* and an MST1/2 inhibitor has been shown to augment liver and intestinal repair in mice (Hong et al. 2019; Fan et al. 2016). In addition, YAP-deficient mice have an impaired intestinal regenerative response in a colitis model and conditional deletion of TAZ in a bleomycin-induced lung injury model reduced AEC1 regeneration during recovery, leading to increased alveolar destruction and fibrosis (Sun et al. 2019). Further, deletion of SAV1 in mouse hearts resulted in enhanced cardiomyocyte regeneration, reduced fibrosis, and recovery of pumping function after myocardial infarction (Leach et al. 2017). In short, dysregulation of canonical Hippo signaling can have effects on cell and tissue development, and may contribute to the pathology of many diseases.

Pharmacological Intervention

In the context of cancer, sustained inhibition of the core kinase cassette members is problematic as NF2, Mst1/2, and LATS1/2 are tumor suppressors. Most reports thus far have focused on targeting the Hippo pathway via the transcriptional co-activators YAP/TAZ and TEAD. While YAP/TAZ are known to be mostly intrinsically disordered proteins that lack binding pockets for ligands, one YAP ligand – verteporfin – has been reported. Statins have also been evidenced to inhibit YAP nuclear translocation by inducing YAP phosphorylation (Bae et al. 2016); however,

the precise molecular mechanisms and thus the utility of both verteporfin and statins as physiological tools remains unproven. Despite the many challenges associated with targeting YAP/TAZ transcriptional activity in cancer or wound healing, multiple groups have identified molecules that lower YAP/TAZ activity. Interestingly, the vast majority of the small molecules reported thus far bind to the TEAD palmitoylation site. This lipidated pocket site is hydrophobic and seems to accommodate a range of small hydrophobic molecules that have been shown to lower target gene transcription in vitro (Crawford et al. 2018). Recent evidence indicates some of these molecules function as a surrogate for palmitoylation to affect TEAD protein levels and induce a dominant-negative phenotype (Holden et al. 2020). Overall, targeting TEAD to affect YAP/TAZ transcription presents an interesting opportunity for the development of new anticancer therapeutics.

Taken together, modulation of the Hippo kinase cassette activity, YAP/TAZ localization, and YAP/TAZ targeted gene transcription play an indisputable role in both development and tumorigenesis. It appears certain that the identification of tool compounds capable of altering YAP/TAZ activity will remain of high interest. Therefore, advances leading to physiological control of nuclear YAP/TAZ activity will enable important discoveries of the Hippo pathway, clinically relevant processes, and the identification of therapeutics for a range of diseases.

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Histamine Receptors

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Synonyms

Histaminergic receptors

Definition

Histamine receptors are transmembrane proteins that bind to the endogenous ligand histamine and activate a sequence of intracellular events ("Transmembrane Signalling") that lead to all the cellular and physiological responses attributed to histamine. They are part of the histaminergic system ("Histaminergic System").

Basic Characteristics

Classification

Four subtypes of histamine receptors have been described in the literature, namely, H_1, H_2, H_3, and H_4 receptors. This classification is based on their sequential discovery by either pharmacological characterization or molecular identification (Alexander et al. 2019).

Expression and Function

H_1 Receptor

Histamine H_1 receptor is widely expressed throughout the body. It is found mostly in the smooth muscle of the airways and blood vessels, endothelium, and cells of the immune system such as dendritic cells, monocytes, neutrophils, and T and B cells. Here, activation of H_1 receptor mediates most actions of histamine related to allergy, asthma, and inflammation (▶ "Inflammation") including bronchoconstriction, mucus secretion, mucosal edema, pruritus, pain, increased vascular permeability, hypotension, flushing, and tachycardia. Most of the clinical relevance of antihistamines rely on the treatment of these allergic/anaphylactic and asthmatic physiological reactions (▶ "Bronchial Asthma"). High levels of H_1 receptors are also found in neurons in several brain regions involved in neuroendocrine, behavioral, and nutritional regulation, such as the cortex, hypothalamus, thalamus, septum, hippocampus, brain stem, and olfactory bulb where it controls the sleep-wake cycle ("Sleep"), food intake, aggressive behavior, memory, and learning. Apart from these systems, the H_1 receptor is also expressed in chondrocytes, hepatocytes, and in the heart where its function is less clear (Panula et al. 2015).

H_2 Receptor

The **H_2 receptor** is predominantly expressed in mucosa parietal cells where it mediates gastric acid secretion. In order to block histamine action at this level, H_2 receptor antagonists have been widely used for the treatment of duodenal and gastric ulcers until they were replaced by proton pump inhibitors ("Proton Pump Inhibitors and Acid Pump Antagonists"). Strong expression of H_2 receptor is also found in almost all brain areas in both neurons and astrocytes and, more modestly, in cells of the immune system such as neutrophils, eosinophils, monocytes, macrophages, dendritic cells, and T and B cells. In addition, H_2 receptor can be found in the heart, chondrocytes, smooth muscle, and endothelial and epithelial cells. Here, activation of the H_2 receptor results in hypotension, bronchoconstriction, flushing,

increased vascular permeability, and tachycardia (Panula et al. 2015).

H_3 Receptor

The **H_3 receptor** is mainly expressed in the central nervous system where it functions as autoreceptor in histaminergic neurons, regulating the synthesis and release of histamine and as a heteroreceptor regulating transmission by GABA, glutamate, and biogenic amines, such as acetylcholine, serotonin, noradrenaline, and dopamine. By doing so, the H_3 receptor is involved in the regulation of the sleep-wake cycle ("Sleep"), cognition and attention, circadian rhythms, homeostasis, arousal, appetite, satiety, and energy expenditure. The H_3 receptor also modulates histamine release from mast cells (Panula et al. 2015).

H_4 Receptor

The last histamine receptor to have been discovered is the **H_4 receptor**. Although it is mainly expressed in bone marrow, it can also be also detected in peripheral hematopoietic cells, the spleen, and the thymus, where it regulates the maturation and differentiation of blasts and the chemotaxis of blood cells. It appears to be an important mediator of inflammation, and its expression is also upregulated by inflammatory stimuli. Therefore, its expression pattern is complicated because it changes in response to the state of the cell. Despite these limitations and considerations, evidence of H_4 receptor expression can be found in the lung, small intestine, colon, heart, liver, brain, skeletal muscle, gastrointestinal tract, and epidermal tissue (Panula et al. 2015).

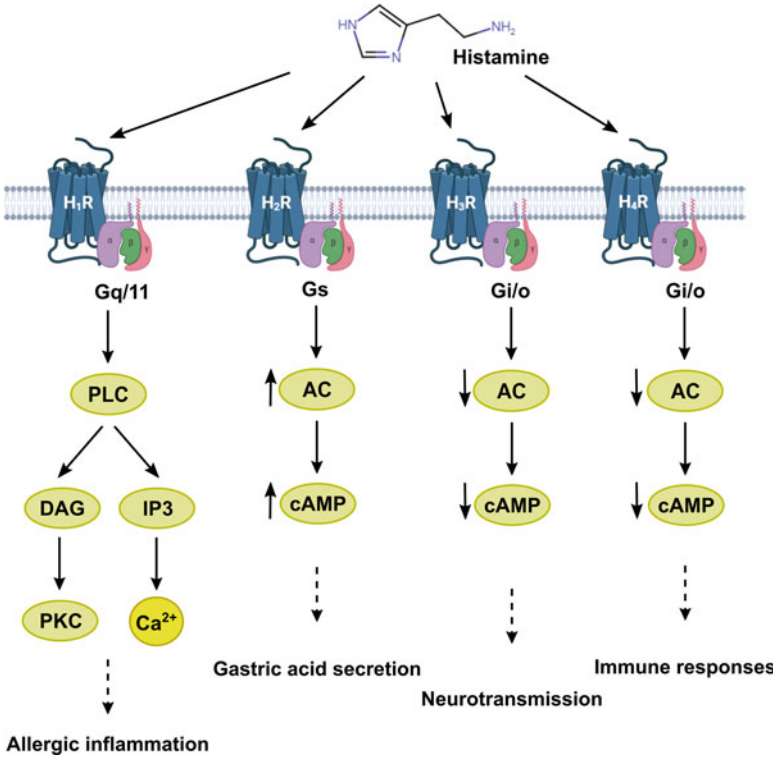
Structure and Signaling

Histamine receptors belong to the superfamily of G protein-coupled receptors (▶ "G-Protein-Coupled Receptors") which are structurally characterized by an amino-terminal extracellular domain, a carboxy-terminal intracellular domain, and seven transmembrane-spanning helices connected by three intracellular loops and three extracellular loops. Functionally, when activated, these receptors signal through coupling to heterotrimeric G -protein and lead to the modulation of cytoplasmic second messengers (Fig. 1).

H_1 Receptor

The **H_1 receptor** human gene (HRH1, 126.16 kb, 3 exons) is located in chromosome 3p25.3. The protein contains 487 amino acids and there are described 20 single nucleotide polymorphisms: 2 of them are relatively common, while some act as predictors of therapeutic efficacy or are associated with the severity of antihistamines' side effects. Crystallographic studies showed that ligands bind deeply in the binding pocket defined by transmembrane helices 3, 5, and 6. Specifically, it has been proposed the interaction of the $Asp107^{3.32}$ with the protonated amine group of histamine that would be conserved among all histamine receptors subtypes (Shimamura et al. 2011).

In most systems, the H_1 receptor is coupled to Gq/11 protein, and its stimulation leads to phospholipase C (PLC) activation, with a consequent increase in diacylglycerol (DAG) that activates protein kinase C (PKC) (▶ "Protein Kinase C"), a serine-threonine kinase that phosphorylates different downstream effectors, and inositol-1,4,5-trisphosphate (IP_3) that stimulates the IP_3 receptor ("IP_3 Receptor") triggering calcium mobilization that binds calmodulin (▶ "Ca^{2+}-Binding Proteins"). One of the main PKC targets is the NF-kB (▶ "NF-κB, Molecular Target") transcription factor which is tightly related to inflammation. In the central nervous system, both calmodulin and PKC modulate, among others, potassium voltage-gated type 7 (Kv7) channels (▶ "Voltage-Gated K^+ Channels") resulting in depolarization and increased nociception (Obara et al. 2020). Human H_1 receptor presents high levels of spontaneous activation ("Constitutively Active Receptor States"). Receptors exist in at least two states: an inactive state and an active state which triggers a response. Under basal conditions, the equilibrium between these two states largely favors the inactive state. Agonists normally bind with higher affinity to the active form of the receptor and modify the equilibrium increasing the proportion



Histamine Receptors, Fig. 1 Main signal transduction mechanisms of histamine receptors and the major physiological processes they regulate

of receptors in the active state. Sometimes, however, under basal conditions, there is enough proportion of the receptor in the active state to produce an appreciable response in the absence of agonist. In this situation the receptor is termed to have constitutive or spontaneous activity) in the absence of an agonist, and many drugs considered to act only by blocking the receptor are actually inverse agonists of this signaling pathway as they reduce the spontaneous basal activity of the receptor. Although coupling to Gq/11 is the canonical signaling mechanism of H_1 receptor, it may also signal through small G proteins Rac and RhoA (► "Small GTPases") or through Gi/o increasing nitric oxide production and cGMP levels. In some cellular systems, it may also stimulate cAMP production by adenylyl cyclase (► "Adenylyl Cyclases") (Monczor and Fernandez 2016).

H_2 Receptor

The **H_2 receptor** was first cloned in 1991. Its gene is located in chromosome 5q35.2 and spans over 28.31 kb. The gene has an open reading frame of 1194 bp including 3 exons and codifies for a 359 amino acid polypeptide. Several polymorphisms have been described. Although there is no direct structural information of this receptor, based on mutagenesis data and homology modelling (► "Molecular Modelling"), it was inferred that histamine binds with a different orientation compared to H_1 receptor (Panula et al. 2015).

In humans, H_2 receptor stimulates adenylyl cyclase-mediated cAMP production by coupling to G proteins. Elevated cAMP, in turn, activates the serine-threonine kinase dependent of cAMP (PKA) which can phosphorylate several targets as Ca^{2+}-activated potassium channels (K_{Ca}), ligand-gated α-AMPA receptors, modulating its activity (Obara et al. 2020).

Histamine H_2 receptor displays high constitutive activity ("Constitutive Activity") toward cAMP production, and many ligands used in clinical practice actually behave as inverse agonists for this signaling pathway. Alternatively, in many

cellular systems, though not in all, the H_2 receptor can also couple to Gq/11 proteins, resulting in inositol-1,4,5-trisphosphate formation and increasing cytosolic calcium levels. Some functions of the H_2 receptor are mediated by modulation of signaling cascades associated with tyrosine kinase receptors such as the extracellular signal-regulated kinase (ERK1/2) and phosphoinositide 3-kinase pathways. H_2 receptors may also inhibit the generation of reactive oxygen species (ROS) by NADPH oxidase (► "NADPH Oxidase") inhibition (Monczor and Fernandez 2016).

H_3 Receptor

The **H_3 receptor** was firstly described on a pharmacology basis in 1983, but due to its poor sequence homology (~20%) with respect to H_1 and H_2 receptors, it was cloned in 1999. The gene covers 5.31 kb, is located in chromosome 20q13.33, and codifies for a polypeptide of 445 amino acids. Alternative splicing has been described for the H_3 receptor producing multiple receptor isoforms that could account for its pharmacological heterogeneity. Several genetic polymorphisms have been identified which may additionally confer pharmacological heterogeneity (Panula et al. 2015).

The H_3 receptor is coupled to Gi/o. Receptor activation results in inhibition of cAMP production and stimulation of PI3K-mediated phosphorylation of protein kinase B (PKB/Akt) via Gi β- and γ-subunits. After that, the downstream signaling pathway may depend on the cellular context. For example, receptor activation may inhibit voltage-gated Ca^{2+} channels (► "Voltage-Dependent Ca^{2+} Channels") in the heart and brain, stimulate G -protein-coupled inwardly rectifying potassium (Kir) channels (► "Inward Rectifier Potassium Channels"), and inhibit the Na^+/H^+ exchange transporter located in the plasmatic membrane of sympathetic nerve endings. Activation of PKB may inactivate neuronal GSK3 and activate MAPK/ERK as a neuroprotective mechanism for inflammation (Obara et al. 2020). The agonist-independent activity of the receptor leads to inhibition of cAMP production and increases arachidonic acid release.

H_4 Receptor

The **H_4 receptor** was the last histamine receptor to be discovered. Its gene was mapped at chromosome 18q11.2 and covers 19.33 kb containing 3 exons and 2 large introns. The open reading frame is of 1172 bp and encodes for 390 amino acid protein. Also, several polymorphisms have been described and at least two truncated isoforms were confirmed. Stimulation of the human H_4 receptor diminishes cAMP and increases Ca^{2+} mobilization from intracellular stores by Gi/o coupling and activation. Although H_4 receptor signaling pathways are thought to be similar to the described for the H_3 receptor, they are still being explored. Human H_4 receptors exhibit high constitutive activity ("Constitutive Activity") diminishing cAMP levels in transfected systems, but this observation was not reproduced in naive systems (Panula et al. 2015).

Drugs

H_1 Receptor Ligands

Those ligands that bind the H_1 receptor and block histamine function are important actors in the pharmacology of inflammation, typically used to treat different allergic and inflammatory-related conditions such as anaphylactic shock and allergic rhinitis and conjunctivitis. They are usually known as antihistamines and are among the most widely prescribed and over-the-counter-sold drugs in the world (Arrowsmith 2012). Most, if not all, of these compounds are now defined as inverse agonists possessing negative efficacy ("Inverse Agonism" and ► "Drug–Receptor Interaction"). Moreover, some of them were described as possessing pluridimensional efficacy, specifically activating different signaling pathways. Whether their pharmacological utility depends on this has to be established (Monczor and Fernandez 2016).

First-generation antihistamines massively marketed since the 1950s (e.g., mepyramine, diphenhydramine, and chlorpheniramine, among

the most common) are poorly selective ligands that also have significant affinity for cholinergic, alpha-adrenergic, dopaminergic, and serotonergic receptors. Chemically first-generation antihistamines include aminoalkyl ethers, ethylenediamines, piperazines, propylamines, phenothiazines, and dibenzocycloheptenes. Their major disadvantage is that they are able to cross the blood-brain barrier (► "Blood-Brain Barrier") and hamper histaminergic neurotransmission causing daytime somnolence and sedation.

A great advance occurred in the 1980s when second-generation antihistamines (e.g., cetirizine, desloratadine, and fexofenadine) were introduced. The second-generation agents share some structural similarity to the first-generation agents but have been modified to be more specific in action and limited in their distribution profiles. While possessing restricted ability to penetrate the central nervous system, minimal or no adverse effects were reported for these ligands. Despite their success, terfenadine and astemizole were withdrawn because they prolong QT interval and can be associated with the occurrence of potentially fatal cardiac arrhythmias.

The development of ligands acting as H_1 receptor agonists has been neglected due to their lack of clinical interest. A few molecules were developed, mostly as laboratory tools to determine H_1 receptor expression and function, and are generally obtained by modifications of histamine (Timmerman et al. 2017).

H_2 Receptor Ligands

H_2 antagonists were launched in the 1970s to treat patients presenting gastric or duodenal ulcers, dyspepsia, and gastroesophageal reflux disease (GERD). There are many ligands approved for their use (ranitidine, cimetidine, famotidine) although they were surpassed by proton pump inhibitors ("Proton Pump Inhibitors and Acid Pump Antagonists") and antibiotic therapies for *Helicobacter pylori*. The chemical structure of H_2 receptor blockers is based on the structure of histamine. Most of them contain an imidazole ring which may be replaced by 1) a furan ring or an aryl or heteroaryl ring bearing a basic substituent; 2) a so-called urea equivalent capable of undergoing H-bonding; and 3) a flexible chain connecting both π-electron systems (Dove et al. 2004). H_2 receptor antagonist treatment is considered safe, and lower doses of these ligands are available over the counter in pharmacies to be used for mild heartburn. Their safety profile is related to the inability of them to cross the blood-brain barrier and to act centrally. As with the H_1 receptor antagonists, H_2 receptor ligands were reclassified as inverse agonists or even more as possessing differential efficacies according to the intracellular pathway considered (Monczor and Fernandez 2016). Since H_2 receptor agonists have no clinical relevance, they were developed only to study the mechanisms of receptor activation or receptor expression. Histamine dihydrochloride has been approved for remission maintenance immunotherapy in acute leukemia (► "Cancer Immunotherapy") since, via H_2 receptor activation, it is able to potentiate IL2-promoted activation of T and NK cells conferring protection from tumor-induced immunosuppression (Martner et al. 2010).

H_3 Receptor Ligands

A considerable number of H_3 receptor selective ligands have been developed. Since the H_3 receptor inhibits the release of several neurotransmitters, those ligands that reach the central nervous system are potentially useful. The first H_3 receptor antagonists were imidazole based. However, non-imidazole molecules have become the main subject of the H_3 receptor antagonist design, because these structures deal with the issues noted with the imidazole-based ligands, especially their relatively poor central nervous system penetration (Timmerman et al. 2017). It has been observed that ligand pharmacology could be dependent on H_3 receptor isoform. H_3 receptor antagonists have the potential to increase the release of several neurotransmitters, and, remarkably, pitolisant, an orally active H_3 receptor inverse agonist, was recently approved for the treatment of narcolepsy. While agonists have been suggested for treating

insomnia, pain, and asthma, there are no currently available clinically useful agonists (Łażewska and Kieć-Kononowicz 2018).

H_4 Receptor Ligands

Since its discovery, the therapeutic benefits of targeting the H_4 receptor have been subjected to intensive research. Many ligands of the H_3 receptor, particularly imidazole-containing molecules, have high affinity for the H_4 receptor as well. With time, several selective non-imidazole H_4 receptor antagonists were developed (Timmerman et al. 2017). H_4 receptor ligands could have applications in the treatment of chronic inflammatory and immune diseases. The antagonist JNJ-39758979 significantly reduced histamine-induced itch in human subjects and reached phase 2 in a controlled clinical trial carried out in patients with moderate atopic dermatitis; however, the study was prematurely discontinued due to the appearance of serious adverse effects. Several clinical studies are being conducted to establish the putative therapeutic use of H_4 ligands in inflammatory and allergic disorders such as atopic dermatitis, pruritus, asthma, rheumatoid arthritis ("► Rheumatoid Arthritis"), and vestibular disease (Mehta et al. 2020).

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Histaminergic Receptors

► Histamine Receptors

Histone Acetylation

► Epigenetics

Histone Deacetylases

► Sirtuins

Histone Methylation

► Epigenetics

Homeostatic Appetite

► Ghrelin, Physiological Roles and Clinical Relevance of

Homologous Recombination Deficiency

► DNA Damage Response

Hormonal Contraceptives

► Contraceptives

Hormonal Regulation of Transcription

► Transcriptional Regulation

Hybridoma-Plasmacytoma Growth Factor, 26 kDa Protein

► Interleukin-6

Hydroxyaryl-Protein Kinase

► Tyrosine Kinases

5-Hydroxytryptamine = 5-HT (= Enteramine)

► Serotoninergic System

Hypoglycaemic Agents

► Glucose-Lowering Drugs Other than Insulin

IL-1

► Interleukin-1 (IL-1)

IL-17

► Interleukin-17

IL-17A

► Interleukin-17

IL-1R

► Interleukin-1 (IL-1)

IL-23

► Interleukin-23

IL23A

► Interleukin-23

IL-23p19

► Interleukin-23

IL-5

► Interleukin-5

Immune Checkpoint Blockade

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Synonyms

Checkpoint inhibitors (CPI); Immune checkpoint inhibitors

Definition

Immune checkpoint blockade identifies a form of ► cancer immunotherapy that acts by unmasking the tumor cells from the host's immune system. The so-called checkpoint proteins present on cancer cells and/or on cells of the immune system have the function to keep the immune response

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under control in physiological condition. In pathological conditions, such as in cancer, tumor cells can use this system to evade the immune response and proliferate undisturbed. Immune checkpoint blockade blocks this inhibitory signaling, thus restoring the immune system response against the tumor.

Basic Mechanism

Mechanism of Action

To prevent self-reactivity and autoimmunity, the immune system employs checkpoint pathways that regulate immune responses and maintain homeostasis. The activation of T cell response is a two-step process. First, the T cell recognizes its target cell toward the interaction between the T cell receptor (TCR) and the antigen presented by an antigen-presenting cell (APC); secondly the interaction between a co-stimulatory receptor on the T cell and its ligand on the target or on the APC is needed to fully activate the T cell. If this second step does not occur, the T cell is not activated and becomes anergic (Hawiger et al. 2001). Co-stimulatory receptors that can be upregulated during T cell activation are CD28, CD40, OX40, and CD137. Next to these co-stimulatory receptors, T cells also express inhibitory receptors that have the opposite function, inhibit T cell activation, and protect the host from uncontrolled immune response and autoreactivity. The inhibitory receptors cytotoxic T lymphocyte-associated protein 4 (CTLA-4), programmed cell death 1 (PD-1), lymphocyte activation gene 3 (LAG-3), T cell immunoglobulin and mucin 3 (TIM-3), and programmed death-1 homologue (PD-1H or VISTA) maintain the cells in a resting state. Cancer cells are able to use this system to escape immune surveillance and promote tumor growth. CTLA-4 and PD-1, both members of the immunoglobulin superfamily, are the better characterized among checkpoint proteins. The interaction between PD-1 (on T cells) and PD-L1 (on tumor cells) and the interaction between CTLA-4 (on T cells) and its ligand on an APC bring the T cell in an anergic state, effectively "hiding" the tumor from the host's immune system and preventing the immune mediate killing of tumor cells. That this pathway is one of the main mechanisms of tumor escape of the immune response is demonstrated by the fact that tumor-infiltrating lymphocytes (TIL) express higher levels of PD-1 as compared to lymphocytes present in normal tissue or in peripheral blood (Granier et al. 2017).

After binding to its ligand PDL-1 (expressed on immune cells and tumor cells), PD-1 clusters with the T cell receptor (TCR) and induces T cell inactivation via dephosphorylation of the proximal TCR signaling molecules (Yokosuka et al. 2012). Not only PD-1 but also its ligand PDL1 has an important role in tumor survival and progression. PD-L1 is not only responsible for PD-1-dependent activation of inhibitory signaling within the T cell but also has an intrinsic anti-apoptotic activity. It has been demonstrated that PD-L1 can send an anti-apoptotic signaling into the tumor cells upon binding with PD-1 (Clark et al. 2016). Monoclonal antibodies (MoAbs, ▶ "Cancer Immunotherapy") directed against PD-1 or PD-L1 are able to interrupt this pathway by impairing the binding of PD-1 and PD-L1, on one side restoring T cell activity and on the other side blocking the anti-apoptotic signal within the tumor cell. Increased levels of interferon gamma (IFN-γ), C-X-C motif ligand chemokine (CXCL)-9, and CXCL-10 (IFN-γ induced chemokines) are present in the serum of patients treated with MoAbs anti-PD-1 and PD-L1, confirming that checkpoint blockade can reactivate the adoptive immune system (Choueiri et al. 2016).

CTLA-4 shares the same ligands with the co-stimulatory receptor CD28, namely, CD80 and CD86. It therefore reduces T cell activation by competitive binding to the same ligands of a stimulatory receptor, preventing the link between CD28 and CD80/CD86. In addition to this competition, the binding of CTLA-4 to its ligands inhibits IL-2 production and T cell proliferation (Granier et al. 2017). Administration of MoAbs anti-CTLA-4 is able to restore the activation of the immune system, in analogy to what seen with PD-1/PD-L1 inhibition. Treatment with anti-CTLA-4 MoAb increases IFN-γ-producing T cells not only in the peripheral blood of the patients but also in the tumor itself (Ng Tang et al. 2013).

Additionally anti-CTLA-4 increases T cell diversity and reactivity, setting the basis for the clinical combination of anti-PD-1 and anti-CTLA-4 (van Rooij et al. 2013). Inhibition of CTLA-4 not only blocks the negative co-stimulatory pathways in T cells but also leaves CD80 and CD86 free for binding with CD28, enhancing positive co-stimulatory pathways.

If the main mechanism of action of immune checkpoint blockade is the unmasking of the neoplastic cells so that the immune system is able again to effectively recognize and kill the tumor, other mechanisms of the activity of checkpoint blockade have been identified as well. IgG1 antibodies anti-CTLA-4 can deplete regulatory T cells (Treg) via antibody-dependent cellular cytotoxicity. As Treg have been shown to inhibit anti-tumor effector T cell, their reduction due to CTLA-4 inhibition further increases the activity of immune checkpoint blockade (Simpson et al. 2013). Activated T cells depend on glucose and aerobic glycolysis to fully express their effector function. As tumor cells are also dependent on high quantity of glucose for their survival, the competitions for nutrients within the tumor microenvironment further reduce effector T cell activity. PDL-1 signaling can inhibit glycolysis through the mTOR/Akt pathway. Blockade of CTLA-4 and PD-1 increases the glycolytic capacity of tumor-infiltrating lymphocytes, thus reestablishing their effector function (Chang et al. 2015).

Biomarkers

The expression of PD-L1 (calculated as general expression, tumor proportion score, or combined positive score) is necessary for the treatment with immune checkpoint blockade in many indications, but it will not identify all the patients that will benefit from this treatment. Together with PD-L1 expression, microsatellite instability (MSI) and mismatch repair deficiency (dMMR) are other approved biomarkers to identify patients that will benefit from PD-1/PD-L1 therapy. MSI is thought to increase tumor mutational burden, and tumor mutational burden can identify patients that will respond to PD-1/PD-L1 blockade, irrespective of PD-1/PD-L1 expression. Unfortunately not all patients with MSI have a high tumor mutational burden, and a high tumor mutational burden can be found also in patients without MSI. Therefore, despite being useful, these markers of response to PD-1/PD-L1 blockade are not interchangeable. Inflammatory gene signatures, such as myeloid inflammatory gene expression, are also being investigated as possible biomarkers to predict which patients will respond to checkpoint blockade (Kruger et al. 2019).

Mechanisms of Resistance

Resistance to immune checkpoint blockade can be classified as intrinsic, i.e., tumor cells are able to escape again the host immune surveillance by altering processes connected to immune recognition, cell signaling, or DNA damage response, or extrinsic, i.e., the T cell activation process is impaired. Tumors with a low tumor mutational burden have a lower antigen expression and are therefore intrinsically more resistant to immune checkpoint blockade. Antigen loss from tumor cell surface or deficiency in antigen presentation can lead to acquired resistance to checkpoint inhibitors. Loss of major histocompatibility complex class I (MHC-I) or mutation in the TCR binding domain of MHC-I is also involved in acquired resistance. Alteration in the JAK/Stat pathway downregulates the expression of MHC-I and PD-L1, further contributing to treatment resistance. Another acquired mechanism of resistance is the upregulation of alternative suppressive checkpoint, such as TIM-3, LAG-3, and VISTA, which leads to T cell exhaustion. Epigenetic changes can also be linked to T cell exhaustion. Tumor microenvironment also plays a role in treatment resistance. Elevated levels of TGF-β have been linked in animal model to poor response to immune checkpoint inhibition. The number of TIL correlates with response to treatment. Regulatory T cells, myeloid-derived suppressor cells, and some cytokines like VEGF, CCL5, CCL17, CCL22, CXCL8, and CXCL12 can promote an immunosuppressive tumor microenvironment, conferring resistance to immune checkpoint inhibitors. VEGF expression can be unregulated also by MAPK signaling or *PTEN* deletion. Loss of *PTEN* results in constitutive activation of the PI3K pathway, and PI3K or

MAPK inhibition can enhance tumor cell cytotoxicity in mouse models. In addition to this, epigenetic silencing of CXCL9 and CXCL10 can also reduce the efficacy of checkpoint blockade (Fares et al. 2019).

Pharmacological Relevance

The importance of checkpoint inhibitors has been acknowledged by the Nobel Prize for Physiology or Medicine 2018 conferred to James P. Allison and Tasuku Honjo for the discovery of CTLA-4 and PD-1/PD-L1, respectively. Checkpoint inhibitors can induce durable remission and prolong progression-free survival in numerous cancer and are now standard therapy in the treatment of melanoma, lung cancer, urothelial cancer, head and neck squamous cell carcinoma (HNSCC), renal cell cancer, and Hodgkin's lymphoma (Gong et al. 2018). Further studies are investigating the role of checkpoint inhibitors also in other malignancies such as acute myeloid leukemia, triple negative breast cancer, hepatocellular carcinoma, or gastrointestinal cancers. Table 1 reports a summary of the main tumor entity treated with immune checkpoint blockade and their clinical indications.

Immune checkpoint inhibitors can be used alone, in combination with chemotherapy, or even combined between themselves. The anti-PD-1 MoAbs are approved as monotherapy in Europe and in the USA for the treatment of the advanced or metastatic solid tumors melanoma, non-small cell lung cancer (NSCLC), renal cancer, HNSCC, and urothelial cancer and for the treatment of relapsed or refractory Hodgkin's lymphoma. Monotherapy with the anti-PD-1 nivolumab is also indicated as adjuvant treatment for melanoma after complete resection of lymphonodal or solid metastasis. Other indications of immune checkpoint blockade vary according to the different lands (Table 1). Combination of immune checkpoint blockade and radiotherapy is also being explored, but results so far have been controversial. While good results have been observed in NSCLC with the combination of PD-L1 inhibition and chemoradiotherapy, results in head and neck cancer were dismal (Kruger et al. 2019). As chemotherapeutic drugs can also have immunological effects (▶ "Cancer Immunotherapy"), the combination of immune checkpoint blockade and conventional chemotherapy was a logical consequence. Polychemotherapeutic protocols including an immune blockade and one or more chemotherapeutic agents have been used with success in NSCLC, head and neck cancer, and triple-negative best cancer (Kruger et al. 2019). The combination of the anti-PD-1 pembrolizumab with a platinum-based chemotherapy is approved for the treatment of metastatic NSCLC. More than 150 clinical studies are investigating the combination of PD-1/PD-L1 blockade with chemotherapy (Tang et al. 2018). Checkpoint blockade can also be combined with other forms of cancer immunotherapy. The most studied is probably the combination of anti-PD-1 and anti-CTLA-4, with about 250 running clinical trials (Tang et al. 2018). The combination of ipilimumab (anti-CTLA-4) and nivolumab (anti-PD1) is approved in the USA and in Europe for the treatment of metastatic melanoma as well as for the treatment of advanced high-risk renal cancer. Despite this approach being extremely intriguing, pitfalls and potential problems should be highlighted. Combining two different agents active in the host's immune system could also have antagonist effects rather than provide a synergism. Toxicity could also be a concern. In this respect clinical trials on the combination of PD-1 inhibitors and immunomodulatory drugs in multiple myeloma were stopped after an excess of death in the PD-1 arm was seen in two phase III studies (KEYNOTE-183 and KEYNOTE-185) comparing the combination of pembrolizumab, immunomodulatory drugs (lenalidomide or pomalidomide), and dexamethasone versus the doublet immunomodulatory drugs and dexamethasone. Results of the trials failed to show an improvement with the addition of pembrolizumab to standard multiple myeloma treatment, raising questions about the future of treatment strategies targeting checkpoint blockade in this disease (Costello 2019).

Side Effects

The use of checkpoint inhibitors might be complicated by the development of autoimmune

reactions, such as pneumonitis, thyroiditis, colitis, or autoimmune hepatitis. Immune reactions due to the administration of immune checkpoint inhibitors have been reported in up to 85% of patients and are more frequent with CTLA-4 inhibition than with PD-1 inhibition. Severe immune adverse events have been reported in percentages as high as 27% with MoAb against CTLA-4 and

Immune Checkpoint Blockade, Table 1 Approved immune checkpoint inhibitors and combinations according to tumor entity

Tumor	Indication	Checkpoint containing treatment	Land of approval
Melanoma	Adjuvant indication	Nivolumab	EU, USA
		Pembrolizumab	USA
		Ipilimumab	USA
	Metastatic or advanced first line	Nivolumab	EU, USA
		Pembrolizumab	EU, USA
		Ipilimumab	EU, USA
		Nivolumab + ipilimumab	EU, USA
Cutaneous squamous cell carcinoma	Metastatic or advanced first line	Cemiplimab	EU, USA
Merkel cell carcinoma	Recurrent locally advanced or metastatic	Pembrolizumab	USA
	Metastatic first line	Avelumab	EU, USA
NSCLC	Metastatic or advanced second line	Nivolumab	EU, USA
		Atezolizumab	EU, USA
	Metastatic with PD-L1 TPS $\geq$50% (Europe) or $\geq$ 1% (USA) and no EGFR or ALK mutation first line	Pembrolizumab	EU, USA
	Metastatic with PD-L1 TPS $\geq$1% second line	Pembrolizumab	EU, USA
	Metastatic with PD-L1 expression $\geq$1% second line responsive to platinum	Durvalumab	EU, USA
	Metastatic without EGFR or ALK mutations first line	Pembrolizumab + pemetrexed + platinum chemotherapy	EU, USA
	Metastatic first line	Pembrolizumab + carboplatin +paclitaxel/ nab-paclitaxel	EU, USA
		Atezolizumab + bevacizumab + paclitaxel + carboplatin	EU, USA
		Atezolizumab + carboplatin + nab-paclitaxel	EU, USA
SCLC	Advanced first line	Atezolizumab + carboplatin+ etoposide	EU
cHL	Relapsed or refractory after ASCT and brentuximab vedotin	Nivolumab	EU, USA
	Relapsed or refractory after ASCT or ASCT ineligible and after brentuximab vedotin	Pembrolizumab	EU
	Refractory or fourth line	Pembrolizumab	USA
PMLBCL	Refractory or third line	Pembrolizumab	USA
RCC	Advanced second line	Nivolumab	EU, USA
	Advanced intermediate−/high-risk first line	Nivolumab + ipilimumab	EU, USA
	Advanced first line	Pembrolizumab + axitinib	EU, USA
		Avelumab + axitinib	EU, USA

(continued)

Immune Checkpoint Blockade, Table 1 (continued)

Tumor	Indication	Checkpoint containing treatment	Land of approval
Urothelial carcinoma	Metastatic or advanced second line after platinum-based therapy	Nivolumab	EU, USA
		Pembrolizumab	USA
		Atezolizumab	EU, USA
		Avelumab	USA
		Durvalumab	USA
	Metastatic or advanced not eligible for cisplatin and with PD-L1 CPS $\geq$ 10	Pembrolizumab	EU
	Metastatic or advanced not eligible for cisplatin and with PD-L1 expression $\geq$5%	Atezolizumab	EU, USA
HNSCC	Metastatic or advanced second line after platinum-based therapy	Nivolumab	USA
		Pembrolizumab	USA
	Metastatic or advanced with PD-L1 CPS $\geq$1 first line	Pembrolizumab	EU
		Pembrolizumab + platinum +5-FU	EU
	Metastatic with PD-L1 TPS $\geq$50% second line after platinum-based therapy	Pembrolizumab	EU
HCC	Second line after sorafenib	Nivolumab	USA
		Pembrolizumab	USA
MSI-H cancer	Second line or without other treatment options (also in pediatric patients)	Pembrolizumab	USA
Gastric cancer	Advanced or metastatic with PD-L1 CPS $\geq$1 third line after fluoropyrimidine and platinum	Pembrolizumab	USA
CRC	Metastatic MSI-H or dMMR second line after fluoropyrimidine, oxaliplatin, and irinotecan (from 12 years of age)	Nivolumab	USA
		Pembrolizumab	USA
Cervical cancer	Metastatic with PD-L1 CPS $\geq$1 second line	Pembrolizumab	USA
TNBC	Metastatic first line with PD-L1 expression >1% (USA)	Atezolizumab + nab-paclitaxel	EU, USA

NSCLC: non-small cell lung cancer; RCC: renal cell carcinoma; cHL: classical Hodgkin's lymphoma; PMLBCL: primary mediastinal large B cell lymphoma; TNBC: triple-negative breast cancer; TPS: tumor proportion score; CPS: combined positive score; MSI-H: microsatellite instability-high; dMMR: mismatch repair deficient; 5-FU: 5-fluorouracil; nivolumab, pembrolizumab, and cemiplimab: anti PD-1; avelumab, durvalumab, and atezolizumab: anti PD-L1; Ipilimumab: anti-CTLA-4; axitinib: tyrosine kinase inhibitor

19% with MoAb against PD-1. The frequency of adverse events varies not only based on the type of checkpoint inhibitor used (CTLA-4 or PD-1) but also according to the type of cancer in which treatment is used, with the lower incidence of adverse events being reported for NSCLC patients treated with the anti-PD-1 nivolumab as monotherapy. As expected the incidence of immune reaction is higher with the combination of anti-PD-1 and anti-CTLA (95% overall and 55% severe). Various organs can be involved in an autoimmune reaction. The skin is usually the first organ to be involved, with the onset of rash or pruritus in the first 4 weeks and a peak at about 6 weeks on treatment. Skin reactions are rare after the first 10 weeks of treatment. Autoimmune hepatitis and hypophysitis occur later on treatment, with an onset that starts usually after 7 weeks of treatment. Autoimmune hepatitis rarely occurs after the first 3 months of treatment, while the

risk of developing a hypophysitis remains unchanged during treatment. Colitis and diarrhea usually are evident between 5 and 10 weeks of treatment. Despite usually occurring during treatment, autoimmune reaction can become evident also once the treatment has been stopped. Anecdotal reports describe autoimmune reactions developing as far as 1 year after the end of checkpoint blockade therapy. When anti-PD-1 and anti-CTLA-4 are used together, immune-related side effects can occur earlier or can develop over a prolonged period of time. Treatment of immune-related adverse reaction requires the administration of glucocorticoids, although a generalized used of steroids should be avoided to prevent hindering the effectiveness of checkpoint inhibitor therapy. The management varies according to the organ involved. For example, in case of mild skin reaction the administration of topical emollients and corticosteroids might be sufficient, whereas in case of a pneumonitis treatment with checkpoint inhibitors should be discontinued event for low-grade events. The rare cases of myocarditis require immediate hospital admission for intravenous steroids. If steroid treatment is not sufficient to resolve the immune-related reaction, other types of immunosuppressive therapy should be considered. Mycophenolat mofetil (MMF) can be used in case of autoimmune hepatitis, pneumonitis, or severe myocarditis. Other immunosuppressive drugs to consider in case of insufficient response to steroids are tacrolimus or anti-thymoglobulin in case of autoimmune hepatitis, the MoAb anti-TNF-α infliximab in case of colitis or pneumonitis, and cyclophosphamide. Tacrolimus can have a role also in severe myocarditis. In case of neurological toxicity such as myasthenia or Guillain-Barré syndrome, plasmapheresis and administration of intravenous immunoglobulins should be considered. For any immune-related grade IV toxicity, treatment should be permanently discontinued (Haanen et al. 2017).

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► Cancer Immunotherapy

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Immune Checkpoint Inhibitors

▶ Immune Checkpoint Blockade

Immune Treatment

▶ Cancer Immunotherapy

Immuno-oncology

▶ Cancer Immunotherapy

Immunoresolvents

▶ Pro-resolving Mediators

Immunotherapy

▶ Cancer Immunotherapy

Infection

▶ Sepsis

Inflammasome

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Definition

Inflammasomes are cytoplasmic, multiprotein complexes that consist of a sensor molecule, the adaptor protein ASC, and the downstream protease pro-caspase-1. In response to microbial infection, environmental stimuli, or endogenous "danger signals," several innate immune receptors, known as pattern recognition receptors (PRRs), can act as sensor molecules to initiate inflammasome assembly. Inflammasome assembly and activation lead to the autocatalytic cleavage of pro-caspase-1 into activated caspase-1. The activation of caspase-1 not only initiates the maturation and release of interleukins 1β (IL-1β) and IL-18 but also induces inflammatory cell death, termed "pyroptosis."

Role of Inflammasomes in Infection and Diseases

The most well-described innate immune receptors initiating inflammasome assembly are NLRP1 (NLR family pyrin domain-containing 1), NLRP3, NLRP9b, NLRC4 (NLR family CARD domain-containing protein 4), IFI16 (interferon gamma-inducible protein 16), AIM2 (absent in melanoma 2), and pyrin (Gong et al. 2018a). Most inflammasomes are activated by specific pathogen-associated molecular patterns (PAMPs) derived from microorganisms. For example, bacterial flagellin and T3SS rod/needle proteins induce NLRC4 inflammasome activation through neuronal apoptosis inhibitory proteins (NAIPs). Microbial double-stranded DNA (dsDNA) can bind to AIM2 and activate the AIM2 inflammasome. Inflammasome activation not only initiates the maturation and release of interleukins 1β (IL-1β) and IL-18 but also induces

inflammatory cell death, termed "pyroptosis." IL-1β and IL-18 are important proinflammatory cytokines that play crucial roles in the activation and recruitment of inflammatory cells. Additionally, pyroptosis can also lead to the release of intracellular pathogenic microorganisms and endogenous pro-inflammatory mediators, amplifying inflammation. Thus, the activation of inflammasomes plays a crucial role in host defense against infection.

Although IL-1β and IL-18 release and pyroptosis play positive roles in host defense and promote the removal of pathogenic microorganisms, excessive inflammasome activation can elicit acute or chronic inflammation, which may lead to inflammatory diseases. In contrast to most inflammasomes, which are triggered by a limited number of PAMPs, the NLRP3 inflammasome can not only be activated by several PAMPs, such as viral RNA and bacterial toxins, but also by the widest array of damage-associated molecular patterns (DAMPs), including extracellular ATP, monosodium urate (MSU) crystals, cholesterol crystals amyloid-β, etc. Given that NLRP3 recognizes the broadest range DAMPs, the NLRP3 inflammasome is one of the most important inflammasomes and is broadly implicated in the pathogenesis of various acute and chronic inflammatory diseases, including ischemia-reperfusion injury-associated diseases (such as stroke and myocardial infarction), metabolic disorders (such as gout, type 2 diabetes and atherosclerosis), and neurodegenerative diseases (such as Alzheimer's disease and Parkinson's disease) (Table 1) (Mangan et al. 2018). In addition, gain-of-function NLRP3 mutations can lead to the autonomous activation of the inflammasome; these mutations are correlated with a group of autoimmune diseases termed cryopyrin-associated periodic syndromes (CAPS).

Inflammasome, Table 1 Sterile NLRP3 activators and associated inflammatory diseases

NLRP3 activator	Associated disease
Endogenous danger signal	
ATP	Ischemia-reperfusion injury (IRI), such as stroke and myocardial infarction
Monosodium urate (MSU) crystals	Gout
Calcium pyrophosphate dihydrate (CPPD)	Pseudogout
Free fatty acids (FFA), islet amyloid polypeptide (IAPP), glucose	Type 2 diabetes
Oxidized low-density lipoprotein (LDL), cholesterol crystals	Atherosclerosis
β-Amyloid (Aβ)	Alzheimer's disease
α-Synuclein	Parkinson's disease
Alu-RNA	Age-related macular degeneration (AMD)
Environmental insult	
Silica	Silicosis
Asbestos	Asbestosis
Skin irritants	Contact hypersensitivity reactions
Ultraviolet light	Sunburn

Mechanisms of NLRP3 Inflammasome Activation

Because the NLRP3 inflammasome can be activated by numerous endogenous and environmental stimuli, tremendous efforts have been made to investigate the molecular mechanisms of NLRP3 inflammasome activation. Generally, the activation of the NLRP3 inflammasome needs two signals, known as the "priming" signal and the "activation" signal. The priming signal (signal 1), provided by microbial or endogenous molecules, can activate the Toll-like receptor (TLR) signaling pathway, which not only increases the expression of the IL-1β precursor and NLRP3 but also facilitates the posttranscriptional modification of NLRP3. Once the process is primed, various DAMPs and PAMPs can act as activation signal (signal 2) to initiate the assembly of the NLRP3 inflammasome. Given the broad range of NLRP3 stimuli, it is impossible for NLRP3 to directly sense all of these activators. Previous studies have suggested that multiple cellular events, including ion flux, mitochondrial damage, lysosomal rupture, and Golgi dispersal, may act as the intracellular "second messenger" to induce

NLRP3 inflammasome assembly and activation (Fig. 1) (Gong et al. 2018b; Swanson et al. 2019).

Multiple NLRP3 agonists can induce the efflux of intracellular K^+ and Cl^-. Both K^+-free and Cl^--free media were sufficient to induce NLRP3 inflammasome activation in the absence of any NLRP3 agonist. In addition, intracellular Ca^{2+} can increase in response to various NLRP3 activators. Several inhibitors targeting either extracellular or intracellular Ca^{2+} storage have been reported to prevent intracellular Ca^{2+} mobilization and to inhibit the activation of the NLRP3 inflammasome.

Several studies have connected mitochondria to NLRP3 inflammasome activation. Although the exact role of mitochondria in NLRP3 activation needs to be further verified, NLRP3 activators have been shown to induce mitochondrial damage, thus promoting mitochondrial reactive oxygen species (mtROS) generation and oxidized mtDNA accumulation. Oxidized mtDNA has been shown to interact with NLRP3 and to promote NLRP3 inflammasome assembly.

Another well-known hypothesis to explain particulate-induced NLRP3 inflammasome is "lysosomal rupture." In this context, particle agonists, such as silica particles, cholesterol crystals, and amyloid-β, are taken up by macrophages or DCs into intracellular phagolysosomes, leading to lysosome rupture, lysosomal protease release, and NLRP3 inflammasome activation.

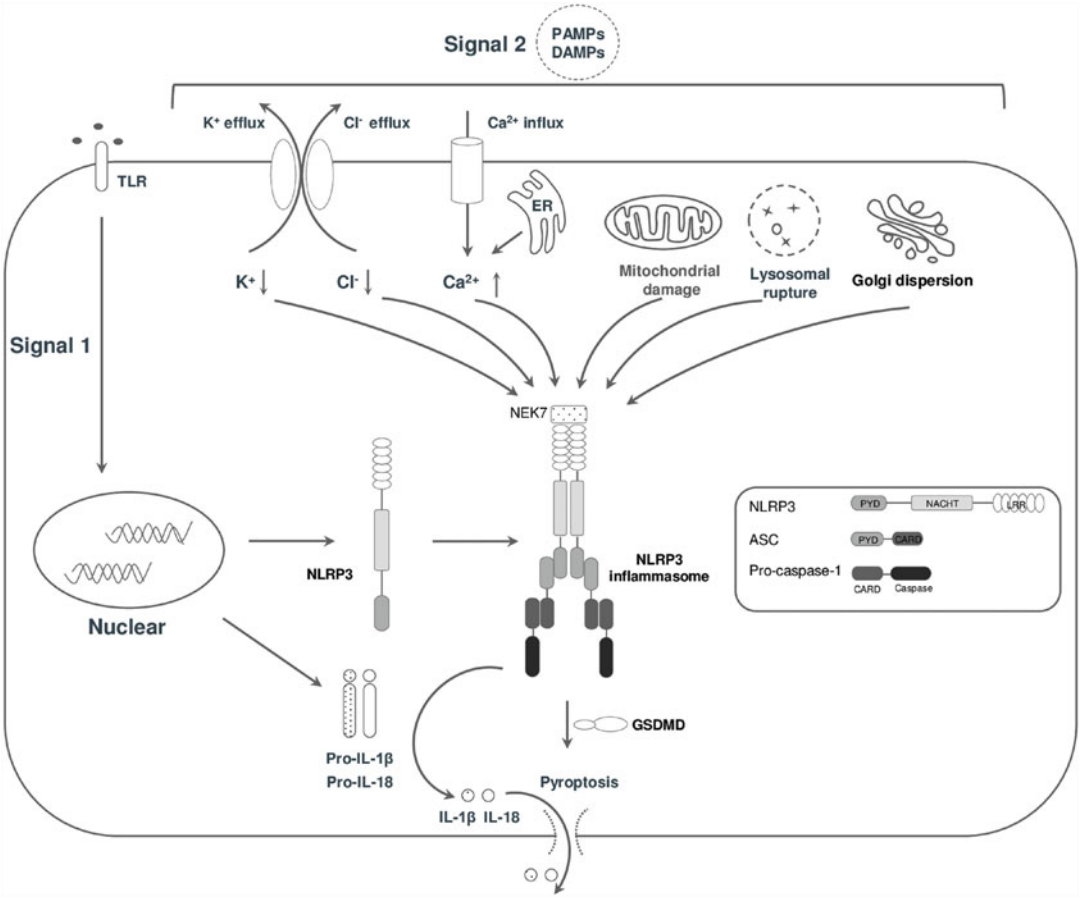
Recent research has also shown a crucial role of the Golgi apparatus in NLRP3 activation. Various NLRP3 activators can induce the disassembly of the trans-Golgi network (TGN), which then functions as a scaffold for the aggregation and polymerization of the NLRP3 inflammasome complex.

In response to various NLRP3 activators, these cellular events can induce the interaction between NEK7 and NLRP3, which facilitates the self-oligomerization of NLRP3. Oligomeric NLRP3 then recruits ASC and promotes its clustering via the homotypic pyrin domain (PYD). Clustered ASC interacts with pro-caspase-1 via the homotypic caspase recruitment domain (CARD), eventually leading to the autocleavage of pro-caspase-1. Activated caspase-1 cleaves the IL-1β precursor and IL-18 precursor, as well as gasdermin D (GSDMD). The cleaved N-terminal domain of GSDMD (GSDMD-N) can insert into the plasma membrane to form cytotoxic pores, which not only induce inflammatory cell death (pyroptosis) but also facilitate mature IL-1β and IL-18 release (Fig. 1).

In addition to canonical NLRP3 inflammasome activation, caspase-11-dependent noncanonical NLRP3 inflammasome activation can also be induced in response to intracellular LPS, a principal membrane component of gram-negative bacteria. Intracellular LPS has been shown to directly interact with and activate mouse caspase-11 (in human caspase-4/5). Activated caspase-4/5/11 directly cleaves GSDMD and pannexin-1, which induce cytotoxic pore formation and nonselective channel opening, respectively, promoting subsequent NLRP3 inflammasome activation and pyroptosis.

Therapies Targeting the NLRP3 Inflammasome

Excessive activation of the NLRP3 inflammasome in response to numerous endogenous or environmental stimuli has been implicated in the pathogenesis of various inflammatory diseases; therefore, inhibition of the NLRP3 inflammasome may be a promising strategy for the treatment of these inflammatory diseases (Mangan et al. 2018). Indeed, several reagents targeting the inflammasome products IL-1β and IL-18 have been developed to treat NLRP3-related diseases. For example, canakinumab, a neutralizing monoclonal anti-IL-1β antibody, significantly reduced the incidence of arthritis and gout clinically. In addition to targeting inflammasome products, several inhibitors can also inhibit NLRP3 inflammasome activation by targeting upstream activating events, the adaptor ASC or caspase-1. However, these inhibitors inevitably result in unexpected cascade reactions in vivo. Thus, numerous scientists have now turned their efforts to controlling NLRP3 inflammasome activation by directly targeting the NLRP3 protein. Several NLRP3-targeting



Inflammasome, Fig. 1 Priming and activation of the NLRP3 inflammasome. Generally, the activation of the NLRP3 inflammasome requires two signals, the "priming" signal (signal 1) and the "activation" signal (signal 2). The priming signal, provided by microbial or endogenous molecules, activates the Toll-like receptor (TLR) signaling pathway, which not only increases the expression of the IL-1β precursor and NLRP3 but also facilitates the post-transcriptional modification of NLRP3. Once the pathway is primed, various DAMPs and PAMPs function as activation signals to initiate the assembly of the NLRP3 inflammasome. Multiple cellular events, including K^+ efflux, Cl^- efflux, Ca^{2+} mobilization, mitochondrial damage, lysosome rupture, and Golgi dispersal, can act as the intracellular "second messenger" to induce NLRP3 inflammasome assembly and activation. Activated NLRP3 inflammasomes process cytokine precursors (pro-IL-1β/pro-IL-18) into their mature forms and induce pyroptosis in a caspase-1-dependent manner

compounds have been recently developed; these compounds not only exhibit anti-NLRP3 activity in vitro but also show excellent anti-inflammatory function in several NLRP3-dependent disease models (Table 2) (Bertinaria et al. 2019).

The ATPase activity of NLRP3 within its NACHT domain is necessary for self-oligomerization, and it has been recognized as a feasible target for the development of NLRP3 inhibitors. Previous studies have shown that multiple inhibitors can bind to NLRP3 and block its ATPase activity (Bertinaria et al. 2019). Several alkylating agents, including Bay11-7082, 3,4-methylenedioxy-β-nitrostyrene (MNS), and 2-cyclohexylimino-6-methyl-6,7-dihydro-5H-benzo [1,3]oxathiol-4-one (BOT-4-one), have been reported to inhibit NLRP3 ATPase activity via direct alkylation of NLRP3. All three NLRP3-

Inflammasome, Table 2 Pharmacological inhibitors of NLRP3

Inhibitor	Target domain or site	Inhibitory mechanism	Disease model
BAY 11–7082	–	Induces NLRP3 alkylation and inhibits the ATPase activity of NLRP3	Diabesity
MNS	LRR or NACHT domain		–
BOT-4-one	–		Peritonitis
MCC950	Within or close to the Walker B motif	Inhibits the ATPase activity of NLRP3	Cryopyrin-associated periodic syndromes (CAPS), Alzheimer's disease (AD), stroke, multiple sclerosis (MS), atherosclerosis, asthma, nonalcoholic fatty liver disease (NAFLD)
CY-09	ATP-binding site		Gouty arthritis, CAPS, type 2 diabetes (T2D)
OLT1177	–		LPS-induced peritoneal inflammation
INF39	–		Colitis
INF58	–		–
Oridonin	Cys279	Inhibits the NEK7-NLRP3 interaction	Peritonitis, gouty arthritis, T2D
NBC6/13	–	Inhibits NLRP3 assembly	LPS-induced peritoneal inflammation
Tranilast	NACHT domain	Inhibits NLRP3 oligomerization	Gouty arthritis, CAPS, T2D

alkylating agents inhibit NLRP3 inflammasome activation and IL-1β secretion in vitro. In addition, BAY 11-7082 was recently shown to inhibit NLRP3-dependent inflammation in a mouse model of high-fat, high-fructose diet-induced metabolic disorder. The anti-NLRP3 activity of BOT-4-one was confirmed in MSU-induced acute peritonitis in mice. One of the most difficult challenges limiting the clinical use of these alkylating agents is that they all have been reported to inhibit other kinases or proteins. For example, BOT-4-one completely inhibits IKKβ activity at 30 μM. Additionally, the exact alkylation site in NLRP3 remains to be determined.

In addition to NLRP3-alkylating agents, multiple small molecules, such as MCC950, CY-09, OLT1177, and INF39, have been developed to inhibit NLRP3 inflammasome activation by blocking the ATPase activity of NLRP3. MCC950, also known as CP456,773, is the best studied inhibitor of NLRP3 (Mangan et al. 2018). It specifically inhibits the activation of the NLRP3 inflammasome at an extremely low dose (IC50 = 8.1 nM based on ATP-induced IL-1β secretion in human monocytes). Mechanistic studies have indicated that MCC950 directly binds to the NLRP3 protein at a site within or close to its Walker B motif, which could inhibit ATP hydrolysis and NLRP3 oligomerization. Several studies have shown that MCC950 not only inhibits NLRP3 inflammasome activation in vitro but also displays anti-inflammatory activity in a wide range of NLRP3-related disease models, such as murine models of CAPS, stroke, atherosclerosis, multiple sclerosis (MS), AD, asthma, and nonalcoholic fatty liver disease (NAFLD). In addition, MCC950 has been tested in phase II clinical trials for the treatment of rheumatoid arthritis. However, these studies revealed that MCC950 may induce liver injury in some cases, and the clinical trials were not continued further. The reason for this liver toxicity needs to be further investigated. CY-09, a novel small molecule, was developed to specifically bind to the ATP-binding site on the NACHT domain of NLRP3, thereby inhibiting the ATPase activity of NLRP3. In vivo data suggest that CY-09 inhibits

NLRP3-dependent systemic inflammation and has remarkable preventive or therapeutic effects in mouse models of gouty arthritis, CAPS, and type 2 diabetes (T2D) (Mangan et al. 2018). More importantly, ex vivo data show that CY-09 also inhibits the preactivated NLRP3 inflammasome in synovial fluid cells (SFCs) from patients with gout, indicating the potential of CY-09 for the clinical treatment of rheumatic diseases. OLT1177, also known as dapansutrile, is another interesting NLRP3 inhibitor that blocks NLRP3 ATPase activity. OLT1177 is now under development by Olatec Therapeutics and is in phase II clinical trials for the treatment of osteoarthritis (Bertinaria et al. 2019). INF39 (ethyl 2-(2-chlorobenzyl)acrylate) and INF58 are two acrylic acid derivatives that inhibit NLRP3 inflammasome activation by inhibiting the ATPase activity of NLRP3. INF39 was shown to reduce inflammatory cytokine release and to exert a protective effect in a rat model of experimental colitis (Bertinaria et al. 2019).

Oridonin, a bioactive ent-kaurane diterpenoid, is the major active constituent of *Rabdosia rubescens*, which is often used in traditional Chinese medicine. Oridonin can covalently bind to cysteine 279 in the NACHT domain of NLRP3, thus inhibiting the interaction between NEK7 and NLRP3 (Bertinaria et al. 2019). In vitro data showed that oridonin can specifically inhibit the assembly and activation of the NLRP3 inflammasome at a very low dose (IC50 $\approx$ 0.5 μM). In addition, oridonin suppresses NLRP3 activation and alleviates disease progression in mouse models of multiple NLRP3-driven diseases, such as peritonitis, gouty arthritis, and T2D.

A series of boron-based inhibitors have been shown to inhibit NLRP3 inflammasome activation by directly targeting the NLRP3 protein (Bertinaria et al. 2019). It has been shown that the minimal pharmacophore for these inhibitors is a boron atom linked to an oxygen atom. Among these compounds, 5-acetyl-6-amino-2,2-diphenyl-4-(trichloromethyl)-2,3-dihydro-1,3,2-oxazaborinin-1-ium-2-uide (NBC6) is the most promising, with limited cytotoxicity and a low inhibitory dose (IC50 = 0.574 μM based on nigericin-induced NLRP3 activation in human THP-1 cell line). Indeed, NBC13, an analog of NBC6, has been evaluated for anti-inflammatory effects in vivo using an LPS-driven peritoneal inflammation model.

Tranilast, an analog of a tryptophan metabolite, is a relatively safe and well-tolerated drug in clinical use. It is used to treat various inflammatory diseases, such as bronchial asthma, atypical dermatitis, and hypertrophic scars. Most recently, tranilast has been shown to specifically bind to NLRP3 and inhibit the assembly and activation of the NLRP3 inflammasome in vitro. In vivo data revealed that tranilast has preventive or therapeutic effects in mouse models of NLRP3-dependent inflammatory diseases, including gouty arthritis, CAPS, and T2D (Bertinaria et al. 2019). Although the exact binding site of tranilast to NLRP3 has not been identified, the NACHT domain of NLRP3 is responsible for the interaction between NLRP3 and tranilast. More efforts are necessary to ascertain the clinical therapeutic effect of tranilast on NLRP3-related diseases.

Taken together, data indicate that targeting NLRP3 may be a feasible and effective strategy for the clinical treatment of NLRP3-related inflammatory diseases. It is time to accelerate the development of drugs targeting NLRP3 and to evaluate them in clinical trials.

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Inflammation

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Definition

Inflammation occurs when a living tissue is injured or infected by microorganisms. It is a beneficial, self-limited response that requires phagocytic cells and elements of circulating plasma to enter the affected area. In principle, it may achieve resolution and repair as the ideal outcome of inflammation. The persistent accumulation and activation of leukocytes is a hallmark of chronic inflammation.

Basic Mechanisms

Introduction

The clinical characteristics of acute inflammation are familiar to anyone who has suffered from a burned or infected finger. The account comprises the cardinal symptoms of inflammation with heat, redness, swelling, and pain that were reported by the Roman encyclopedist Aulus Cornelius Celsus (25 BC–50 AD) as calor, rubor, tumor, and dolor. Galen of Pergamon (129–199 AD) added a 5th cardinal symptom, the impaired function, or functio laesa. Though unpleasant, these signs are indicators of useful processes going on with the aim of limiting tissue damage and infection and initiating repair. Inflammation starts after an initial injury by mechanical trauma, infections, UV irradiation, burns, ischemia, and many others (Ley et al. 2007). It initially comprises the release of chemical mediators like histamine from a population of cells that are distributed throughout the connective tissue and are extremely sensitive to injury, the mast cells (Kubo 2018). The same is true for the blood basophil, which in many aspects resembles the mast cell (Kubo 2018). Histamine and secondary mediators like eicosanoids and nitric oxide cause vasodilation and increase the diameter of arterioles, capillaries, and venules, which results in increased blood flow to the injured area and consequently redness and heat. An increase in the vascular permeability causes the loss of solutes and proteins from the blood plasma, a process called exsudation, which leads to swelling and edema formation (tumor). The local increase in tissue turgor and the activation of the kinin cascade with the generation of particularly bradykinin are major factors causing pain, the fourth cardinal symptom of inflammation. Besides these changes of the microcirculation, the acute inflammatory response essentially requires the extravasation of leukocytes and phagocytosis of microorganism and cell or tissue debris.

Cells and Mediators of Acute Inflammation

The migration of phagocytic cells to the site of damage is one of the most fundamental components of the acute inflammatory response, and the key players in this process will be presented next.

The Endothelial Cell

The vascular endothelium plays an important role in regulation of vascular tone and permeability. Dilation of arterioles to increase blood flow and constriction of endothelial cells of postcapillary venules causing exsudation of plasma constituents illustrates the complex nature of this cell type. Moreover, by expression of adhesion molecules and secretion of chemokines, endothelial cells play an important role in the recruitment of leukocytes to the inflamed area. Endothelial cells express two basic types of adhesion molecules on their surface:

1. Selectins (E-selectin, P-selectin)
2. Members of the immunoglobulin gene superfamily (VCAM-1, ICAM-1, ICAM-2)

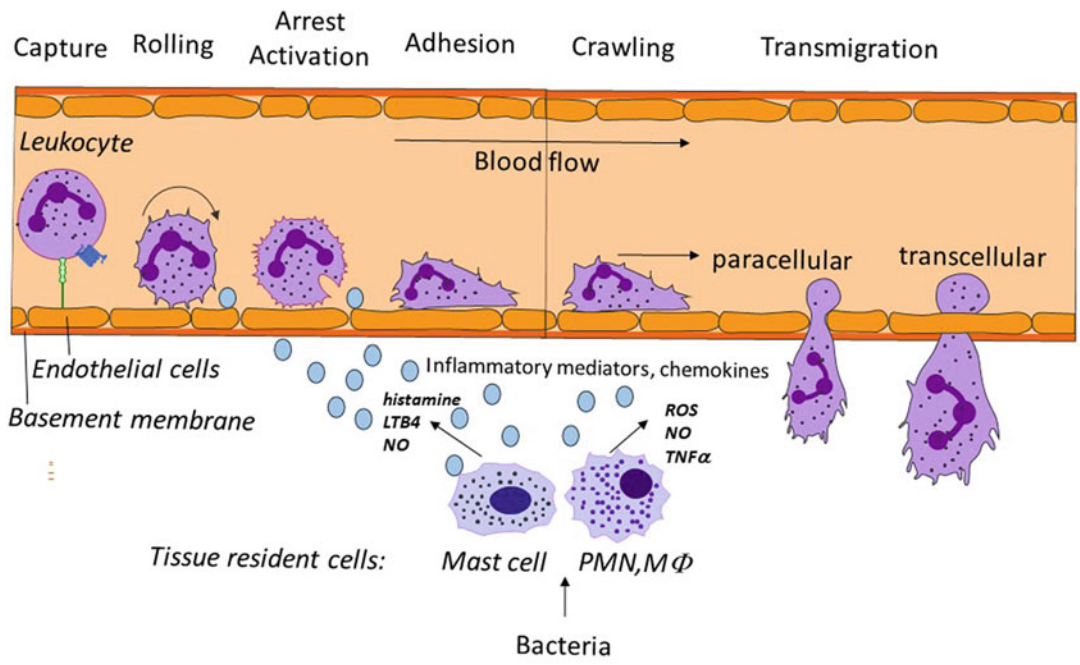
Selectins are a family of glycoproteins that allow the initial attachment and rolling of leukocytes on endothelial cells. Selectins are not expressed on the surface of resting endothelial cells but are exposed upon activation with a number of mediators like interleukin-1 (IL-1), tumor necrosis factor α (TNFα), lipopolysaccharide, or thrombin. Two endothelial selectins have been reported. E-selectin is synthesized de novo and expressed on endothelial cell surface following stimulation. The specific ligand for E-selectin is stored in Weibel–Palade bodies of endothelial cells and in α-granules of platelets and is translocated within minutes to the plasma membrane following exposure to inflammatory mediators. The ligand for P-selectin is the so-called P-selectin glycoprotein ligand-1 (PSGL-1), which has the sugar lacto-n-fucopentaose III in its core domain and is present on hematopoietic cells.

The second class of adhesion molecules expressed on endothelial cells belong to the immunoglobulin gene superfamily. These transmembrane glycoproteins structurally resemble in certain parts the structure of immunoglobulins. Whereas intercellular cell adhesion molecule-1 (ICAM-1) and ICAM-2 are constitutively expressed on endothelial cells, the third member vascular cell adhesion molecule-1 (VCAM-1) is not present on resting endothelial cells but is upregulated together with ICAM-1 in the course of acute inflammation by cytokines like IL-1 or TNFα within a few hours. The counterreceptor on the cell membrane of leukocytes that binds to ICAMs and VCAM-1 belongs to the integrin family of adhesion molecules.

The Neutrophil

In the very early phases of the acute inflammatory response, most of the cells invading the damaged area are polymorphonuclear neutrophils, also denoted as PMNs, which serve as initial line of defense and source of proinflammatory cytokines (Liew and Kubes 2019). These cells, which usually live for 4–5 days, circulate in the blood until they are attracted by chemokines into injured tissues. Whereas physical injury does not recruit many neutrophils, infections with bacteria or fungi elicit a striking neutrophil response. Under inflammation, the life span of neutrophils increases by several fold, which is important to ensure their continuous presence at the site of inflammation. The characteristic pus of a bacterial abscess is composed mainly of apoptotic (*apoptosis*) and necrotic PMNs. Emigration of neutrophils from the blood starts with a process denoted as margination where neutrophils come to lie at the periphery of the cross-sectional lumen of the blood vessel and adhere to endothelial cells (Fig. 1). L-Selectin is expressed constitutively on most leukocytes and interacts with ligands that are induced in endothelial cells exposed to inflammatory cytokines. The second class of surface molecules that mediate adhesion of neutrophils and monocytes to endothelial cells are the integrins. Leukocyte integrins are heterodimeric transmembrane proteins consisting of α and β chains with short cytoplasmic domains that are engaged in signal transduction. Many cells express integrins, and some of the known integrins are cell-type specific. Integrins mediate firm adhesion that follows the initial rolling of leukocytes along the endothelial cell lining. All the leukocyte integrins share a common small subunit, the β_2 subunit. There are at least four larger α subunits known that can associate with the β_2 subunit to form unique receptors. These β_2-integrins will adhere to the endothelial ICAMs in the course of acute inflammation. Once the neutrophils have made firm contact to the endothelial cells, they protrude pseudopodia and leave the blood vessel by transmigration either by a paracellular route (between endothelial cells), or a transcellular route (through an endothelial cell), and subsequently pass the basement membrane (Fig. 1) (Liew and Kubes 2019). The whole process takes a few minutes.

The most important function of neutrophils is the killing of bacteria, which is executed by various mechanisms including phagocytosis, generation of reactive oxygen species, degranulation of cytotoxic and antimicrobial proteases, and by release of neutrophil extracellular traps (NET). The latter consist of DNA, associated citrullinated histones, and a series of proteases (Sorensen and Borregaard 2016; Liew and Kubes 2019).



Inflammation, Fig. 1 Sequence of events in the recruitment of leukocytes in postcapillary venules adjacent to injured tissue. At the site of lesion, diverse reactive substances stimulate the endothelium to produce inflammatory cytokines, chemoattractants, and other inflammatory mediators. The cytokine-activated endothelium expresses adhesion molecules that lead to the low affinity interactions between leukocytes and endothelium, which is mediated by selectins and described as rolling. Subsequently integrins mediate the firm adhesion of leukocytes, which allows emigration of the cells from venules into the interstitial compartment. Activated mast cells, PMNs, and macrophages secrete cytokines (TNFα), lipid mediators (LTB_4), and other inflammatory players (histamine, NO)

The Monocyte/Macrophage

The very early peak of neutrophil invasion into an inflamed area is followed several hours later by a wave of a second class of phagocytic cells, the macrophages. This biphasic pattern of inflammatory cell movement and accumulation is observed in most acute inflammatory responses. The mononuclear phagocyte in the blood is known as the monocyte and differentiates into the macrophage upon entering into tissues. Depending on the local cytokine and growth factor milieu, monocytes and tissue-resident macrophages differentiate into macrophages of different phenotypes, an event that is also known as macrophage polarization (Shapouri-Moghaddam et al. 2018). Two main phenotypes exist, which include the classically activated or inflammatory (M1) macrophage and the alternatively activated or anti-inflammatory (M2) macrophage. M1 macrophages are typically induced by interferon-γ, TNFα, or the bacterial product LPS, and, in turn, produce and secrete pro-inflammatory cytokines. M1 macrophages have an important function in removing pathogens during infection, thus are antimicrobial, but also induce tissue damage, impair tissue regeneration and wound healing, and have antitumoral activity. M2 macrophages are induced by IL-4, IL-13, and IL-33, and trigger rather anti-inflammatory cytokine and chemokine production, like IL-10 and transforming growth factor β (TGFβ). M2 macrophages have an anti-inflammatory function and show a potent phagocytic and tissue remodeling capacity.

Chemotactic factors that act on macrophages include complement cleavage products, membrane components of microorganisms, and fibrin degradation products that also attract neutrophils. In addition, there are specific chemokines that act

exclusively on macrophages. Macrophages in turn release large amounts of growth factors and cytokines.

Over the last decade, evidence has accumulated that not all the tissue macrophages derive from invaded blood monocytes, but that a subpool of macrophages are established during embryonic development to reside in the different organs and persist to adulthood independent of blood monocytes (Davies et al. 2013). These include Kupffer cells in the liver, osteoclasts in the bone, microglia in the central nervous system, alveolar macrophages in the lung, and dermal macrophages in the skin. These tissue-resident macrophages are classified as M2-like and exert specialized organ-specific functions such as immune surveillance of the organ, clearance of cell debris, and maintenance of homeostasis.

Mediators of Inflammation

Many low-weight compounds produced by microorganisms, like formylated peptides as well as endogenous mediators, are chemotactic for leukocytes and promote the inflammatory process. The main endogenous compounds are listed in Table 1 and are derived from activated plasma protein cascades that function as amplification mechanisms, are performed and released from activated cells, or are de novo synthesized on demand by cells participating in or being affected by inflammatory events. The major modulators of leukocyte adhesion to endothelial cells are listed in Table 2.

Inflammation, Table 1 Endogenously produced mediators of inflammation

Category	Mediators
Preformed mediators released from activated cells	Histamine
	Serotonin
	Lysosomal enzymes
Mediators derived from activated plasma protein cascades	Complement system
	Kinin cascade
	Clotting pathway
	Fibrinolytic system
De novo synthesized mediators	Prostaglandins
	Leukotrienes
	Cytokines
	Reactive oxygen species
	Gasotransmitters (NO, CO, H_2S)

Healing

The objectives of the inflammatory response can be viewed as a hierarchical ordered panel of events. The most successful consequence of an inflammatory response is the complete restoration of function and structure of the affected tissue, also denoted as resolution. If this is not possible, inflammation aims for healing by repair and replacement of lost tissue by scar tissue.

For many years, the mechanism of resolution was thought to solely include proinflammatory mediator catabolism that fades out the inflammatory response. This is, however, only the one side of the story as recent discoveries highlight the involvement of an active program of resolution that is activated during inflammation (Serhan and Levy 2018; Serhan et al. 2007). Certainly, the cessation of initiating stimuli, e.g., the killing of invading bacteria and microorganisms and the complete removal of inflammatory exudate, is an important event to slow down inflammation. Neutrophils carry out the killing of invading microorganisms that subsequently die by apoptosis, whereas macrophages are mainly responsible for clearing dead neutrophils and fluid phase debris by phagocytosis and extremely active pinocytosis. It should be emphasized that the clearing of apoptotic cells by phagocytes is extremely fast and efficient, and dying cells release chemokines to speed up their removal by attracting macrophages. However, cytokines, like interleukin-6 and anti-inflammatory lipid mediators, such as the specialized pro-resolving mediators (SPM), including resolvins, protectins, maresins, and lipoxins (Serhan and Levy 2018; Serhan et al. 2007), or prostaglandins D_2 and E_2, play important roles in providing stop signals for acute inflammatory processes. Most macrophages emigrate from the inflamed site to reach draining lymph nodes. Subsequently tissue that has been

Inflammation, Table 2 Modulation of leukocyte adhesion to endothelial cells

Target cell	Endothelial cell	Leukocyte
Antiadhesive	Interleukin-4	Interleukin-4
	Interleukin-10	Interleukin-10
	Interleukin-13	Interleukin-13
		Prostacyclin (PGI_2)
	Adenosine	Adenosine
	Gasotransmitters (NO, CO, H_2S)	Gasotransmitters (NO, CO, H_2S)
	Glucocorticoids	Glucocorticoids
	Lipoxins, resolvins, protectins, maresins	Lipoxins, resolvins, protectins, maresins
Proadhesive	Tumor necrosis factor α (TNFα)	Tumor necrosis factor α (TNFα)
	Interleukin-1	Interleukin-1
	Interferon γ	Interferon γ
		Interleukin-8
		MCP-1
	Leukotriene B_4	Leukotriene B_4
	PAF	PAF
	Endotoxin	Endotoxin C3b, C5a
	Histamine	Neuropeptides

lost must be replaced in an orderly fashion. The replacement of lost cells by compensatory proliferation and phenotypic change of surviving resident cells is part of the healing process. The exsudate is invaded by macrophages and fibroblasts and the formation of new blood vessels (angiogenesis) is initiated. This series of events finally results in fibrosis and the formation of tissue scar, which may be considered as an essential component of wound healing and repair (Wynn and Ramalingam 2012).

Chronic Inflammation

If the endogenous control mechanisms of inflammation fail and resolution or healing by repair cannot be achieved, the inflammatory process may persist for weeks, months, or even years and is termed chronic. The inflammatory macrophage is not only a ringmaster for safe resolution and repair of inflammation but also for chronicity of the disease. Some of the products secreted by macrophages are relevant to chronic inflammation such as IL-1, TNFα, IL-6, or IL-10, just to name a few. A further characteristic feature is the presence of activated B and T lymphocytes, which represent a local immune response to antigens presented to them by macrophages or dendritic cells. B cells differentiate upon activation to plasma cells that release immunoglobulins which in most cases is a good indicator of chronicity of inflammation.

There are now several diseases described as chronic inflammatory, where no clearly defined initiating trigger is known. These are of particular interest as they affect a great proportion of people of Western countries and include type 2 diabetes mellitus, obesity, atherosclerosis, and cancer (Medzhitov 2010).

Cross-References

- ▶ Allergy
- ▶ Anti-integrins
- ▶ Ca^{2+}-Binding Proteins
- ▶ Glucocorticoids
- ▶ S100 Proteins

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Inhibitors

► Monoamine Oxidases and Their Inhibitors

Inhibitory Glycine Receptor

► Glycine Receptors

Inhibitory Neurotransmission

► GABAergic System

INSL3

► Relaxin Family Peptides and Their Receptors

INSL5

► Relaxin Family Peptides and Their Receptors

INSR (Gene Name)

► Insulin Receptor

Insulin Receptor

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Synonyms

INSR (gene name)

Definition

The insulin receptor (INSR) is a transmembrane receptor tyrosine kinase located in the plasma membrane of insulin-sensitive cells (e.g., adipocytes, myocytes, hepatocytes, neurons). It mediates the effect of insulin on specific cellular responses (e.g., glucose transport, glycogen synthesis, lipid synthesis, protein synthesis).

Basic Characteristics

Structure and Function

The INSR is a heterotetrameric protein which consists of two extracellular α-subunits (molecular weight 135 kDa) and two membrane spanning

β-subunits (molecular weight 95 kDa). These subunits are covalently linked by disulfide bonds ($\alpha_2\beta_2$-structure) (Fig. 1). The receptor is encoded by a single gene which is located on human chromosome 19 and consists of 22 exons (exon 1–11 = α-subunit, exon 12–22 = β-subunit). The single chain polypeptide precursor is posttranslationally cleaved in the endoplasmic reticulum into the α- and β-subunits which subsequently dimerize. Both subunits are glycosylated in the Golgi apparatus.

In addition to insulin, the INSR can also bind insulin-like growth factors (IGF1 and IGF2, the latter activates INSR predominantly during the prenatal period) albeit with considerably lower affinity (2 and 3 orders of magnitude, respectively).

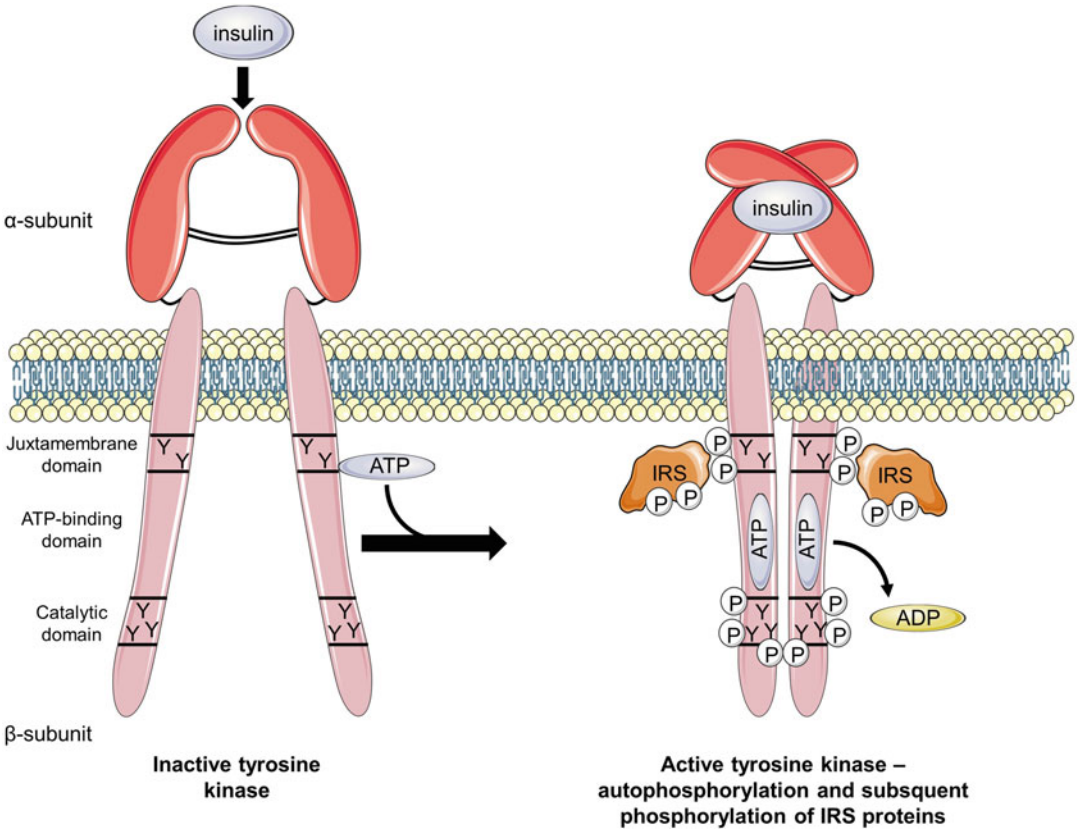
The insulin-binding domain of the INSR is located within a cysteine-rich region of the α-subunits. Alternative splicing of exon 11 generates two isoforms of the α-subunit which differ in their C-terminus and in their tissue distribution (type A: brain; type B: liver; type A and B: skeletal muscle and fat). The isoforms differ in their affinity to insulin (A > B), but their relevance for normal and impaired insulin action is not entirely clear. It has been suggested that INSR A isoform evokes more mitogenic and INSR B isoform more metabolic effects (Belfiore et al. 2009).

Activation of the tyrosine kinase activity of the INSR is essential for the receptor function. The tyrosine kinase domain of the INSR is localized in the cytoplasmic region of the β-subunit. In the absence of insulin, the α-subunits strongly repress the tyrosine kinase activity of the β-subunits. Binding of insulin releases this block through a conformational change and induces dimerization and/or oligomerization of β-subunits which results in receptor transautophosphorylation. While several phosphorylated tyrosine residues in the catalytic domain (Y1158, Y1162, Y1163 of human INSR B isoform) are essential for the kinase activity of the receptor, phosphorylation of two tyrosine residues in the juxtamembrane domain (Y965 and Y975 of human INSR) is critical for the interaction of the receptor with other signaling components of the insulin receptor signaling cascade (Fig. 1) (Kido et al. 2001; Frojdo et al. 2009).

Signaling through the INSR systems has evolved early and has been highly conserved during evolution. Proteins related to the human INSR gene product have been found in *Caenorhabditis elegans* (DAF-2) as well as in *Drosophila melanogaster* (DIR). Insulin-like signaling peptides (e.g., bombyxin) have been found in such distantly related organisms as the silkworm (Kido et al. 2001).

Structurally and functionally related receptors to the INSR in mammalian organisms are the insulin-like growth factor 1 receptor (IGF1R) and the insulin receptor-related receptor (INSRR). Although the function and signal transduction of the IGF1R and the INSRR resembles that of the INSR, major differences appear in the tissue distribution of the receptors. In contrast to the INSR, the IGF1R is expressed in adipocytes only at low levels and is almost absent in hepatocytes, yet more abundant expressed in brain compared to the INSR. The INSRR is an orphan receptor whose endogenous ligand is yet unknown and acts as an extracellular alkali sensor. It has been found in adrenal glands, kidney, and testis and a few neuronal cell types (e.g., dorsal root ganglion and trigeminal neurons).

Deletion of the INSR gene results in normal development but early postnatal lethality because of ketoacidosis. Organ-specific INSR-knockout models (>95% reduction of the receptor protein content in the organ) exhibit less severe phenotypes. Mice with a **l**iver-specific **i**nsulin **r**eceptor **k**nock **o**ut (LIRKO) showed severe insulin resistance and hyperglycemia due to an increased hepatic gluconeogenesis, whereas the tissue-specific knockout of the insulin receptor in pancreatic β-cells (β-IRKO) results in an altered insulin secretion comparable to that seen in type-2-diabetes. Specific knockout of the IGF1R in β-cells results in a similar phenotype. In contrast, mice with a skeletal **m**uscle-specific **i**nsulin **r**eceptor **k**nock **o**ut (MIRKO) had normal blood glucose and normal glucose tolerance but elevated serum-free fatty acids and triglycerides. Mice deficient for insulin receptor in adipose tissue exhibited a lipodystrophic-like phenotype with a strong reduction of white adipose tissue mass, lipid accumulation in peripheral tissues, hyperglycemia,



Insulin Receptor, Fig. 1 Structure and function of the INSR. Binding of insulin to the α-subunits (red) leads to activation of the intracellular tyrosine kinase (β-subunit) by autophosphorylation. The insulin receptor substrates (IRS) bind via a phosphor-tyrosine binding domain to phosphorylated tyrosine residues in the juxtamembrane domain of the β-subunit. The receptor tyrosine kinase then phosphorylates specific tyrosine motifs (YMxM) within the IRS. These tyrosine phosphorylated motifs serve as docking sites for various adaptor proteins with SRC homology 2 (SH2) domains like the regulatory subunit of PI 3-kinase

hyperlipidemia, and hyperinsulinemia, a phenotype worsened by the loss of INSR and IGF1R in adipose tissue. Immense progress has been made in understanding the role of INSR in the brain. Mice with a neuron-specific INSR knockout (NIRKO) developed an obese phenotype with mild insulin resistance and impaired fertility. These central effects of insulin fine tune peripheral metabolism via, for example, regulation of orexigenic and anorexigenic neuronal populations (NPY, Agrp, and POMC) in the brain. In addition, brain INSR signaling regulates peripheral metabolism, by, for example, suppressing hepatic glucose production, regulating brain glucose sensing mechanisms, and altering the reward system. Combined deletion of the INSR, IGF1R, and the INSRR in male (XY) mice results in a female phenotype with ovaries, thereby providing evidence that the insulin receptor tyrosine kinase family is essential for male sex differentiation.

Taken together, these data emphasize the importance of insulin action in the liver and adipose tissue for glucose homeostasis, in the regulation of insulin secretion from β-cells, and indicate that the INSR plays an important role in the central regulation of body weight and reproduction (Kleinridders et al. 2014; Kubota et al. 2017).

There are a few clinical syndromes with an impaired function of the INSR: Leprechaunism

and Rabson-Mendenhall syndrome are rare genetic diseases characterized by growth retardation, hyperinsulinemia, and insulin resistance due to mutations in the INSR gene. Acanthosis nigricans is a syndrome of hyperpigmentation and hyperandrogenism associated with hyperinsulinemia and diabetes mellitus. Insulin resistance in this syndrome is either due to mutations in the insulin receptor gene (type A) or to autoantibodies to the INSR (type B). Some patients with insulin resistance due to decreased expression, increased degradation, or impaired insulin binding of the INSR, as well as mutations in insulin signaling molecules have been described (Boucher et al. 2014).

Signal Transduction and Insulin Action

Stimulation of the insulin receptor results in the activation of two major pathways (Taniguchi et al. 2006): (i) the **m**itogen- **a**ctivated **p**rotein (MAP) kinase cascade (discussed in chapter MAP kinase cascade) and (ii) the phosphatidylinositol 3-kinase (PI 3-kinase) pathway which has been extensively studied in the context of the metabolic responses to insulin (summarized in Table 1 and Fig. 2).

Other, more general effects of insulin on cellular function include stimulation of cell growth (increase in DNA and protein synthesis), inhibition of apoptosis, and modulation of ion-channel activity.

The major intracellular target molecules of the tyrosine kinase activity of the INSR β-subunit are the **i**nsulin **r**eceptor **s**ubstrates (IRS). Interaction of phosphotyrosine-binding domains (PTB) within the N-terminal region of an IRS with the juxtamembrane phosphotyrosines of the INSR β-subunit results in tyrosine phosphorylation of consensus motifs (YMXM) in the C-terminus of IRS (Fig. 1). Six different mammalian IRS isotypes have been identified (IRS1-6), with IRS3 being a pseudogene in humans. IRS1 knockout mice develop a mild state of insulin resistance (impaired glucose intolerance) without diabetes, whereas knockout of the IRS2 gene causes a phenotype with severe insulin resistance (liver > muscle), diabetes, and impaired pancreatic β-cell function. IRS1 and IRS2 knockout mice exhibit growth retardation (IRS1 > IRS2). Disruption of the IRS4 gene produces mild glucose intolerance and growth retardation in male animals. While IRS1 and IRS2 are ubiquitously expressed, IRS4 mRNA is detected in thymus, brain, and kidney, while IRS3 is expressed only in adipocytes, β-cells, and hepatocytes in rodents but is not expressed in humans. Taken together, the data suggest that the major effects of insulin on metabolism are mediated via IRS1 and IRS2 (Boucher et al. 2014).

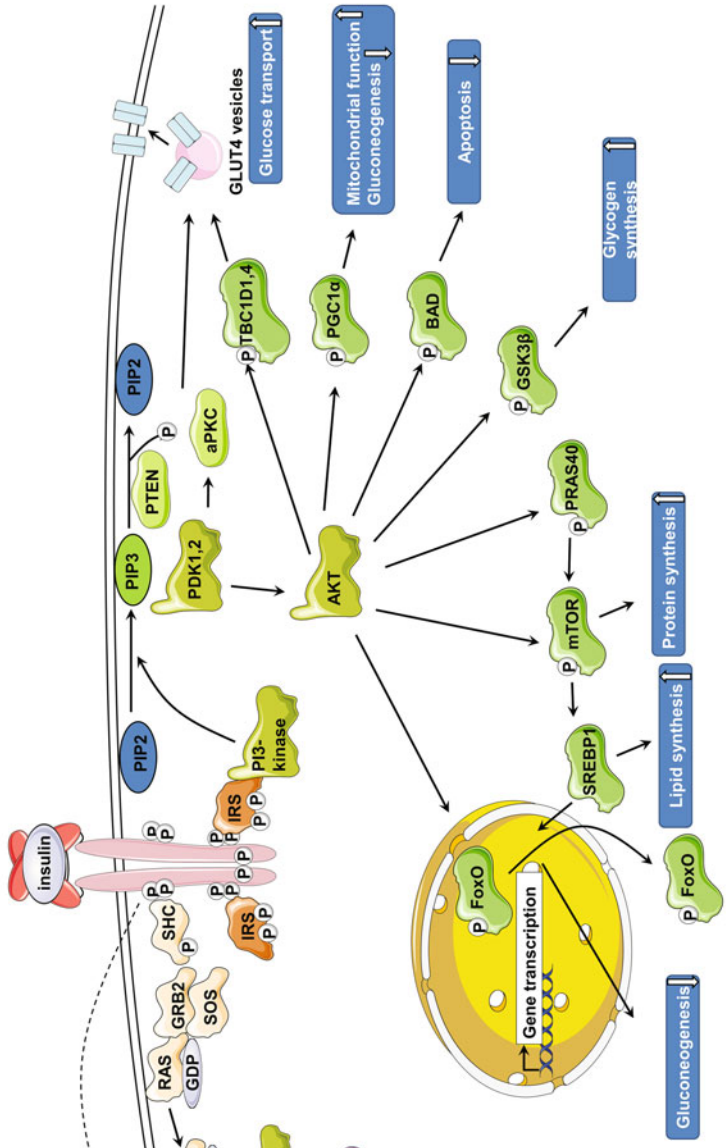
Tyrosine phosphorylated IRS interacts with and activates PI 3-kinase (Taniguchi et al. 2006). Binding takes place via the **S**RC **h**omology **2** (SH2) domain of the PI 3-kinase regulatory subunit. The resulting complex consisting of INSR, IRS, and PI 3-kinase facilitates interaction of the activated PI 3-kinase catalytic subunit with the phospholipid substrates in the plasma membrane. Generation of PI 3-phosphates in the plasma membrane recruits **p**hospholipid-**d**ependent **k**inases (PDK1 and PDK2) which subsequently phosphorylate and activate the serine/threonine kinase Akt (synonym: **p**rotein **k**inase **B**, PKB). Three isotypes of Akt have been identified (Akt1, 2, 3). AKT1-knockout mice exhibit growth retardation and increased apoptosis, but no increased prevalence in diabetes. Disruption of the Akt2 gene produces diabetes due to insulin resistance of skeletal muscle and liver. Double knockout of AKT1 and 2 showed severe growth deficiency and died shortly after birth. There is solid evidence that activation of Akt is essential for the effect of insulin on glucose transport (GLUT4), glycogen synthesis, gluconeogenesis, protein synthesis, and gene expression (Boucher et al. 2014; Taniguchi et al. 2006) (summarized in Table 1 and Fig. 2). Substrates of Akt are glycogen synthase kinase 3 (GSK3) and FoxO1a (previously known as FKHR), a transcription factor regulating the expression of gluconeogenic enzymes. Also, phosphorylation of Akt results in activation of sterol regulatory-element binding protein 1 (SREBP1), a key transcription factor involved in regulation of lipogenic enzymes. In

Insulin Receptor, Table 1 The effect of insulin on energy and glucose homeostasis

Organ	Effect	Mechanism	Comments
Liver	Activation of glycogen synthesis	Activation of glycogen synthase (GS)	Inactivation of glycogen synthase kinase 3 (GSK3) through phosphorylation by Akt
	Inhibition of gluconeogenesis/ increased glycolysis	Inhibition of gluconeogenic gene expression/activation of some glycolytic enzymes	Akt-dependent inhibition of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) gene expression; Akt-dependent inhibition of PGC1α
			Decrease of cAMP, the second messenger of glucagon. Induction of pyruvate kinase and glyceraldehyde-3-phosphate dehydrogenase
Fat	Increased glucose transport	GLUT4-translocation	PI 3-kinase/Akt mediated translocation of GLUT4 into the plasma membrane. Potential involvement of atypical forms of protein kinase C (PKC ζ and λ)
	Increased lipid synthesis/ inhibition of lipolysis	Activation of lipoprotein lipase (LPL)/ induction of fatty acid synthase (FAS)/ inactivation of hormone sensitive lipase (HSL)	Facilitated uptake of fatty acids by LPL-dependent hydrolysis of triacylglycerol from circulating lipoproteins. Increased lipid synthesis through Akt-mediated FAS-expression. Inhibition of lipolysis by preventing cAMP-dependent activation of HSL
Skeletal muscle	Increased glucose transport	GLUT4-translocation	See above (fat)
	Activation of glycogen synthesis	Activation of glycogen synthase	See above (liver)
	Increased protein synthesis/ decreased protein degradation	Increased amino acid uptake/increased translation of mRNA/ reduced proteasomal and autophagy-lysosomal degradation	Akt-mediated stimulation of system A amino acid transporter and stimulation of mRNA-translation through activation of p70S6kinase and elongation initiation factor 4 (eIF4). Possible involvement of atypical PKCs
Central nervous system	Reduction of body weight	Reduced appetite and reward behavior	Changed expression of anorexigenic (e.g., POMC) and orexigenic (e.g., NPY) hypothalamic neuropeptides and alterations in the dopaminergic system, potential involvement of mitochondrial function

addition, some of the effects of insulin on cell proliferation and survival may be explained by an Akt-dependent inhibition of apoptosis through phosphorylation and inactivation of proapoptotic proteins (e.g., BAD, Caspase 9).

INSR signaling is terminated by specific phosphotyrosine phosphatases (e.g., PTP1B) (tyrosine phosphatases), and mice lacking the PTP1B gene exhibit increased insulin sensitivity. Furthermore, it has been suggested that the lipid phosphatases PTEN and SHIP2 act as negative modulators of insulin signaling. The ligand-induced endocytosis and degradation of the activated INSR and the degradation of insulin by insulin-degrading enzyme are involved in the termination of insulin signaling.



Insulin Receptor, Fig. 2 Signal transduction of the insulin receptor. Activation of the insulin receptor leads to stimulation of the PI 3-kinase and MAP kinase pathway. Generation of 3′-phosphorylated PI-phospholipids mediates the effect of insulin on glucose transport, cell survival, protein synthesis, glycogen- and lipid synthesis, gene expression, and gluconeogenesis. The protein kinase Akt plays a central role in the regulation of these PI 3-kinase-dependent processes. The activation of MAP kinase cascade regulates mainly proliferation and mitochondrial function

Drugs

Agents Stimulating the Receptor or the Signaling Pathway

Insulin Analogs. At present, the only known ligands of the insulin receptors are insulin isotypes from different species and a number of synthetic analogs with insulin-like activity. Five analogs generated by site-directed DNA mutagenesis are used in clinical practice because of their pharmacokinetic characteristics: insulin lispro (swap of proline B28 and lysine B29), insulin aspart (generated by exchange of proline B28 for aspartate), and insulin glulisin (generated by exchange of lysine B29 for glutamate) are rapid and short-acting insulins (diabetes mellitus). Insulin glargine and insulin detemir are long-acting analogs. Because of two additional arginines at B31 and B32, insulin glargine is soluble at low pH and precipitates after injection, forming a stable, long-acting depot. Insulin detemir is a truncated insulin derivative (threonine B30) that has been coupled to a long-chain fatty acid at lysine B29. This results in the formation of a slowly dissociating complex and an increased binding to albumin, which further delays its inactivation. An ultra-long lasting insulin analog is degludec with a duration of action of up to 40 hours. Its protein sequence is based on human insulin, but modified by acylating DesB30 at the ε-amino group of LysB29.

Thiazolidinediones (synonyms glitazones, insulin sensitizers; rosiglitazone, pioglitazone) are a class of oral antidiabetic drugs that activate the transcription factor peroxisome proliferator-activated receptor (PPARγ). Thiazolidinediones ameliorate insulin resistance in obese animal models and in individuals with type-2 diabetes. They enhance the insulin-dependent activation of the PI 3-kinase/Akt pathway, stimulate differentiation of preadipocytes, and redistribute intra-abdominal and hepatic triglycerides to subcutaneous adipose tissue stores. The marked reduction of hepatosteatosis is believed to be the primary reason for its antidiabetic effect. Furthermore, anti-inflammatory properties of thiazolidinediones have been suggested to contribute to their effect on insulin resistance in type-2 diabetes.

Concanavalin A is a plant lectin from the jack bean (*Canavalia ensiformis*) which binds with high affinity to mannose residues of glycoproteins. Concanavalin A is known to stimulate the tyrosine kinase activity of the INSR β-subunit with consecutive activation of kinases downstream the insulin receptor (IRS, PI 3-kinase). It is believed that Concanavalin A stimulates the activation and autophosphorylation of the INSR kinase through aggregation of the receptor, although the precise mechanism of action is unclear.

Vanadate (sodium orthovanadate or peroxovanadate) exhibits insulin-like effects in vitro (activation of insulin receptor tyrosine kinase, PI 3-kinase, Akt) and in vivo (diabetic rats, humans). These effects can be explained at least in part by the inhibition of phosphotyrosine phosphatases which deactivate the INSR tyrosine kinase.

Hydrogen peroxide (H_2O_2) exhibits insulin-like activity in isolated cells. Like that of vanadate, this effect is thought to be mediated by inhibition of protein-tyrosine phosphatases.

XMetA is a monoclonal antibody, enhances insulin binding to the receptor, and acts as a partial agonist and improves metabolism in a diabetes mouse model.

Agents Inhibiting Insulin Receptor Signaling

Insulin Analogs. Interestingly, several covalently dimerized insulin analogs are partial agonists of the insulin receptor. The intrinsic activity of the dimers decreases with the length of the spacer, with B29-B29'-suberoyl-insulin exhibiting the highest antagonist efficacy at the receptor (Weiland et al. 1990).

Wortmannin is a fungus-derived inhibitor of PI 3-kinase. The agent binds and inhibits the enzyme covalently and irreversibly. It is very potent and considered to be highly specific (IC_{50} in most cells in the low nanomolar range).

LY294002 is a synthetic drug which reversibly inhibits PI 3-kinases. It is less toxic and also less potent than wortmannin. The IC_{50} in most cells is in the micromolar range.

Rapamycin is an immunosuppressive drug and an inhibitor of S6K1 (also known as p70S6-kinase) which phosphorylates ribosomal S6 protein. S6K1 is activated in response to insulin via activation of Akt. Rapamycin binds to a specific target protein (mTOR, mammalian target of rapamycin) which is functionally located downstream of Akt, but upstream of S6K1. The IC_{50} in most cells is in the high nanomolar range.

S961 is an insulin receptor antagonist peptide of 43 amino acids and causes an overt diabetes-like phenotype in rodents. But when used in low nanomolar concentrations, S961 can also act as a partial agonist.

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Insulin Secretagogues

▶ Glucose-Lowering Drugs Other than Insulin

Integrins as Drug Targets: Is There a Future?

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Background

In 1981 fibroblasts were found to adhere to a surface coated in fibronectin and by 1984 the amino acid sequence Arg-Gly-Asp-Ser (RGDS) was identified in a 30-mer fragment of fibronectin as the site essential for supporting adhesion. The RGD sequence was subsequently found to be a common motif with many proteins containing this sequence such as fibronectin, vitronectin, and fibrinogen suggesting a family of RGD-containing proteins that were involved in cell adhesion (Humphries et al. 2006).

The initial characterization of the RGD-dependent fibronectin receptor identified a 140 kDa protein. At the same time work on immune cell antigens showed that two antigens, LFA-1 (T cells) and Mac-1 (macrophages), while expressed on different cells had similar structures. Both were composed of an α- and β-subunits. While both α-subunits were different both β-subunits were probably identical. The platelet fibronectin receptor was identified as a complex of glycoproteins (GP) IIb and IIIa. The vitronectin receptor was also found to have two subunits and while it bound RGD peptides it did not bind to fibronectin. Thus, there was a picture developing of a family of heterodimeric adhesion proteins that interact with RGD-containing proteins. While all bound RGD there was evidence that some of the receptors were specific for certain RGD-

containing proteins. It was also evident that leucocytes have a family of three α/β heterodimeric proteins that share a common β-subunit. Evidence that an anti-GPIIb/IIIa antibody also bound to leucocytes suggested that GPIIb/IIIa may be related to the leucocyte surface markers. This was confirmed in a study which showed that all of the α-subunits came from the same gene family. The cDNA for an 89 kDa subunit of a glycoprotein that mediates cell adhesion was isolated and characterized and this protein was called integrin. By 1987 it had become clear that these three different receptor families – leucocyte surface receptors, platelet GPIIb/IIIa (αIIbβ3), and matrix adhesion receptors – were all related. They were grouped together into a super family of distinct but related receptors called Integrins (Hynes 1987). Some but not all of these receptors bound to a family RGD-containing proteins in an RGD-dependent manner.

Initial evidence suggested that α-subunits only interacted with a single β-subunit (only 3 β-subunits were known) and that ligand specificity was conferred by the α-subunit. Thus, integrins were thought to consist of three families based around the unique β-subunits. However, there are currently 18 identified α-subunits and 8 β-subunits and it is clear that some α-subunits, especially αV, are capable of interacting with multiple β-subunits. Thus, integrins can be grouped into sub-families, although the precise arrangement is complex (Cox et al. 2010) (Fig. 1). One approach to categorizing integrins is to use a phylogenetic tree where integrin subunits can be grouped according to similarity in their gene sequences (Hight-Warburton and Parsons 2019).

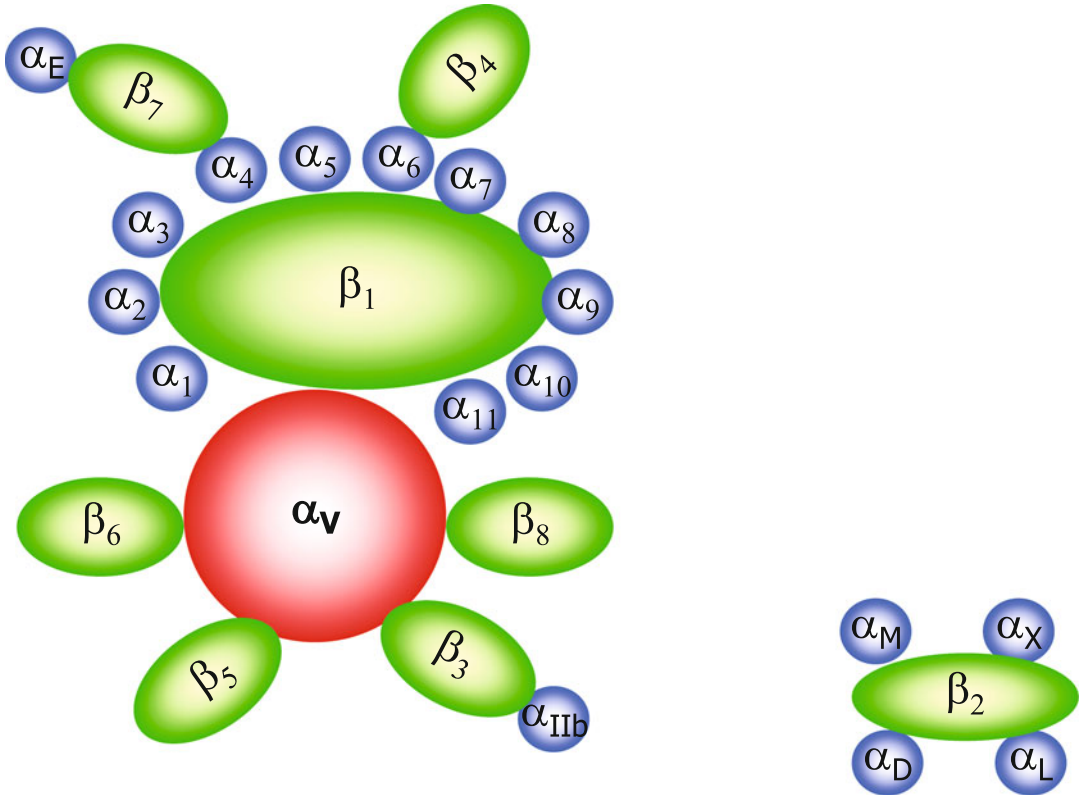
This discovery of integrins created great opportunity for drug discovery. Integrins were associated with many major diseases with unmet needs. Their presence on leucocytes suggested a role in autoimmune diseases. Their role as cell adhesion molecules suggested a potential role in cancer and their presence on platelets suggested a role in thrombosis and cardiovascular disease. Furthermore the RGD-dependent nature of the interactions provided a hit compound for a small molecule discovery program and the discovery of inhibitory monoclonal antibodies provided the potential for a biotechnology solution. While monoclonal antibody technology had been around for over 10 years they had yet to be deployed clinically.

Thrombosis

Not surprisingly thrombosis was the first target for anti-integrin therapy. Around the time of the discovery of integrins platelets were shown to be the critical factor in ischemic heart disease. Considering that cardiovascular disease was the major cause of death, that platelets played a critical role in the pathogenesis of heart disease, and that targeting platelets was shown to improve outcome (25% reduction in events with aspirin), there was a lot of commercial interest in developing new and improved antiplatelet agents (Cox 2019).

The discovery that GPIIb/IIIa was the fibrinogen receptor on platelets and an RGD-dependent integrin (Hawiger et al. 1989) clearly identified GPIIb/IIIa as a drug target. Platelet agonists are divided into strong and weak agonists. Weak agonists such as ADP activate platelets via phospholipase (PL) A2 and cyclooxygenase (COX) while strong agonists such as thrombin activate platelets via PLC. Thus, antiplatelet agents such as aspirin (COX inhibitor) were always going to be hampered in their antithrombotic activity because they only inhibited one platelet activation pathway. It was believed that this could account for their limited clinical benefit and that a stronger agent would be more effective. GPIIb/IIIa antagonists were seen as the ideal agent because no matter what agonist was involved platelet activation resulted in activation of GPIIb/IIIa with subsequent fibrinogen binding that cross-linked platelets into a thrombus. This was often described as the "final common pathway" in aggregation. Everybody was so enthusiastic about this new class of antiplatelet agent that they were known as "super-aspirin" in the media.

The first approach to discovering a GPIIb/IIIa antagonist was to take advantage of the



Integrins as Drug Targets: Is There a Future?, Fig. 1 Integrin receptors

monoclonal antibodies that had been generated to characterize GPIIb/IIIa. One of these that attracted a lot of attention was 7E3 (Coller and Scudder 1985). Abciximab is a chimeric antibody generated from 7E3 and commercialized as ReoPro®. It was only the second monoclonal antibody to come to the market (Investigators 1994). However, as of 2019 it appears to be withdrawn from the market by the manufacturer for commercial reasons.

As an alternative approach to antibodies there were efforts to discover small molecule inhibitors and the obvious approach was to use RGDS as a hit compound with modest potency (IC_{50} 60 μM (Cox et al. 1992)). However, the discovery of disintegrins – RGD-containing snake venom peptides – was critical in developing potent small molecules (Huang et al. 2016). Unlike the simple RGDS peptide these disintegrins are very potent and thus a good starting point for a drug discovery program. Barbourin, a disintegrin from *Sistrurus m. barbouri*, was used as the starting point for a cyclic-peptide inhibitor. Unusually, barbourin had a KGD sequence rather than an RGD sequence. Modification of barbourin led to the cyclic peptide eptifibatide (Scarborough 1999), which was commercialized as Integrilin®. A second approach was based on the idea that disintegrins were cyclic peptides in a highly constrained conformation which was optimum for activity. Echisatin, a disintegrin from *Echis carinatus*, was purified, crystallized and subjected to NMR analysis to determine the conformation of the RGD sequence. This was then mapped onto chemical scaffolds and used to discover small molecule peptidemimetics (Garsky et al. 1989). This led to the discovery of tirofiban, which was commercialized as Aggrestat®.

While all of these agents were very potent (low nanomolar Kd) none were orally active and thus were only useful for acute conditions such as acute myocardial infarction, stenting, etc. As the market for acute use is relatively small, companies searched for bioavailable small molecules. One approach was to make incremental changes to RGDS to both stabilize the molecule and increase its potency. This led to the discovery of xemilofiban and orbofiban (Anders et al. 2001) and sibrafiban (Refino et al. 1998). Another approach was the identification of compounds with a profile suggestive of anti-GPIIb/IIIa activity such as the anti-parasite agent pentamidine (Cox et al. 1992) (an early example of repurposing existing drugs), which led to the discovery of FK633 (Cox and Seki 1998). The result was a family of very potent compounds with varying levels of oral activity.

So within a few years of the discovery of GPIIb/IIIa there was a plethora of agents – antibodies, peptides, and non-peptides – entering clinical trials. Ultimately the IV agents were shown to be beneficial in preventing thrombosis during percutaneous coronary intervention (PCI) (Boersma et al. 2002). So while there was evidence of efficacy it was clear that these agents were hampered by the short duration of administration due to the need for intravenous infusion. All eyes were then on the oral agents as they could be administered for longer duration and thus more likely to be of use.

A number of orally active agents made it to PIII trials – xemilofiban, orbofiban, and sibrafiban. Despite large clinical trials with all of these agents the results were disappointing. They all showed an increase in mortality and surprisingly this was due to an increase in cardiovascular events rather than some off-target toxicity (Chew et al. 2001). Ultimately none of the oral agents made it to market and the key question was how everybody got it so wrong. This failure was multifactorial and involved both pharmacokinetic and pharmacodynamic properties of the drugs (Cox et al. 2010; Cox 2004).

In hind-sight the problem was obvious. While all drugs were described as GPIIb/IIIa antagonists because they inhibited fibrinogen binding and platelet aggregation they were in fact agonists on the receptor. This was seen with increased platelet activation with these agents (Cox et al. 2000) and increased fibrinogen binding when these agents dissociate from GPIIb/IIIa (Cox et al. 1996). This is hardly surprisingly as they were all modeled on RGDS which is the natural agonist for the receptor. One reason for this was that integrins were not considered to be true receptors as there were no obvious signaling domains in the receptor and thus thought not to generate a signal. Thus, GPIIb/IIIa was considered to be a fibrinogen-binding protein rather than a fibrinogen receptor. Furthermore, the two outputs that were used to assess the actions of these agents were fibrinogen binding and platelet aggregation both of which were entirely dependent on fibrinogen binding and did not involve signaling. In reality even if there was a wish to investigate signaling potential it was not possible as no signaling pathway had been identified.

Signaling

GPIIb/IIIa is the best studied integrin due to the interest in it as a drug target. However, the principles of signaling associated with GPIIb/IIIa apply to other integrins (Li et al. 2010). Like most integrins GPIIb/IIIa exists in a resting conformation that does not allow it to bind to its ligand – fibrinogen – and must be activated to support aggregation and to generate signals. In the case of GPIIb/IIIa this can occur in two ways. Firstly, the receptor can be directly activated by certain ligands. For instance RGD peptide binding to GPIIb/IIIa induces a conformational change (Quinn et al. 2002) leading to receptor activation (Cox et al. 1996; Peter et al. 2001). While resting GPIIb/IIIa cannot bind soluble fibrinogen it can bind to immobilized fibrinogen. In this case interaction with the Asn-Gly-Arg (NGR) sequence in fibrinogen leads to receptor activation (Moriarty et al. 2015). Inside-out signaling is the process by which integrins are converted to an active form and become capable of binding their ligand in response to activation of other receptors on the cell surface which in the case of platelets are

usually G-protein-coupled receptors. Activation is mediated by binding of proteins such as talin, paxillin, and kindilin leading to an increased affinity for the ligands and also the formation of adhesion complexes which are essential for the signal transduction process (Humphries et al. 2019). The intracellular cytoplasmic tails of both the α- and β-subunits are critical for his activation process as they support the binding of the signaling molecules (Coller and Shattil 2008).

Outside-in signaling is the signaling that occurs upon ligand binding and the transmission of the signal into the cell and altering cellular processes as a result. This outside-in signaling is critical to the concept of integrins as receptors rather than adhesion molecules. Activated GPIIb/IIIa binds Src family kinases such as Src, Lyn, and Fyn which in turn phosphorylate focal adhesion kinase (FAK), Syk (tyrosine kinase), etc. (Shattil et al. 2010). Integrin clustering both with other integrins and other receptors is important to generate a signal. For instance, Syk can bind to both β3 cytoplasmic tail and to the immunoreceptor tyrosine-based activation motif (ITAM) domain of FcγRIIa. Interestingly, *Staphylococcus aureus* can utilize this by crosslinking both GPIIb/IIIa and FcγRIIa to trigger platelet activation (Fitzgerald et al. 2006). Resting GPIIb/IIIa cannot bind soluble fibrinogen – it must be converted into an active conformation usually by an inside-out signaling mechanism. As agonists, when the GPIIb/IIIa inhibitors bind they induce a conformational change in GPIIb/IIIa converting it into the active conformation of the receptor. Since these agents bind reversibly they leave the receptor but there is a delay for the receptor to return back to the resting conformation. Prior to this return to a resting conformation fibrinogen can bind (there are high levels of fibrinogen in plasma) and once fibrinogen binds it can support aggregate formation (Peter et al. 2001).

So while there were clear pharmacodynamic problems with GPIIb/IIIa antagonists there were also pharmacokinetic problems as well. While all of these agents were used on a once-per-day schedule many would have been better used with a twice daily dosing regimen (Quinn et al. 2000). Thus, there were very significant trough periods with these agents. Furthermore there were significant numbers of spare receptors on platelets. There are around 50–80,000 GPIIb/IIIa molecules on a platelet (Quinn et al. 2000) and clinical studies measuring receptor occupancy and inhibition of platelet aggregation showed that 90% inhibition was the target for benefit. While IV agents are dosed as infusions to maintain this level of inhibition the oral agents with their PK profile were incapable of delivering that level of inhibition over 24 h. With a large number of spare receptors GPIIb/IIIa antagonists must occupy a large percentage of receptors but as agonists they can exert their full activity with the occupation of a small percentage of receptors. This was confirmed when it was shown that orbofiban at doses too low to inhibit platelet aggregation could enhance aggregation to low dose platelet agonists (Cox et al. 2000).

Thus, oral GPIIb/IIIa antagonists failed because there was a lack of understanding of both the PK and PD properties of these agents. While there were clear signals of potential problems speed was (and still is) essential in the drug development timeline. Every effort is made to get the drug through clinical trials and onto the market as soon as possible – this includes overlapping timelines for the different phases of the clinical trials. Considering that RGD was only shown to be the GPIIb/IIIa recognition domain on fibrinogen in 1989 and 5 years later we have the first clinical trials of GPIIb/IIIa antagonists, there was no time to understand the structure and function of this new family of receptors. Since then agents have been discovered with high bioavailability, no agonist-like properties and are truly once-a-day agents. However, the pharmaceutical industry has lost all interest in oral GPIIb/IIIa antagonists so these will never make it to market. Despite the fact that the arguments for an oral GPIIb/IIIa antagonist are as valid today as 20 years ago and that agents with the ideal profile are available, the pharmaceutical industry will not go there.

Cancer

Cell adhesion and its regulation is critical in the pathogenesis of cancer especially in the metastatic process where primary tumor cells must downregulate their ability to adhere to allow cells to breakaway but must regain this ability to attach to a new site. As integrins are important cell adhesion molecules it is reasonable to assume that they are potential drug targets in cancer. They play important roles in tumor metastasis where regulating the ability to adhere is critical. They also play a significant role in angiogenesis which is an essential step in tumor growth (Hamidi and Ivaska 2018). The importance of integrins in cancer became more obvious as it became clear that as true receptors integrins were far more significant than simple cell adhesion molecules. For instance, integrins play an important role in the metabolic reprogramming in cancer cells (Ahmad et al. 2019). This of course creates problems similar to that seen with GPIIb/IIIa antagonists – drugs based on RGD may be agonists rather than antagonists (Alghisi et al. 2009). Thus, while they would prevent metastatic cell adhesion they could also stimulate tumor growth (Paladino et al. 2019). While numerous integrins have been associated with cancer, the αV integrins attracted the most attention as the most important family of cancer-associated integrins. However, unlike platelets which have a single integrin associated with thrombosis, many cancers are associated with multiple integrins, for example, breast cancer is associated with at least 10 different integrins and all cannot play critical roles. Inhibiting a single receptor may have little effect as other integrins can compensate. Thus, there may be a need to screen a patient's tumor to determine if it is sensitive to a specific anti-integrin. Another approach is to develop nonspecific inhibitors that would target multiple integrins. However, this also greatly increases the chance of adverse effects.

As tumor growth is complex it may be unreasonable that inhibiting a single integrin prevents tumor growth. An alternative possibility is that anti-integrin therapy may be effective in combination with other treatment options. This can even by synergistic and act to sensitize the tumor to other agents. However, there are many anti-cancer agents and a lack of clarity on which ones would best respond to anti-integrin therapy. With the failure of anti-integrin monotherapy there were studies to investigate the role of anti-integrin therapy as part of combination therapy. Anti-integrin therapy does alter the sensitivity to other chemotherapeutic agents. However, even then the results were unclear as in breast cancer inhibition of αV enhanced sensitivity to chemotherapy while in melanoma it decreased sensitivity to chemotherapy.

Table 1 shows the anti-integrin agents that were investigated in cancer. Despite initial promising PII success none progressed to the market due to lack of efficacy. All of these failed studies in cancer must raise the question of whether there is a role for anti-integrin therapy in cancer. Certainly, numerous questions still remain unanswered: as multiple integrins are involved in carcinogenesis are other integrins more appropriate targets? Furthermore, it is not clear how they should be used: monotherapy or part of combination therapy. Is the aim of treatment to inhibit

Integrins as Drug Targets: Is There a Future?, Table 1 Integrin targeted agents in cancer

Drug	Integrin	Target	Reference
Cilengitide	αVβ3 αVβ5	Glioblastoma	Stupp et al. (2014)
Volociximab	α5β1	Ovarian cancer	Bell-McGuinn et al. (2011)
Intetumumab	αV	Melanoma	O'Day et al. (2011)
Etaracizumab	αV	Melanoma	Hersey et al. (2010)
Abituzumab	αV	Prostate cancer	Hussain et al. (2016)

tumor growth, metastasis, or angiogenesis? In reality until these questions are answered, it is unlikely that there will be a successful anti-integrin in cancer.

Infection and Immunity

One of the most promising areas in integrin pharmacology is immunology especially since the original integrins that were identified were the leucocyte adhesion molecules and are now known as the β2 integrins. While the β2 integrins are restricted to leucocytes there are other integrins that are involved in immune cell function as well. A key function of integrins in immune cells is to localize the inflammatory response to a site of infection. Integrins mediate the adhesion of immune cells to a site of injury and trigger a co-stimulatory signal necessary for full immune cell activation (Smith-Garvin et al. 2009).

Pharmacologically there are a few integrins that are important in the immune system. When complexed with β1 the α1 subunit plays an important role in T-cell adhesion and migration (Hight-Warburton and Parsons 2019). However, integrins are not just adhesion receptors, especially in the immune system. For instance complement receptors 3 and 4 are integrins – αMβ2 and αXβ2, and αIIbβ3 is a receptor for C-reactive protein (Brennan et al. 2008).

The role of α4 integrins in T-cell migration (Hight-Warburton and Parsons 2019) suggested that it might be a suitable target in autoimmune disease. T-cell migration across the blood-brain barrier is important in multiple sclerosis and across the gut in Crohn's disease. Monoclonal antibodies to α4 (natalizumab and vedozilumab) were generated and tested and shown to be effective in these two diseases. Furthermore, the β7 integrin subunit, a common binding partner for the α4 subunit, was also targeted (etrolizumab). The other integrin that was targeted by efalizumab was αLβ2 which proved to be effective in treating psoriasis (Gordon et al. 2003). Table 2 shows the anti-integrins that were developed as immunomodulators.

A limitation with the anti-integrins used as immunomodulators are their safety profiles. It appears that integrins are very important co-stimulatory receptors in the immune system and when inhibited this leads to a significant immune suppression that puts patients at risk of infection. One very serious adverse effect is progressive multifocal leukoencephalopathy (PML) which is of particular concern with the use of natalizumab in multiple sclerosis and efalizumab in psoriasis. This is due to reactivation of JC virus infection leading to lysis of neurons and permanent destruction of the myelin sheath (Khalili et al. 2019). In patients that are JC virus positive the incidence of PML is 1% with a 25% mortality rate and 30% of survivors will have severe neurological complications. As a result, efalizumab was withdrawn from the market although natalizumab remained on the market subject to a risk evaluation and mitigation strategy (REMS) and became a blockbuster. The one exception appears to be vedozilumab, which targets α4β7. It may be the case that the localization of this receptor to the gut ensures that there

Integrins as Drug Targets: Is There a Future?, Table 2 Integrin-targeted agents as immunomodulators

Drug	Integrin	Target	Reference
Natalizumab	α4β1 α4β7	Multiple sclerosis Crohn's disease	Pucci et al. (2011) and Nelson et al. (2018)
Vedozilumab	α4β7	Ulcerative colitis Crohn's disease	Feagan et al. (2013) and Sandborn et al. (2013)
Etrolizumab	β7	Ulcerative colitis Crohn's disease	Vermeire et al. (2014)
Efalizumab	αLβ2	Psoriasis	Gordon et al. (2003)
Firategrast	α4	Multiple sclerosis	Miller et al. (2012)

are no systemic effects from the immune suppression.

Conclusion

There is no doubt that the discovery of integrins and their important roles in the most significant human diseases created great opportunities for the pharmaceutical industry. Thirty years later there must be great disappointment in this class of agents as, despite many clinical trials, few agents made it to the market and those that did all have limited sales. There were multiple reasons for these failures including poor PK profile, lack of efficacy, adverse effects, and poorly defined targets. In the case of cardiovascular disease the lack of orally active agents meant that they were restricted to short-term use in acute patients. The discovery of P2Y12 antagonists such as clopidogrel, prasugrel, and ticagrelor soon displaced GPIIb/IIIa antagonists as they were every bit as effective and orally active. As a result the peak annual global sales for all GPIIb/IIIa antagonists are equivalent to the peak global sales for 1 month for clopidogrel. Efficacy was a problem with the cancer target and none of the agents proved to be effective. In this case the lack of a validated target played a key role. It is not clear what integrin should be targeted and even what part of the cancer process should be targeted. Adverse effects are a major problem with many anti-integrins. Those used as immune suppressants are associated with very serious adverse effects such as PML and the oral antiplatelet agents were associated with increased cardiovascular mortality. While natalizumab sales were initially very promising the advent of orally active disease modifying agents such as fingolimod and dimethyl fumarate which did not have a significant toxicity problem soon relegated natalizumab to a second tier agent.

So is there a future for anti-integrins? Existing antiplatelet agents are so successful and cheap (many are now generic) and it is hard to see any role for anti-integrin agents in thrombosis. The lack of success in cancer suggests that industry will lose interest in this area soon unless there is a validated integrin target with a clear role in cancer. The most promising area for anti-integrin agents is immunomodulation. The agents such as natalizumab were game changers in the treatment of multiple sclerosis, although with significant adverse effects. However, like with GPIIb/IIIa antagonists newer agents became available without the adverse effects relegating the anti-integrins to second-line agents. The best opportunity is specifically targeting integrins that are highly localized such as α4β7 in the gut. As they are not widely distributed it minimizes systemic effects.

Anti-integrins, once thought of as the next big blockbuster drugs, have been relegated to minor, second level therapeutic agents. The pharmaceutical industry has stuck with this family of agents for over 30 years with little to show for it. With a lack of clarity on a future direction it is not clear for how long the pharmaceutical industry will retain an interest in anti-integrin agents.

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Interferons

► Cytokines

Interleukin 11 (IL-11)

► Hematopoietic Growth Factors

Interleukin-1 (IL-1)

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Synonyms

Anakinra; Autoinflammation; Canakinumab; IL-1; IL-1R

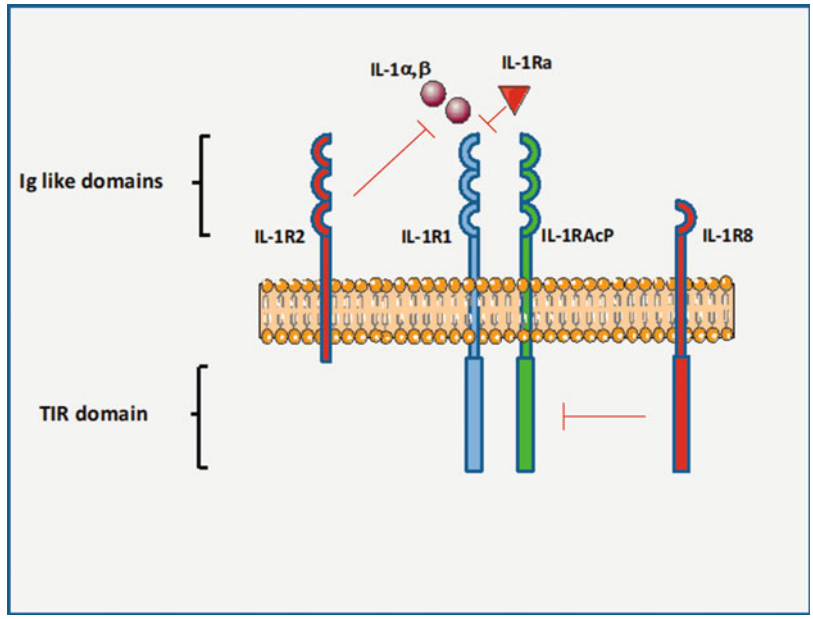
Definition

Interleukin-1 (IL-1) refers to two of the earliest identified cytokines, which act primarily as intercellular protein messengers with important roles in mediating the inflammatory response. Originally described as an endogenous pyrogenic factor, Interleukin-1 in fact refers to two distinct cytokines, encoded by separate genes, designated as Interleukin-1α and Interleukin-1β (Dinarello et al. 1977; Dinarello 2010). Although both cytokines engage the same Interlukin-1 receptor to elicit pro-inflammatory responses on a diverse array of target cells, their modes of expression and activation are distinct, underlying several notable differences in terms of their roles in human physiology and disease. Since the original description of IL-1 over 40 years ago, subsequent discoveries have identified an expanded family of IL-1 related cytokines with a broad range of functions as mediators of tissue homeostasis and inflammation (Hernandez-Santana et al. 2019). As the prototypical members of this cytokine family, efforts aimed at modulating the activity of IL-1α and Il-1β in human disease have, to date, yielded the most progress.

Basic Characteristics

The IL-1 Receptor

Both IL-1α and Il-1β bind to the same specific receptor on target cells known as the Interleukin-1 receptor (IL-1R). Upon ligand binding, the Interleukin-1 receptor 1 chain hetero-dimerizes with the Interleukin-1 receptor accessory protein (IL-1RAcP) to form the IL-1R (Fig. 1). Both chains of the IL-1R comprise of extracellular Immunoglobulin (Ig)-like domains and



Interleukin-1 (IL-1), Fig. 1 The heterodimeric IL-1 receptor consisting of IL-1R1 and IL-1AcP chains, each with extracellular Immunoglobulin (Ig) like domains responsible for binding of ligands, IL-1α and IL-1β, and intracellular Toll-IL-1R Resistance (TIR) domains required for activation of downstream signaling. Activation of the receptor can be negatively regulated by endogenous mechanisms including the Interleukin-1 receptor antagonist, the decoy IL-1R2 receptor, and negative regulatory receptors such as IL-1R8

intracellular Toll-IL-1R Resistance (TIR) domains, which upon dimerization facilitate the activation of downstream intracellular signaling pathways. Activation of these pathways is initiated through the recruitment of the TIR domain containing signaling adaptor protein MyD88 which results in the subsequent downstream activation of IL-1R associated kinases (IRAKs) and Tnf receptor associated-factor 6 (Traf6). These events ultimately lead to the induction of pro-inflammatory gene expression through the activation of NF-κB transcription factor and other MAPK dependent transcription pathways (Mantovani et al. 2019). Despite sharing the same receptor and signaling events, significant differences exist with regards to the modes of expression and activation of both IL-1α and IL-1β.

Overlapping and Distinct Features of IL-1α and IL-1β

While IL-1α expression is widespread across many different cell and tissue types, IL-1β expression is largely confined to cells of myeloid origin. Additionally, IL-1α is expressed for the most part on the cell surface, whereas IL-1β is expressed in secreted form. This has led to the hypothesis that while IL-1α acts predominantly in a localized fashion, IL-1β exerts its pro-inflammatory effects more systemically.

Both cytokines are expressed as pro-proteins whose function is regulated through cleavage by specific proteases. While full-length IL-1α has been reported to exhibit functional activity, it can be further processed by proteases, such as calpain leading to enhanced pro-inflammatory capacity. In contrast, full length IL-1β (ProIL-1β) is not biologically active and is required to undergo a highly coordinated process in order to acquire full activity. Firstly, expression of ProIL-1β is induced, either by microbe, or "damage" associated molecular patterns, or by other pro-inflammatory cytokines such as TNF or the IL-1 cytokines themselves. Once expressed, ProIL-1β is activated through proteolytic cleavage by caspase-1 in a process mediated by inflammasomes, such as containing the NOD-like receptor protein 3 (NLRP3) (Garlanda et al. 2013a, b).

As well as acting at the cell surface where it can stimulate the IL-1R, IL-1α is also expressed intracellularly, in both the cytoplasm and the nucleus, in many cell types. Indeed, IL-1α localized to the nucleus has been proposed as a regulator of pro-inflammatory gene transcription. Intracellular IL-1α is also recognized as an "alarmin" molecule and can act as a potent mediator of sterile inflammation, upon its release from injured or necrotic cells. With distinct patterns of expression and activity, both IL-1 cytokines act in a highly regulated fashion to control local and systemic inflammatory responses.

Endogenous Regulators of IL-1 Activity

Underlining the importance of IL-1 cytokines as mediators of inflammation, a number of distinct mechanisms have evolved to ensure that their activity is highly regulated under homeostatic and inflammatory conditions (Garlanda et al. 2013a, b). As well as IL-1α and IL-1β, a separate IL-1 family gene encodes a specific Interluekin-1 receptor antagonist (IL-1Ra). IL-1Ra, when present, binds to the IL-1R1 chain thereby preventing the interaction with IL-1α and IL-1β and inhibiting their activity. Unlike IL-1α and IL-1β, IL-1Ra binding does not result in the recruitment of IL-1RAcP to the receptor complex and does not initiate intracellular signaling. The discovery of this natural antagonist of IL-1 signaling has been exploited with some success for the treatment of several inflammatory diseases in humans discussed in more detail below.

As well as IL-1Ra, there is also a decoy IL-1 receptor chain encoded by the *IL1R2* gene. The extracellular domain of the IL-1R2 receptor shares significant homology with IL-1R1 and can bind IL-1β with high affinity. IL-1R2 can be found expressed both on the cell surface and in soluble form and acts as a molecular "sink" to neutralize the bioavailability of IL-1 cytokines. Although cell surface bound IL-1R2 can heterodimerize with IL-1RAcP upon ligand binding, it lacks an intracellular TIR domain required to initiate downstream signaling pathways and as a result inhibits the activation of pro-inflammatory gene transcription (Fig. 1). Similar to IL-1R2, IL-1R1 can also be expressed in soluble form,

where it may also exert neutralizing effects in a cell extrinsic fashion. A further layer of regulation of IL-1 activity is provided by negative regulatory receptors of the IL-1 family such as IL-1R8 (Garlanda et al. 2013b; Russell et al. 2013). IL-1R8 can act to restrict IL-1 activity through interfering with appropriate assembly of the IL-1R heterodimer complex as well as through preferential sequestering of intracellular signaling intermediates such as MyD88 (Qin et al. 2005).

These multiple regulatory mechanisms point to the undesirable effects associated with uncontrolled IL-1 activity, which are recognized as a characteristic of several human inflammatory disease conditions.

IL-1 in Human Disease

IL-1 cytokines have been implicated as important mediators of a range of inflammatory diseases. Several of these diseases are associated with disruption of the endogenous regulatory mechanisms of IL-1 activity described above. These include autoinflammatory conditions resulting from monogenic mutations in key genes, which regulate IL-1β secretion and activation such as the genes, which encode IL-1RA and NLRP3. Perhaps, unsurprisingly these disorders which include deficiency of the IL-1R antagonist (DIRA), familial Mediterranean fever (FMF), and cryopyrin-associated periodic syndrome (CAPS) have been found to respond relatively well to therapeutic strategies aimed at targeting IL-1 described in detail below. As well as these rare autoinflammatory disorders, IL-1 activity has been described as an important pathogenic factor in more prevalent inflammatory disorders such as rheumatoid arthritis, gout, metabolic syndrome, cardiovascular disease, and certain types of cancer (Dinarello et al. 2012).

Drugs

Specific therapeutic strategies targeting IL-1 cytokines in rare autoinflammatory disorders have proven relatively successful. Currently, three different therapeutic strategies targeting IL-1 cytokines have been licensed to date (Table 1), with more in clinical development with potential for treating an expanding number of systemic inflammatory diseases.

Anakinra, a recombinant human IL-1Ra, which inhibits inflammation mediated by both IL-1α and IL-1β, first received regulatory approval in 2001 for the treatment of patients with adult rheumatoid arthritis (RA) and the autoinflammatory disorder neonatal-onset multisystem inflammatory disease (NOMID). Another approved agent, Rilonacept, is in clinical use for the treatment of CAPS. Rilonacept, also referred to as "IL-1Trap," is a dimeric fusion protein consisting of portions of IL-1R1 and the IL-1RAcP. As such, it not only binds and neutralizes IL-1α and IL-1β, but also IL-1Ra. A third approach is an anti-IL-1β monoclonal antibody, Canakinumab, which binds to and neutralizes human IL-1β by blocking its interaction with IL-1 receptors without effecting IL-1α or IL-1Ra. Canakinumab is indicated to treat CAPS, including familial cold autoinflammatory syndrome (FCAS) and Muckle Wells syndrome (MWS), as well as systemic juvenile idiopathic arthritis (sJIA) (Dinarello et al. 2012; Hernandez-Santana et al. 2019).

Other IL-1 targeting therapeutic strategies which have not yet been approved but are currently in clinical development include MABp1, a monoclonal antibody specifically targeting IL-1α, CAN-04 a first-in-class fully humanized monoclonal antibody targeting IL-1RaAcP, thereby blocking IL-1α and β signaling, and OSP-101, the first and only inhaled IL-1Ra. In addition,

Interleukin-1 (IL-1), Table 1 Currently approved therapies targeting Interleukin-1

Drug name	Therapeutic strategy	Mechanism of action
Anakinra	Recombinant Interleukin-1R antagonist	Binding to IL-1R
Rilanocept (IL-1Trap)	Recombinant IL-1R fusion protein	Binding to IL-1α, IL-1β and IL-1Ra
Canakinumab	Monoclonal Ab specific to human IL-1β	Neutralizing IL-1β

small molecule inhibitors of inflammasome components such as NLRP3 are showing considerable promise in preclinical development (Dinarello et al. 2012; Coll et al. 2015). Notwithstanding the clear efficacy of these drugs in the treatment of monogenic autoinflammatory disorders associated with unregulated and enhanced IL-1 activity, the evidence that they may also provide benefit in other more prevalent disease settings is also accumulating. For example, IL-1 has long been at the center of hypotheses linking inflammation with the pathogenesis of atherosclerosis. This is now more clearly evident from the recently reported CANTOS study, one of the largest phase III clinical trials ever established, investigating the efficacy of the monoclonal anti IL-1β antibody Canakinumab, to prevent secondary cardiac events among cardiovascular disease patients (Ridker et al. 2017a). The results of this trial not only confirmed the contributory role for inflammation, through an IL-1β mediated process, as an important driving factor in the pathogenesis of atherosclerosis, but opens up the possibility of adding more specific modes of anti-inflammatory therapeutic intervention to cardiovascular disease patients. IL-1 has also been implicated as mediating pathological events that can occur in the heart following a myocardial infarction, including inflammation and remodeling processes which further weaken viable heart muscle tissue contributing to subsequent heart failure. Pilot studies, examining anakinra administration following myocardial infarction, led to lowering of CRP levels, as well as a reduced progression to heart failure. In other cardio-metabolic disease states, such as Type 2 diabetes, administration of the anti-IL-Iα monoclonal antibody, MABp1, reduced levels of glycosylated hemoglobin (HbA1c), an indicator of the average blood glucose levels. The beneficial effects of targeting IL-1 in patients with an array of cardiovascular diseases will likely prompt further investigations, as more information concerning the role of IL-1 in cardiovascular pathology is uncovered.

The significance of IL-1 in tumourigenesis is also currently under investigation. IL-1α and IL-1β have been found to be abundantly expressed in advanced tumors and associated with higher tumor grade and invasiveness. Anakinra, which neutralizes both IL-1 ligands, has been reported to inhibit colon tumor growth in preclinical studies and has been investigated as a therapy across various malignancies. Due to its relatively shot half-life, anakinra requires continuous administration, which may lead to reduced patient adherence. Therefore, the anti-IL-1 neutralizing antibodies may represent a more suitable approach and are currently being investigated. Interestingly, during the CANTOS trial, incident cancers were tracked and a reduced incidence of lung cancer and deaths from all cancers was reported among patients administered Canakinumab (Ridker et al. 2017b). This has subsequently led to the design of a combination trial investigating its use alongside a programmed cell death protein 1 (PD-1) immune checkpoint inhibitor in patients with non small cell lung cancer. Other unlicensed therapeutics, such as CAN-04 and MABp1, are also under clinical investigation to determine whether they may improve outcomes for patients with different types of tumor (Mantovani et al. 2018). As more trial results are reported, greater evidence supporting a role for targeting IL-1 in cancer therapy programs will likely be revealed.

Beyond cardiovascular disease and cancer, the therapeutic benefit of targeting IL-1 is also under investigation across a wide range of human inflammatory conditions (Dinarello 2014). These include chronic inflammatory skin conditions such as hidradenitis suppurativa and severe atopic dermatitis, inflammatory lung conditions such as chronic obstructive pulmonary disease, and chronic rheumatologic disorders such as osteoarthritis. As the results of such investigations are revealed, the benefits of targeting IL-1 activity in the clinic are likely to further expand. To date targeting IL-1 has not been associated with an increased risk of opportunistic infections; however, continued vigilance is required for the risk of potential serious infections with chronic therapy, particularly as IL-1 plays an important role in host defense against invading pathogens.

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Interleukin-17

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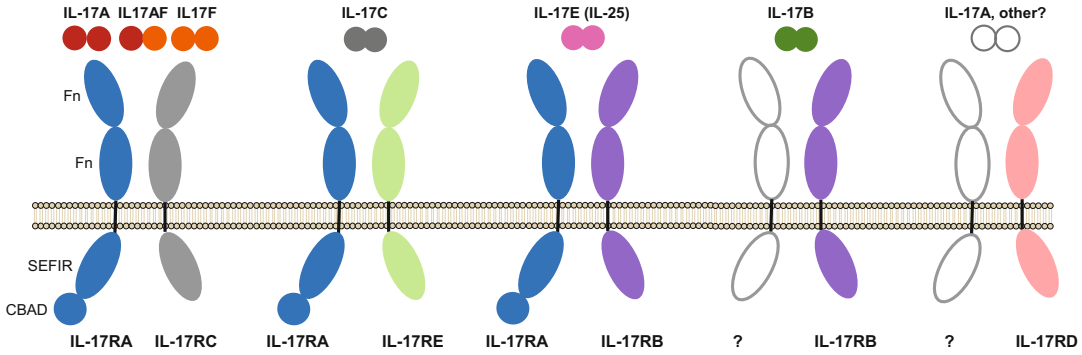
Synonyms

CTLA8; IL-17; IL-17A

Definition

Interleukin-17 (IL-17) is a cytokine, that is, a low molecular weight cellular messenger contributing to the regulation of immune responses. IL-17, also referred to as IL-17A, is the founding member of a whole family of six cytokines (IL-17A to IL-17F). These proteins form mostly homodimers, with the exception of IL-17A and IL-17F that can also form the heterodimer IL-17A/F. IL-17 family members signal through specific IL-17 receptor complexes consisting of two different subunits (IL-17RA to IL-17RE) that also constitute their own receptor family. IL-17A, the most widely characterized member, has mainly pro-inflammatory effects. It is produced by Th17 and other immune cells upon their activation, and stimulates a range of cells including epithelial cells, keratinocytes, fibroblasts, and endothelial cells to produce inflammatory mediators, which in turn attract and activate immune cells to combat pathogens and to ensure barrier integrity. The chronically dysregulated pathway plays a pivotal role in several inflammatory/autoimmune diseases including psoriasis, psoriatic arthritis, and ankylosing spondylitis, as underscored by the beneficial effect of IL-17 blockade in the clinic.



Interleukin-17, Fig. 1 IL-17 cytokine and receptor family. Schematic overview of the known IL-17 receptor complexes with their respective IL-17 cytokines. Unknown co-receptors and ligands are indicated with non-colored symbols. CBAD, C/EBPβ activation domain; Fn, Fibronectin-like domain; SEFIR, SEF/IL-17R domain

Basic Characteristics

IL-17 Cytokine and Receptor Family

The rodent *IL17* gene and IL-17 protein were discovered 25 years ago in T cells, and were originally named as cytotoxic T lymphocyte-associated antigen 8 (CTLA8). Human IL-17 was identified 2 years later, and renamed IL-17A when additional family members, that is, IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25), and IL-17F, were identified based on sequence homology (for review see McGeachy et al. 2019). IL-17A shares the highest homology (55%) with IL-17F. The cytokines exert their functions mostly as homodimers, with the exception of IL-17A and IL-17F that can form also the heterodimer IL-17A/F (Fig. 1). IL-17A also commonly known as IL-17 is the founding member of this family, and also the best characterized. The biology of IL-17A and its closest relative IL-17F is linked, and IL-17F is often but not exclusively co-expressed with IL-17A. In cellular systems, the effects of IL-17A, IL-17F and the heterodimer largely overlap, while their potency is IL-17A > IL-17A/F > IL-17F.

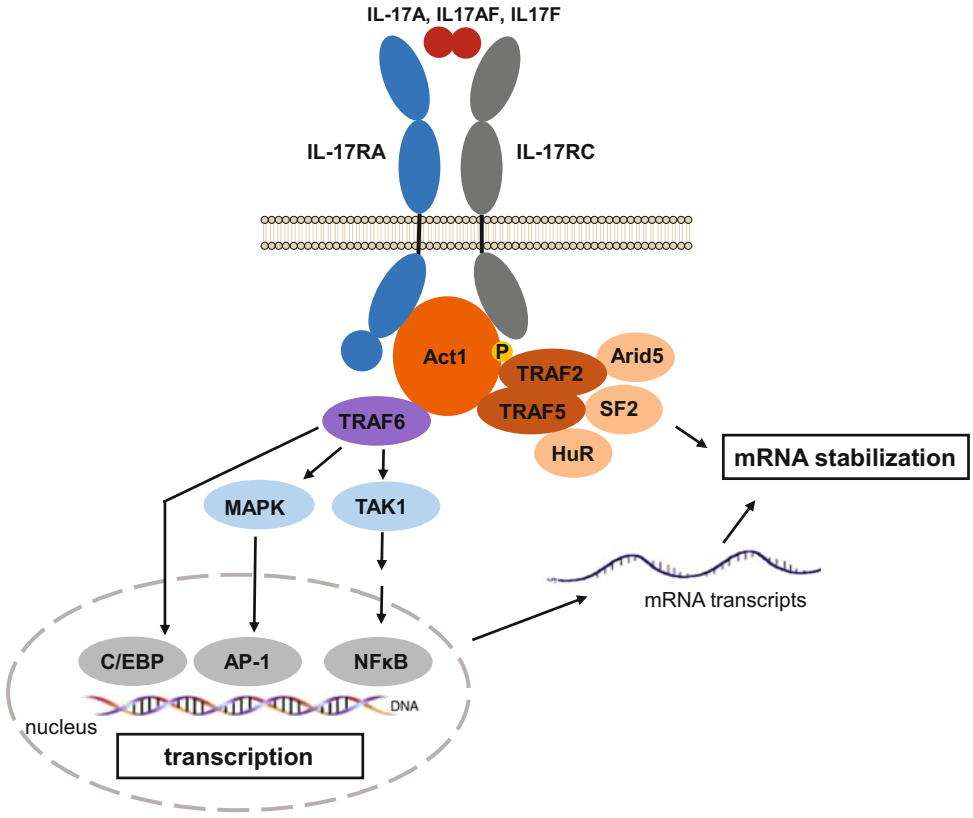
In analogy to the IL-17 family, the receptors for IL-17 (IL-17R) also form their own family, with five homologous subunits, IL-17RA to IL-17RE, identified (Fig. 1). IL-17RA, the founding member, was discovered in 1995. The receptors share an intracellular conserved signaling motif termed SEFIR (similar expression of fibroblast growth factor genes and IL-17 receptor) which is distantly related to the Toll/Interleukin-1 receptor (TIR) domain from Toll-like receptors (TLRs) and IL-1 receptors (IL-1Rs). IL-17RA and IL-17RC contain also an extension of the SEFIR domain (SEFEX, SEFIR extension), and IL-17RA harbors in addition a CBAD (C/EBPβ activation domain) at the C terminus. All IL-17 receptors express two extracellular fibronectin-like (FN) domains to mediate protein-protein interaction and ligand binding, and a single transmembrane domain. The IL-17 cytokines signal via a receptor complex consisting of two different subunits. IL-17A and IL-17F homodimers as well as the IL-17A/F heterodimer bind to the same receptor complex consisting of the IL-17RA and IL-RC subunit (Fig. 1). A recent publication suggests that IL-17A may also bind and signal through IL-17RD (Su et al. 2019). IL-17RA is also the co-receptor used by two other IL-17 family members: IL-17C binds to the IL-17RA/IL-17RE complex, and IL-17E to the IL-17RA/IL-17RB complex. In addition, there is evidence for interaction of the IL-17RA/IL-17RC complex with the epidermal growth factor receptor family. Little is known, however, about the other IL-17 homodimers and the IL-17RD complex.

IL-17 Signaling

While members of the IL-17R family are defined by the conserved SEFIR domain, the only other known protein with a SEFIR domain is the

adaptor protein Act1, the usage of which is a unique feature of IL-17R signaling. IL-17RA/IL-17RC signaling has been studied most comprehensively (McGeachy et al. 2019). Binding of the ligand (IL-17A, IL-17AF, IL-17F) to the receptor complex induces conformational changes that enable the recruitment of the cytosolic adapter protein Act1 via homotypic SEFIR-SEFIR domain interaction (Fig. 2). The central role of Act1 in IL-17A signaling has been shown by the deletion of the *act1* gene, which abrogates all responses to IL-17A. Act1 directs the two main signaling cascades and functions of IL-17, a transcriptional arm and an mRNA stabilization arm. To direct these two downstream signaling cascades, Act1 interacts via its TNF receptor-associated factor (TRAF) binding motif with TRAF proteins. The cascade leading to (overall weakly increased) gene transcription is TRAF6 dependent. Act1 activates TRAF6 via K63 polyubiquitinylation, which leads to initiation of the canonical NF-κB pathway via TAK1, MAPK signaling cascades (p38, JNK and ERK) and activation of C/EBP transcription factor. C/EBPβ was recently also shown to be directly activated by IL-17RA. Of note, the atypical IκB family member IκBζ emerged as an important signaling node of the IL-17 pathway. Its expression is dependent on IL-17 and it acts in the nucleus as a transcription factor modulator to increase a set of NF-κB-driven genes that constitute a major portion of IL-17 target genes.

The signaling cascade resulting in mRNA stabilization involves TRAF2 and TRAF5 and is initiated by the phosphorylation of Act1 by activated inducible IκB kinase IKKi (also known as IKKε) or TANK binding kinase 1 (TBK1). The phosphorylated Act1 recruits TRAF2 and TRAF5



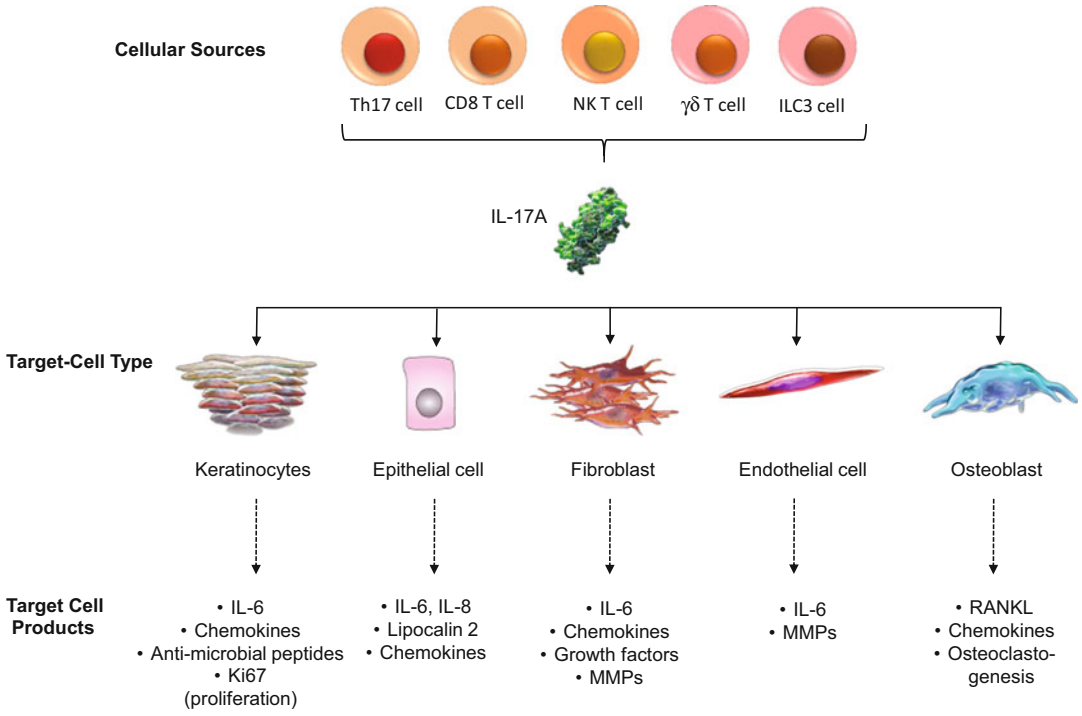
Interleukin-17, Fig. 2 Simplified scheme of IL-17 signaling. The adaptor protein Act1 plays a central role to direct the two main signaling cascades: initiation of transcription involving TRAF6, and mRNA stabilization mediated via TRAF2, TRAF5, and mRNA binding proteins. See text for details

to form a complex with several proteins involved in mRNA stabilization, for example, preventing the mRNA destabilizing SF2 from mRNA binding, and promoting binding of the mRNA stabilizing proteins HuR or Arid5 to act on a subset of IL-17 target gene transcripts. Arid5 stabilizes mRNA by counteracting mRNA degradation mediated by the endoribonuclease Regnase-1. The net effect is increased stability of transcripts from a set of inflammatory genes dependent on IL-17. IL-17 signaling is tightly controlled at several levels, that is, TRAFs such as TRAF3 and TRAF4 disrupt signaling complex formation, enzymes including A20 or USP25 modulate the ubiquitination status of TRAF5 or TRAF6, and binding of the heat shock protein 90 (hsp90) modulates IL-17 signaling by enhancing proteasomal degradation of Act1. Importantly, IL-17A alone is only a moderate transcription and NF-κB activator, and a major role of IL-17A is to act cooperatively with other cytokines such as tumor necrosis factor (TNF) or IFN-γ which are upregulated during inflammation and autoimmune disease.

While IL-17RA is ubiquitously expressed, the expression of IL-17RC appears less prominently on hematopoietic cells and is more evident on epithelial cells including keratinocytes, fibroblasts, and endothelial cells, which thus constitute the main target cells of IL-17 action (Fig. 3).

Cellular Sources of IL-17

IL-17A is produced by several immune cells upon stimulation (Fig. 3). IL-17 is the hallmark cytokine of Th17 cells, a subset of $CD4^+$ T helper (Th) cells, and led to the identification of this new Th cell subset in addition to the already established Th1 and Th2 cells. The differentiation of naive T cells to inflammatory Th17 cells is driven by pro-inflammatory cytokines (IL-1β, IL-6, IL-21) to promote the transcription of the Th17 master regulator retinoid-related orphan receptor-γt (RORγt), which subsequently instructs the cell to increase IL-23R expression. In a feed forward loop, IL-23 signaling then results in the stabilization of Th17 cells with expression of the associated receptor CCR6 and ligand CCL20, and



Interleukin-17, Fig. 3 Cellular sources of IL-17 and main targets of IL-17 with respective responses. MMP, matrix metalloproteinase; RANKL, receptor activator of NF-κB ligand

production of their associated pro-inflammatory type 17 signature cytokines such as IL-17A, IL-17F, granulocyte macrophage colony stimulating factor (GM-CSF), TNF, IL-6, and IL-22. Other immune cells beyond Th17 cells have also emerged as producers of IL-17 depending on the pathological conditions. Those include $CD8^+$ cytotoxic T cells, γδ T cells, natural killer T (NKT) cells, and innate lymphoid cells of the subtype 3 (ILC3). These cells release IL-17A and the aforementioned pro-inflammatory cytokines upon activation by IL-23 or other stimuli. The evidence of IL-23- induced activation of Th17 cells and other leukocytes to release IL-17 has led to an association between these two cytokines and establishment of the IL-23/IL-17 axis. However, IL-17 production and activity without the involvement of IL-23 has also been described, and depends on the cellular, organ, and disease context.

IL-17 Roles in Health and Disease

In healthy individuals, IL-17A and other family members including IL-17F play a role in skin and mucosal barrier homeostasis and in host defense against specific fungal and bacterial infections. Innate immune cells, especially dendritic cells located throughout the body in non-lymphoid compartments, act as sentinels and respond to foreign insults due to tissue injury, pathogen particles, or other infections by mounting an immune response. They release IL-23 and other mediators to activate immune cells including γδ T cells to elicit locally IL-17A, IL-17F, and other cytokines encompassing the IL-17 signature. IL-17 in turn activates, for example, epithelial cells and keratinocytes (Fig. 3) to release chemokines CXCL-1, CXCL-2, CXCL-5 that attract neutrophils to the infected or injured site. Further, to mount the defense, IL-17 stimulates the cells to release antimicrobial peptides (AMPs) such as β-defensin 2 (BD-2), lipocalin 2, S100A7 (psoriasin), and S100A8. The IL-17 pathway was shown to be especially important to defend against *Candida albicans* infection. This is corroborated by genetic studies indicating that individuals with genetic defects affecting IL-17 immunity (e.g., *IL17F, IL17RA, IL17RC*) have chronic mucocutaneous candidiasis. *Candida albicans* infection is also noted as an adverse side effect in clinical trials with antibodies against IL-17 or the receptor. In the gastrointestinal tract, IL-17 is required to maintain barrier integrity, substantiated by the observation that neutralizing IL-17 in patients with Crohn's disease was not beneficial and in some cases disease worsening was observed.

IL-17 in Autoimmune Disease

When chronically activated during autoimmune disease, the IL-17 pathway can constitute an important driver of the pathophysiology. A pivotal role of the dysregulated pathway has been shown in psoriasis, in psoriatic arthritis (PsA) and ankylosing spondylitis (AS), underscored by the treatment efficacy of therapeutic agents inhibiting IL-17.

Psoriasis

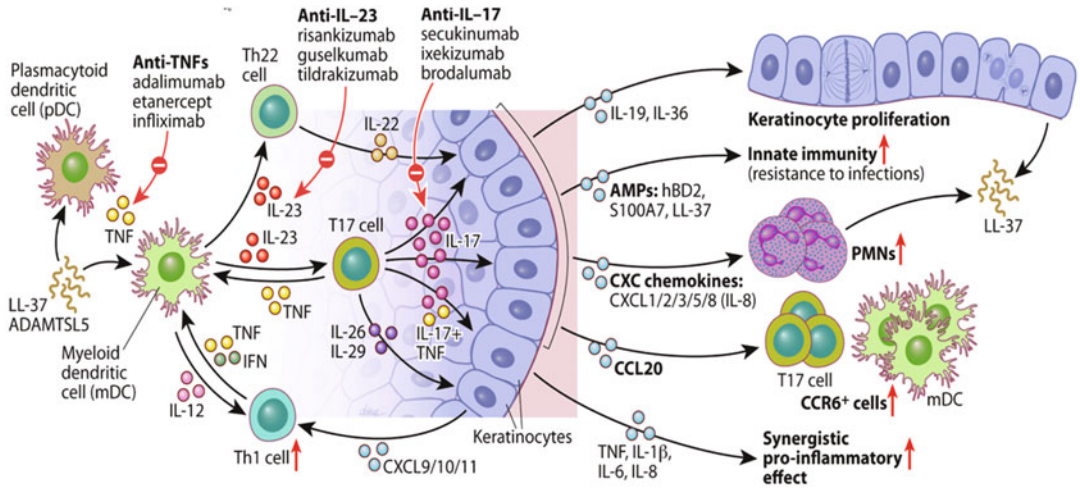
Psoriasis is a chronic autoinflammatory disease with prominent skin involvement that affects approximately 2–4% of the world's population and occurs more commonly in patients with a family history of the disease. The disease is characterized by increased epidermal thickness (acanthosis), thickening of the stratum corneum (hyperkeratosis), retention of nuclei in the cornified layer (parakeratosis), dilated blood vessels, and dense clusters of inflammatory cells composed of T cells and dendritic cells in the dermis, and $CD8^+$ T cells and neutrophils in the epidermis (Hawkes et al. 2018). Psoriasis patients thus develop thick red skin with flaky, silver-white scales. The severity of psoriasis ranges from mild disease with limited number of local inflammatory skin lesions to more severe disease involving widespread plaques that cover more than 10% of the body surface. Especially the moderate and severe forms of psoriasis have been difficult to treat. While the advance of antibodies targeting TNF about 20 years ago was a milestone, neutralizing IL-17 or IL-23 with monoclonal antibodies showed even better results, and these agents have transformed the treatment of moderate-to-severe-psoriasis. Antibodies directed against IL-17A or against IL-17RA showed strong efficacy with 60–

90% of patients achieving a 75% reduction in the psoriasis activity and severity index (PASI75). Depending on trial design, 40% of patients achieved PASI100 indicative of cleared skin. The strong efficacy of both IL-23 and IL-17 blockade suggests a pivotal role of IL-23-dependent IL-17 production in this disease (Fig. 4). Keratinocytes seem to be central to mediate the effects of IL-17, eliciting cytokines including IL-19 and IL-36 that activate keratinocyte proliferation to drive epidermal hyperplasia, and releasing chemokines (CXCL-1, CXCL-2, etc.) to recruit neutrophils, and CCL20 to recruit $CCR6^{+}$ cells, that is, dendritic cells and more Th17 cells. This will in turn lead to more IL-17 production. Furthermore, IL-17-stimulated keratinocytes increase the production of AMPs (β-defensin 2, lipocalin 2, S100A7, S100A8, and LL-37). LL-37 in complex with DNA can activate more dendritic cells, thus perpetuating a vicious circle of events (Fig. 4).

Spondyloarthritides and Other Diseases

The spondyloarthritides comprise related but phenotypically distinct inflammatory diseases including psoriatic arthritis (PsA) and axial spondyloarthritis (axSpA). The clinical manifestations of PsA are diverse, and can manifest as peripheral arthritis, inflammation of the enthesis (i.e., the tendon or ligament insertion sites into the bone), inflammation of an entire digit, skin and nail disease, as well as other manifestations. PsA occurs in a substantial portion of patients with psoriasis. Genetic studies had pointed to a role of the IL-23 pathway in this disease, and components of both IL-23 and IL-17 pathways were shown to be elevated in PsA patients. Subsequently initiated clinical trials with IL-17 blockade demonstrated beneficial treatment responses, and both anti-IL17A antibodies secukinumab and ixekizumab were approved for this indication (Table 1; McGonagle et al. 2019). Blocking IL-23 with guselkumab also showed positive outcomes in PsA phase 3 clinical trials, suggesting that the IL-23/IL-17 axis contributes to a substantial portion to the pathobiology.

AxSpA affects mainly the sacroiliac (SI) joint, and possibly the spine. The subtype ankylosing spondylitis (AS) is also referred to as radiographic axSpA and characterized by the presence of radiographic sacroiliitis. Of note, systemic bone loss and at the same time local bone formation at the entheses can occur. Genetic studies and evidence



Interleukin-17, Fig. 4 IL-23/T17–mediated effects on epidermal keratinocytes in psoriatic skin. Schematic showing the broad downstream effects of increased IL-23 and IL-17 signaling on various immune cell populations and keratinocyte biology. See text for details. PMN, polymorphonuclear cells. (From Hawkes JE, Chan TC and JG Krueger (2017). Psoriasis pathogenesis and the development of novel targeted immune therapies. J Allergy Clin Immunol.;140(3):645–653, with permission from Elsevier)

Interleukin-17, Table 1 Therapeutic agents targeting IL-17 in clinical use or late clinical development. mAb monoclonal antibody, PsA psoriatic arthritis, AS Ankylosing spondylitis/axSpA axial Spondyloarthritis

Target	Agent (trade name)	Type	Approved indication (or as specified)
IL-17A	Secukinumab (Cosentyx®)	Fully human mAb	Psoriasis, PsA, AS/ax SpA
IL-17A	Ixekizumab (Taltz®)	Humanized mAb	Psoriasis, PsA (in development, including AS)
IL-17A	Netakimab (Efleira®, Russia)	Humanized mAb	Psoriasis (in development, including PsA, AS)
IL-17A + IL-17F	Bimekizumab	Humanized mAb	(in development, including psoriasis, PsA, AS)
IL-17RA	Brodalumab (Siliq® (US), Kyntheum® (EU))	Fully human mAb	Psoriasis

of elevated IL-23 and IL-17 in human axSpA as well as data from preclinical models were the basis to initiate clinical trials with IL-23 or IL-17 blockade in AS. While IL-17 blockade showed beneficial treatment effects, the results obtained with IL-23 blockade were surprisingly negative, indicating a divergence of the roles of the two cytokines in this disease. This is an area of further research (Bravo and Kavanaugh 2019; McGonagle et al. 2019).

Initial studies of IL-17 function were performed in synovial fibroblasts from patients with rheumatoid arthritis (RA). They provided evidence of a strong effect of IL-17, especially in combination with TNF, to activate the release of chemokines (CXCL-1, CXCL-2, etc.), cytokines including IL-6, growth factors, and prostaglandin (PGE_2). Furthermore, a key function of IL-17 is to activate matrix metalloproteases resulting in structural damage of tissue, supported by preclinical murine model evidence that a combination of IL-17 and TNF led to cartilage damage. Although there is preclinical evidence of IL-17 pathway upregulation in the synovium from RA patients, IL-17 blockade did not show strong effects in RA clinical trials, although a more detailed analysis of the trial data may identify subsets of responders. Of note, RA is similar to PsA an inflammatory arthritis, yet does not belong to the spondyloarthritides (Bravo and Kavanaugh 2019).

Th17 cells were originally identified in a rodent model for multiple sclerosis (MS), where their ablation caused a strong reduction of the inflammation. This led to a broad investigation of IL-17 roles in this area (Kolbinger et al. 2016), with most evidence pointing to a pathogenic role of IL-17 when produced in the brain. In a phase 2 trial, the anti-IL17 antibody secukinumab significantly reduced the number of cumulative new gadolinium-enhancing T1 lesions, and showed a tendency to reduce the number of active brain lesions on magnetic resonance imaging scans in patients with relapsing-remitting MS. This is an area of further investigation.

Drugs

An overview of the IL-17-directed agents in clinical use or clinical development is given in Table 1. Antibodies targeting IL-17A are the most advanced. Secukinumab is the first-in-class anti-IL17A antibody. It is a fully human monoclonal antibody and was approved for moderate-to-severe psoriasis in 2015 and a year later for PsA and AS/axSpA. Subsequently, Ixekizumab, a humanized monoclonal antibody, was licensed for psoriasis in 2016 and lateron in PsA. This year, Netakimab, a humanized monoclonal antibody, was approved for the treatment of moderate-to-severe psoriasis in Russia. Bimekizumab is a monoclonal antibody directed against IL-17A and IL-17F, and is in late clinical development

for psoriasis, PsA, and AS. Further to the array of IL-17 inhibitors, there is a monoclonal antibody, brodalumab, directed against IL-17RA. As this receptor subunit is shared with other receptor complexes, it inhibits IL-17A, IL-17F, IL-17C, and IL-17E. Brodalumab was approved for moderate-to-severe psoriasis treatment in 2017. Furthermore, efforts to identify low molecular weight compounds that prevent IL-17 pathway activation are ongoing.

Cross-References

- ▶ Bone Metabolism
- ▶ Cytokines
- ▶ Inflammation
- ▶ Interleukin-23
- ▶ Interleukin-6
- ▶ MAP Kinase Cascades
- ▶ Rheumatoid Arthritis
- ▶ S100 Proteins

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Interleukin-23

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Synonyms

IL-23; IL23A; IL-23p19

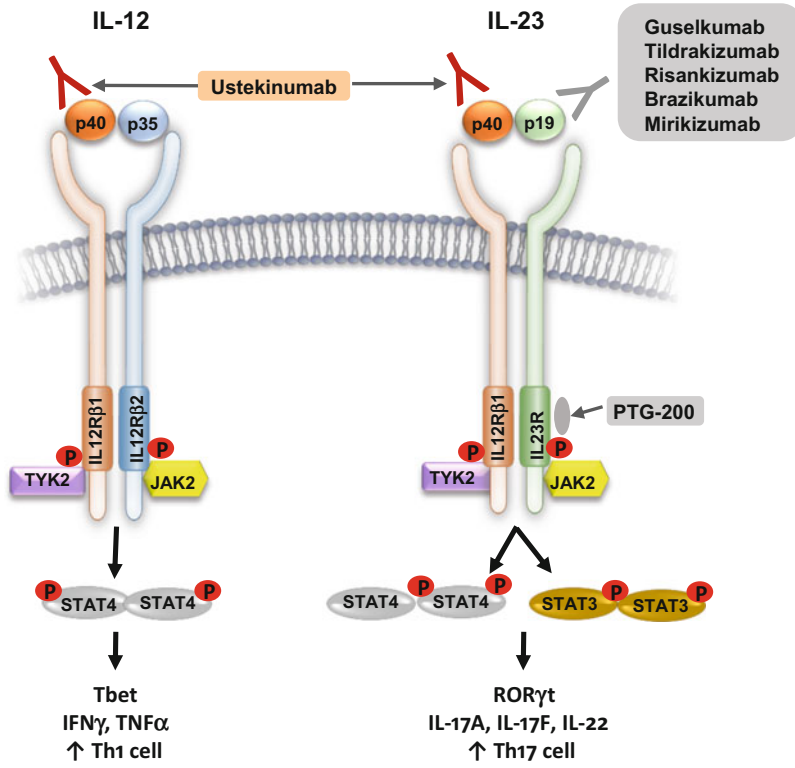
Definition

Interleukins (Latin derivation – communication between leukocytes) are a subset of low molecular weight cellular messengers known as cytokines, which contribute to the regulation of immune responses. Interleukin (IL)-23 is a heterodimer (i.e., consisting of two different subunits). As a member of the IL-12 family, it harbors the common p40 subunit that is characteristic of that family. What distinguishes IL-23 is a p19 component (whereas IL-12 comprises a p35 subunit) covalently bound to p40. In turn, IL-23 binds to a receptor (R) comprised of IL-12Rβ1 and IL23Rα to trigger an array of inflammatory processes. The role of IL-23 in inflammation and autoimmunity has been validated in the clinic by the success of anti-IL-23 antibodies.

Basic Characteristics

IL-23 Discovery and Signaling

IL-23 is a heterodimer consisting of p19 and p40 (Fig. 1). In 2000, Oppmann et al. discovered the p19 subunit, with a 4-helix bundle assembly typical of both IL-6 and IL-12 categories of cytokines via a computational screen (Oppmann et al. 2000). Although p19 alone was inactive, when associated via a disulfide bond with p40, it became a biologically active moiety and was subsequently named IL-23. Given the fact that it shares the p40 subunit with IL-12, IL-23 is recognized as one of the IL-12 family members with the p19 moiety



Interleukin-23, Fig. 1 Organization of the IL-23 receptor complex and signaling cascade in comparison to IL-12. Monoclonal antibodies blocking IL-12 and IL-23 and a peptide IL-23R antagonist which are in clinical use or development are indicated as well. See text for details

showing a distant homology to IL-12's p35 constituent.

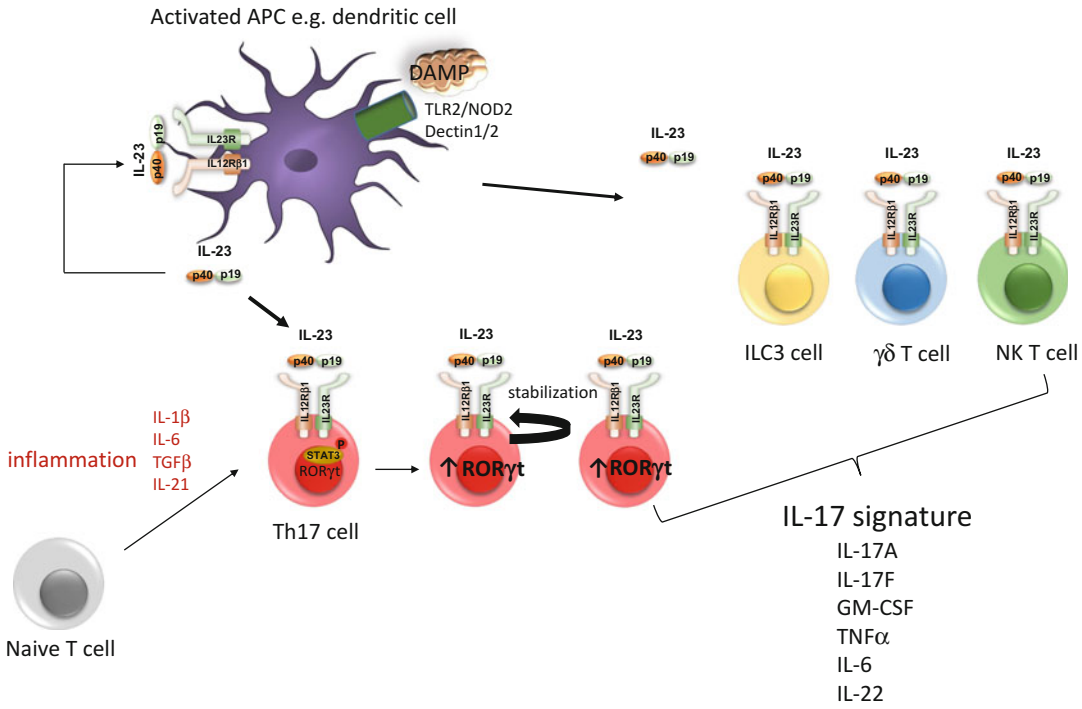
The corresponding receptors are also heterodimeric with both IL-23 and IL-12's p40 subunits binding IL-12Rβ1, but unlike IL-12p35's engagement with IL-12Rβ2, IL-23p19 associates with a subunit termed IL-23R (Fig. 1). By examining the crystal structure of the IL-23 ligand and receptor complex, it was established that for signaling to occur, the N terminal portion of the IL-23p19 subunit first associates with the IL-23R subunit to cause a restructuring of the helical domain that in turn facilitates a high affinity interaction of the p40 subunit to IL-12Rβ1 (Bloch et al. 2018). Binding of IL-23 to its receptor triggers the phosphorylation of several tyrosine residues within the intracellular domains of the receptor by the Janus kinase (JAK) family members JAK2 and TYK2, followed by the recruitment and phosphorylation of the signal transducer and activator of transcription (STAT) family members, especially STAT3. STAT3 subsequently dissociates from the IL23R intracellular portion and translocates to the nucleus to initiate various transcriptional events. IL-23 activates predominantly STAT3 but also more weakly, STAT4, whereas IL-12 signaling mainly triggers the latter. This may account for some of the differential biological effects observed for these two cytokines. Other pathways suggested to be activated by IL-23 engagement with its receptor are mitogen-activated protein kinases (MAPK), and phosphatidylinositol-3-kinase (PI3K) signaling cascades.

Expression of IL-23 and IL-23R

Whether IL-23 is expressed is due to the tight regulation of the p19 subunit from activated cells. p19 is thus understood to be the limiting subunit since p40 can be produced in great quantities. Further, although the p19 subunit is expressed in several cell types such as endothelial cells and polarized T cells, without the p40 moiety, which is absent from these cells, p19 is inactive. Biological activity is only achieved if both p19 and p40 subunits are synthesized within the same cell and these are mainly hematopoietic

derived cells of the myeloid lineage. Antigen-presenting cells (APC), dendritic cells especially, and macrophages release IL-23 upon encounter with triggers associated with host defense against pathogens, inflammatory and wound healing signals. In particular, IL-23 production is elicited via either microbial or damage-associated molecular pattern (DAMP) signals engaging toll-like receptor 2 (TLR2) or nucleotide-binding oligomerization domain-containing protein 2 (NOD2) or dectins 1 and 2 (Fig. 2). ATP and prostaglandin E_2 also act as DAMPs to elicit IL-23 production.

The expression of the IL-23R is limited to certain $CD4^+$ T cell subsets including the inflammatory effector T helper cell 17 (Th17) subset, some γδT cells, memory cells, and innate lymphoid cells (ILC), a small number of B cells and dendritic cells (Moschen et al. 2019). IL-23 is proposed to indirectly generate the Th17 subset from naive T cells as the latter do not express (in mouse) or have absent to negligible levels (in human) of the IL-23R. Under conditions where Th17 cells are generated (i.e., cytokine cocktail of IL-1β, IL-6, IL-21, TGFβ, Fig. 2), the IL-23-IL-23R axis via STAT3 activation promotes the transcription of the Th17 master regulator retinoid-related orphan receptor-γt (RORγt encoded by the RORC gene), which subsequently instructs the cell to increase IL-23R expression (in mouse and human). In a feed forward loop, IL-23 signaling then results in the stabilization of Th17 cells with expression of the associated receptor CCR6 and ligand CCL20, and production of their associated pro-inflammatory type 17 signature cytokines such as IL-17A, IL-17F, granulocyte macrophage colony stimulating factor (GM-CSF), tumor necrosis factor (TNF), IL-6, and IL-22. Th17 cells are thus a major source of the IL-17 signature cytokines (Fig. 2). In addition, as well as stimulating memory T cell proliferation, IL-23 signaling also activates innate and acquired immune cell subtypes including γδT cells, natural killer T (NKT) cells, and ILC, especially ILC3 to release these aforementioned pro-inflammatory



Interleukin-23, Fig. 2 Activated antigen-presenting cells (APC) release IL-23 that results in stabilization of Th17 cells, and together with innate lymphoid cells (ILC3), γδT and natural killer T (NKT) cells, elicits release of an IL-17 pro-inflammatory signature. See text for details

cytokines, particularly IL-17. These observations indicate that IL-23 mediates some of its influence through activating IL-23R positive innate immune cells to elicit pro-inflammatory cytokines including IL-17 (Fig. 2). Of note, however, IL-23 activity without the involvement of IL-17 has also been described as will be discussed below.

Biological Consequences of IL-23 Signaling in Health

IL-23 and its resultant Th17 related cytokine signature are required to both maintain the integrity of mucosal and skin barriers as well as protect against specific bacterial and fungal infections. IL-23R bearing innate cells are distributed throughout the body in nonlymphoid compartments, act as sentinels, and are quick to respond to foreign insults by releasing IL-17 signature cytokines. Consequently IL-17, in turn, triggers especially epithelial, endothelial, and other stromal cells to release chemokines such as CXCL-1, CXCL-2 that attract neutrophils to sites to combat inflammation.

Biological Consequences of IL-23 Signaling in Autoimmune Diseases

However, it has also become evident that the IL-23–IL-23R axis has a strong connection to autoimmune disease pathogenesis. Both genetic evidence from genome-wide association studies (GWAS) and preclinical work in mice have highlighted this. Several polymorphisms in IL-23 and genes along the IL-23 signaling axis such as *IL23R*, *IL12B*, *TYK2,* and *RUNX3* are associated with human autoimmune diseases, including psoriasis, psoriatic arthritis, Crohn's disease (CD), and autoimmune thyroiditis. There has also been a genetic link described for ankylosing spondylitis; however, unexpectedly, blocking IL-23 did not ameliorate this disease.

Many protective functions in autoimmune disease preclinical models originally attributed to inhibition of IL-12 from observations with p40 knockout mice or animals treated with anti-p40 antibody have now been reevaluated in light of the fact that p40 is shared between both IL-12 and IL-23. Indeed, mice lacking the IL-12 subunit p35 showed exacerbated encephalomyelitis, for example, in a mouse model for multiple sclerosis whereas p19 knockout mice were protected in the same model. The fact that IL-23 p19 knockout animals are resistant to several autoimmune disease models including inflammatory bowel disease (IBD) and collagen-induced arthritis, a model for rheumatoid arthritis, further underlines the critical role that dysregulated IL-23 appears to play in autoimmune diseases. Animal models of IBD have provided evidence of IL-23 mediating some of its effects through IL-17 producing cells. ILC and γδT cells via the IL-17/IL-23 axis drive mouse IBD pathology, for example, and in humans, increased numbers of IL-17 producing IL-23R positive ILC and γδT cells are present in tissues of IBD patients. Preclinical data has to be taken with caution, however, as anti-IL-17 therapy in humans with CD was ineffective thus highlighting IL-23 mediated effects independent of IL-17. Nonetheless, there is an abundance of evidence to relate IL-23 to psoriasis preclinically. Mice injected intradermally with IL-23 exhibit psoriasiform changes in the skin accompanied by Th17 infiltration, IL-22 production, and evidence of dendritic cell and γδT cell involvement similar to the human situation. In mice overexpressing STAT3 in keratinocytes, psoriatic-like lesions are formed, and anti-IL-23p19 treatment abrogated disease pathogenesis and reduced Th17 cell signatures. Imiquimod if continuously applied topically causes a psoriasis-like disease in mice with increased epidermal expression of IL-23 and IL-17 in the skin. Anti-IL-23p19 knockout mice are resistant to imiquimod induced psoriasis again strongly implicating this cytokines connection to psoriasis.

Thus, there is an abundance of evidence that the dynamic interplay between IL-23 and IL-17, if dysregulated, can lead to pathological consequences. This association is recognized by various groups and thus the IL-23-IL-23R axis has become an attractive pathway to target therapeutically.

Other Biological Consequences of IL-23 Signaling

IL-23 action is not limited to IL-17 producing immune cells. Other target cells such as the

IL-23R positive bone forming osteoblasts release receptor activator of NF-κB ligand (RANKL) in response to IL-23. Subsequently, RANKL together with M-CSF stimulates osteoclastogenesis and subsequent bone destruction.

More recently, IL-23 driven inflammation is being investigated in terms of cancer biology. Elevated levels of IL-23 are found in serum of patients bearing breast, colorectal, and pancreatic tumors, and it is overexpressed especially in tumors with profound myeloid infiltrates. In preclinical models, IL-23 promoted tumor growth and metastasis but conversely, was reported to have tumor suppressive effects when overexpressed in some tumor lines implanted into mice. Thus, the biology surrounding this is complex and made more so by virtue of the fact that IL-23 can exhibit both direct effects in some IL-23R positive tumor models, and indirect effects, suppressing the activity of $CD8^+$ and NK cells via induction of immunomodulatory agents from IL-23R positive cells. The balance between IL-12 and IL-23 in the tumor microenvironment also seems to be a pivotal factor that needs to be taken into account. Especially tumors with an inflammatory component, at least in humans, appear to have an IL-23 driven component that may be worth considering for therapeutic intervention.

Therapeutic Agents Targeting IL-23

The most advanced therapeutic agents targeting IL-23 are monoclonal antibodies directed against the subunits of this cytokine (Fig. 1). An overview of the IL-23-directed agents in clinical use or clinical development for autoimmune diseases is given in Table 1 and discussed together with the indications that they are registered for below.

Psoriasis is a chronic autoinflammatory disease with prominent skin involvement that affects approximately 2–4% of the world's population. The disease is characterized by increased epidermal thickness (acanthosis), thickening of the stratum corneum (hyperkeratosis), retention of nuclei in the cornified layer (parakeratosis), dilated blood vessels, and dense clusters of inflammatory cells composed of T cells and dendritic cells in the dermis, and $CD8^+$ T cells and neutrophils in the epidermis (Hawkes et al. 2017). Ustekinumab is

Interleukin-23, Table 1 Therapeutic agents targeting IL-23 in clinical use or in clinical development in immune-mediated diseases

Target	Action	Generic name (trade name)	Agent	Approved indications (or as specified)
p40 subunit of IL-12 and IL-23	Inhibits IL-12 and IL-23	Ustekinumab (Stelara®)	Monoclonal antibody, fully human, IgG1κ	Psoriasis, psoriatic arthritis, Crohn's disease, ulcerative colitis
p19 subunit of IL-23	Inhibits IL-23	Guselkumab (Tremfya®)	Monoclonal antibody, fully human, IgG1	Psoriasis
p19 subunit of IL-23	Inhibits IL-23	Tildrakizumab (Ilumya® (USA), Ilumetri® (EU))	Monoclonal antibody, humanized, IgG1	Psoriasis
p19 subunit of IL-23	Inhibits IL-23	Risankizumab (Skyrizi®)	Monoclonal antibody, fully human, IgG1	Psoriasis
p19 subunit of IL-23	Inhibits IL-23	Brazikumab	Monoclonal antibody, fully human, IgG2	In clinical development (incl. Crohn's disease)
p19 subunit of IL-23	Inhibits IL-23	Mirikizumab	Monoclonal antibody, humanized, IgG4	In clinical development (incl. Crohn's disease and ulcerative colitis)
IL-23R	IL-23R antagonist, inhibits IL-23	PTG-200	Oral peptide designed to act locally in the gastrointestinal tract	In early clinical development (incl. Crohn's disease)

one of the first agents that underwent clinical trials for psoriasis. It is a monoclonal antibody directed against the IL-12/IL-23p40 subunit, and thus inhibits both IL-12 and IL-23 action, and reduces responses of both Th1 and Th17 cells as well as other cell types expressing the receptors for these two cytokines (Fig. 1, Table 1). The antibody showed strong efficacy in psoriasis clinical trials with approximately 75% of patients achieving a 75% reduction in the psoriasis activity and severity index (PASI75), leading to its approval for moderate or severe psoriasis in 2009. While the treatment effects of ustekinumab were already substantial, even stronger effects were obtained with selective inhibition of the IL-23/T17cell/IL-17 axis using anti-IL-23p19 or anti-IL-17 antibodies. The first-in-class antibody directed against IL-23p19 and thus targeting IL-23 selectively is guselkumab, a fully human monoclonal antibody (Table 1, Fig. 1). Two clinical phase 3 trials for the treatment of moderate-to-severe plaque psoriasis with guselkumab have been conducted. Results from the two studies were very similar and indicated that 73% and 70% of patients treated with guselkumab achieved a 90% reduction in the psoriasis activity and severity index (PASI90) at week 16. More than 30% of patients achieved a PASI100 response, leading to its approval for moderate-to-severe psoriasis in 2017 (Hawkes et al. 2017). Additional IL-23p19 antibodies have demonstrated excellent efficacy in clinical trials and were recently approved for moderate-to-severe psoriasis, i.e., tildrakizumab which is a humanized, IgG1 monoclonal antibody approved in 2018, and risankizumab, a fully human IgG1 monoclonal antibody which was approved in 2019 (Table 1, Fig. 1). The strong efficacy of both IL-23 and IL-17 blockade suggests that there is a crucial role of IL-23-dependent IL-17 in this disease. IL-17 (in synergy with TNF) is assumed to stimulate keratinocytes, dermal fibroblasts to release pro-inflammatory mediators and chemokines (CXCL-1, CCL20, others), which attract more inflammatory cells and thus perpetuate a vicious circle of events. IL-23 antibodies and IL-17 antibodies have transformed the treatment of psoriasis, especially the moderate and severe forms of the disease.

Psoriatic arthritis (PsA) is an inflammatory arthritis which belongs to the spondyloarthritides (SpA). The clinical manifestations of PsA are diverse and can manifest as peripheral arthritis, enthesitis (inflammation of the tendon or ligament insertion sites into the bone), dactylitis (inflammation of an entire digit), skin and nail disease, and others. PsA occurs in a substantial portion of patients with psoriasis. Ustekinumab was already approved for PsA in 2013. Selectively blocking IL-23 with guselkumab also showed positive outcomes in PsA phase 3 clinical trials. Genetic studies and evidence of elevated IL-23 in human axial spondyloarthritis (axSpA) as well as data from preclinical models were the basis to initiate clinical trials with IL-23 or IL-12/23 blockade in ankylosing spondylitis, a subtype of axSpA. However, the results were negative (Bravo and Kavanaugh 2019). Since IL-17 blockade is an effective treatment, there is obviously a divergence of the roles of the two cytokines which is an area of further research. Interestingly, IL-23 or IL-17 blockade did not show strong effects in rheumatoid arthritis, which is similar to PsA an inflammatory arthritis, yet does not belong to the spondyloarthritides (Bravo and Kavanaugh 2019). While a more detailed analysis of the trial data may identify subsets of responders, the data suggest a crucial role of other drivers.

Inflammatory bowel disease (IBD) encompasses Crohn's disease (CD) and ulcerative colitis (UC) (for review, see Moschen et al. 2019). While the advance of antibodies targeting TNF about 20 years ago was a milestone for IBD treatment, there is still an unmet medical need, as not all patients respond to this agent or lose response over time. CD has been targeted first with anti-IL-12/IL-23 antibodies as it displays characteristics of a Th1-cytokine driven disease with increased production of the hallmark cytokine IFN-γ. Furthermore, there was evidence of cells secreting IL-12 in the lamina propria from CD patients. Ustekinumab showed positive outcome in the treatment of CD patients, including patients who did not respond to TNF blockade or to

conventional drugs, leading to its approval for treatment of moderate-to-severe CD in 2016. While UC and CD are often discussed as divergent (with a Th1 and Th2 feature, respectively), there are shared genetic polymorphisms between both indications suggesting pathogenic similarity. Ustekinumab also showed efficacy in the UC development program and was recently approved in 2019. While the treatment effect seen with ustekinumab could be due to IL-12 and/or IL-23 action, several anti-IL-23 antibodies including brazikumab, risankizumab, mirikizumab, and guselkumab are in clinical development for CD, and in some instances, for UC. Furthermore, an oral peptide selectively antagonizing the IL-23R and thus inhibiting IL-23 action, PTG-200 (Fig. 1, Table 1) designed to act locally in the gastrointestinal tract, has been reported to be in early development for CD (Moschen et al. 2019). Taken together, the treatment of IBD underwent a major improvement with the advance of IL-12/IL-23 and IL-23 blockers.

The advent of antibodies blocking IL-23 or IL-17 in the clinic thus help to delineate the respective roles of the cytokines.

Cross-References

- ▶ Cytokines
- ▶ Inflammation
- ▶ Interleukin-17
- ▶ Interleukin-6
- ▶ JAK-STAT Pathway
- ▶ Rheumatoid Arthritis

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Interleukin-5

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Synonyms

Asthma; Benralizumab; Eosinophils; IL-5; Mepolizumab; Reslizumab; Severe eosinophilic asthma

Introduction

Interleukin (IL)-5 is a cytokine dimer derived from both hematopoietic and non-hematopoietic cell lines that primarily contribute to the proliferation, differentiation, and survival of eosinophils. While other cytokines have been implicated in the maturation, survival, and function of eosinophils (Matucci et al. 2019), IL-5 remains the only known human "eosinophilopoietin," as one of its primary roles includes stimulating production of these cells from the bone marrow (Patterson et al. 2015). Given eosinophilic prominence in a variety of disease states, considerable research has focused on the development of therapeutic drugs targeting IL-5-dependent signaling.

Background

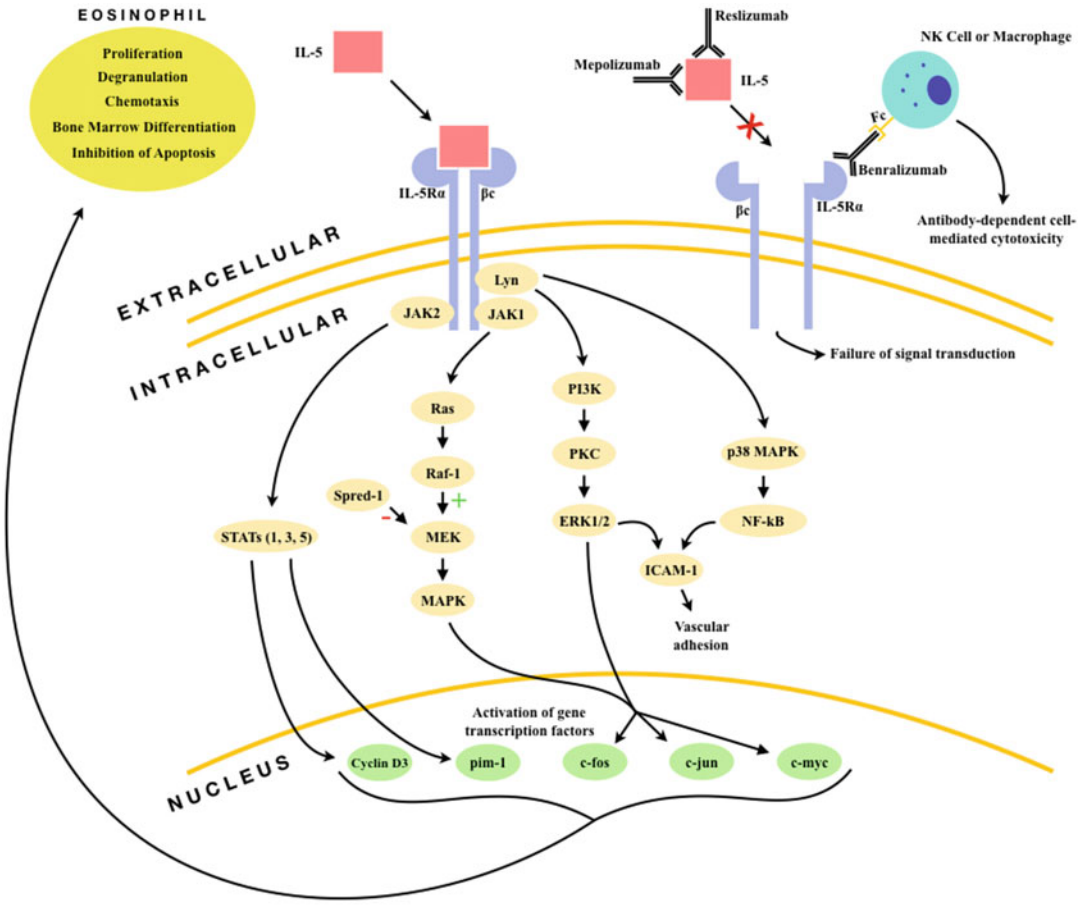
In the late 1870s, German scientist Paul Ehrlich discovered bilobed nucleated cells known as "eosinophils" utilizing a new staining method requiring alkaline dyes. Eosinophils, or "acid-loving," derive their name from their ability to uptake eosin, a dye that binds to cationic proteins within granules located inside the eosinophil (Varricchi et al. 2016). These cells comprise ~1–6% of the white blood cells and are important defenders against parasitic infection (Patterson et al. 2015). They have also been identified as a key cell type involved in the inflammatory process of eosinophilia-associated clinical conditions. However, despite Ehrlich's discovery of eosinophils, over 100 years would pass before understanding of eosinophil regulation would advance. Historically, Campbell et al. cloned eosinophil differentiation factor (EDF) in 1987 using a murine EDF cDNA clone as a probe (Campbell et al. 1987). During the same year, Tanabe et al. cloned the IL-5 gene (Tanabe et al. 1987). Subsequently, IL-5 was identified and determined to be important in every step of the eosinophil life cycle, including the maturation of CD34+ progenitor cells into mature eosinophils within the bone marrow. As IL-5 stimulates eosinophils to transition out of bone marrow into the blood, they are recruited to sites of tissue inflammation or stored until activated (Matucci et al. 2019). In varying disease states, eosinophils may migrate to affected organs such as the nose, esophagus, lung, and skin (Molfino et al. 2012). The generation of IL-5 and other cytokines and chemokines is important in the chemotaxis of eosinophils.

While eosinophils and mast cells are involved in the production of IL-5, the main source is largely from T lymphocytes and type 2 innate lymphoid cells (ILC2). IL-5 production within T lymphocytes and ILC2s is triggered primarily by IL-25 and IL-33, which are potent inducers of the inflammatory and pro-allergic cytokines IL-4, IL-5, and IL-13 (Matucci et al. 2019; Varricchi et al. 2016; Yanagibashi et al. 2017). In vivo analysis demonstrates that IL-33 (compared to IL-25) has a greater effect on proliferation of IL-5-producing ILC2 (Yanagibashi et al. 2017). In addition to IL-5, cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3 are also expressed by activated peripheral blood T cells and may be involved in eosinophil survival, growth, activation, and function (Matucci et al. 2019).

The Axis of IL-5 and IL-5R

IL-5 is comprised of four distinct α-helices on two separate polypeptide chains, a structure similar to cytokines IL-3 and GM-CSF (Matucci et al. 2019; Pelaia et al. 2017; Varricchi et al. 2016). Of the four α-helices, three originate from one of the polypeptide chains, while the fourth helix extends from the second chain (Molfino et al. 2012).These native IL-5 α-helices confer its higher affinity to IL-5R (Molfino et al. 2012). As eosinophils and B cells express IL-5 receptor (IL-5R), selective binding with IL-5 is mediated by IL-5Rα and IL-5Rβc, which form the subcomponents of the receptor. IL-5Rα and IL-5Rβc confer high and low affinity for IL-5, respectively (Pelaia et al. 2017). IL-5Rβc provides the non-specific protein structure, which also allows binding with other cytokines such as IL-3 and GM-CSF (Molfino et al. 2012).

Binding of IL-5 to IL-5Rα leads to complex-associated activation of IL-5Rβc, initiating specific signal transduction pathways that confer IL-5-mediated effects on eosinophils (Pelaia et al. 2017). These signal transduction pathways are characterized in Fig. 1. After complex formation, there is a series of downstream signaling events that originates from the tyrosine kinase-mediated phosphorylation of Janus kinase proteins (JAK1/JAK2). JAK1 and JAK2 are inherently associated with IL-5Rα and IL-5Rβc, respectively, independent of IL-5 activation (Molfino et al. 2012; Pelaia et al. 2017). JAK1 and JAK2 phosphorylate signal transducer and activator of transcription (STAT) proteins, including STAT1, STAT3, and STAT5, interactions that are indispensable to IL-5 signaling within eosinophils. Phosphorylations of STAT1, STAT3, and STAT5 cause gene transcription of pim-1 and cyclin D3, which contribute to eosinophil survival



Interleukin-5, Fig. 1 Current understanding of IL-5 monoclonal antibody blockade and IL-5 signaling pathways, receptor interactions, and effects on eosinophils

via production of leukotrienes, eosinophil cationic protein, major basic protein, and other eosinophil mediators (Molfino et al. 2012; Pelaia et al. 2017; Yanagibashi et al. 2017). In conjunction with JAK2, Lyn and Raf-1 kinases are also involved in survival of eosinophils via inhibition of apoptotic pathways (Molfino et al. 2012; Pelaia et al. 2017; Yanagibashi et al. 2017).

Although the JAK/STAT pathway is invariable to eosinophilic survival and function, mitogen-activated protein kinases (MAPK) (primarily extracellular signal-regulated kinases [ERK]1, ERK2, and p38) play an equally important role. ERK expression is mediated by activation or inhibition from Raf-1 and Spred-1, respectively (Molfino et al. 2012). Raf-1, in particular, contributes to activation of ERK, which leads to induction of c-fos gene transcription and promotion of leukotriene C_4 release (Pelaia et al. 2017). While, both ERK1/2 MAPK and p38 are involved with promoting cytokine production and cell degranulation, p38 (in conjunction with NF-κB) contributes more to eosinophil chemotaxis and cytokine release at sites of allergic inflammation (Pelaia et al. 2017). Prior to p38-mediated chemotaxis, the interaction between eosinophils and intercellular adhesion molecule-1 (ICAM-1) is imperative for eosinophil vascular wall adhesion. This adhesion is primarily mediated by phosphoinositide 3-kinase (PI3K) via protein kinase C (PKC) activation and subsequent phosphorylation of ERK (Molfino et al. 2012; Pelaia et al. 2017).

Ultimately, IL-5-dependent pathways collectively activate multiple gene transcription factors, including c-myc, c-fos, c-jun, cyclin D, and pim-1, which serve to enhance eosinophil proliferation, differentiation, chemotaxis, and degranulation (Molfino et al. 2012).

Eosinophils and Disease

Eosinophils are uniquely associated with a spectrum of diseases, including hypereosinophilic syndromes, nasal polyposis, eosinophilic granulomatosis with polyangiitis, eosinophilic esophagitis, atopic dermatitis, and bronchial asthma (Hassani and Koenderman 2018; Varricchi et al. 2016). Given the ability of IL-5 to dramatically influence eosinophil function, it has been long hypothesized as a susceptible therapeutic target. While eosinophilia and IL-5 are important aspects of the aforementioned conditions, asthma affects many more people, and this disease has historically been identified for its potential benefit concerning therapies directed toward IL-5. Research of asthma serves as a unique template to illustrate the clinical impact of IL-5 antagonism.

Therapeutic Interventions with IL-5 Antagonism

Asthma is a chronic disease characterized by airway inflammation, leading to bronchial hypersensitivity and reversible airway obstruction. Notable clinical symptoms suggestive of asthma include wheezing, cough, and shortness of breath. A wide variety of therapeutic medications have been developed to alleviate these symptoms and/or prevent their recurrence. Asthma, similar to many chronic diseases, demonstrates a spectrum of phenotypic endotypes compounded by variable symptoms, pathophysiology, and pharmacological treatment options (Pelaia et al. 2017). While traditional therapy of inhaled corticosteroids and bronchodilators targeting β2-adrenergic receptors addresses the vast majority of patients, there are subsets of uncontrolled asthmatic individuals that require prolonged oral glucocorticoid treatments.

Eosinophils have been identified as a culprit in severe asthmatics not controlled with standard therapy (Matucci et al. 2019; Molfino et al. 2012; Pelaia et al. 2017). Eosinophilic asthma is characterized by eosinophilia localized throughout the bronchial tract, which causes chronic degranulation and remodeling of airways (Patterson et al. 2015). Remodeling in the context of chronic bronchial inflammation includes basement membrane thickening and mucosal infiltration by eosinophils, Th2 lymphocytes, and mast cells (Matucci et al. 2019; Pelaia et al. 2017). Specifically, Th2 lymphocytic infiltration contributes to two distinct endotypes termed T2 "high" or T2 "low," based on the degree of Th2 infiltration. Due to their proinflammatory nature, blood and sputum eosinophilia contributes largely to the Th2 "high" endotype (Matucci et al. 2019).

Initially, supporting a relationship between IL-5 and asthma severity proved difficult. While elevated concentrations of IL-5 were shown in bronchial biopsies of patients with asthma (Tang et al. 1997), early studies evaluating improvement of lung function in humans provided anti-IL-5 monoclonal antibodies were disappointing (Patterson et al. 2015). In fact, two studies recruited asthmatics to receive anti-IL-5 or placebo with the primary end points of improved lung function and quality of life. While both studies showed significant depression of peripheral eosinophilia, neither showed improvements in the primary outcomes, leaving significant questions regarding the utility of these antibodies (Flood-Page et al. 2007; Leckie et al. 2000; Menzella et al. 2016; Molfino et al. 2012; Patterson et al. 2015; Varricchi et al. 2016). Despite the negative data, the scientific community learned from these studies, especially regarding the importance of defining study populations and primary end points. As more information became available regarding eosinophilic asthma, many recognized this as a phenotype that might better respond to anti-IL-5 medications. Subsequently, various clinical trials emerged involving

the use of monoclonal antibodies targeting IL-5 and IL-5Rα. Currently, there are two biologics that target IL-5, mepolizumab and reslizumab, and one that targets IL-5Rα, benralizumab (Table 1).

Mepolizumab

Mepolizumab (US trade name Nucala), produced by GlaxoSmithKline in Brentford, UK, was approved by the US Food and Drug Administration (FDA) in November 2015 for use in severe asthma with eosinophilic phenotype for ages 12 years and older. Subsequent studies (discussed below) have lowered the age range for administration down to 6 years. Derived from ovaries of Chinese hamsters, it is a selective humanized monoclonal antibody targeting the α-chain of IL-5, which disrupts binding with the α-subunit of IL-5R (Abonia and Putnam 2011).

Mepolizumab debuted in December of 2000. A randomized, double-blind, placebo-controlled study demonstrated decreased eosinophils in both peripheral blood and sputum samples among three distinct groups of eight patients. Unfortunately, clinically significant outcomes pertaining to improvements in forced expiratory volume in one second (FEV1), peak expiratory flow rate, and histamine-responsive airway hyperactivity were not readily apparent when considering all-comers with asthma (Abonia and Putnam 2011; Flood-Page et al. 2007; Leckie et al. 2000). In fact, until 2012, only two studies had shown evidence of clinically significant improvement in asthma exacerbations with marginal increases in FEV1 from baseline for prednisone-dependent severe asthmatics with significant sputum eosinophilia (≥3%) (Haldar et al. 2009; Nair et al. 2009). However, in 2012, Pavord et al. published the Dose Ranging Efficacy and Safety with Mepolizumab (DREAM) clinical trial, which was the single-largest clinical study (621 patients) for severe *eosinophilic* asthma to date (Menzella et al. 2016; Pavord et al. 2012). The DREAM findings showed significant decreases in blood and sputum eosinophil levels and, finally, a reduction in acute exacerbations of asthma. Following the work of Pavord et al., Bel et al. and Ortega et al. published supportive conclusions highlighting improvements in the Asthma Control Questionnaire (ACQ)-5 scores, reduction in exacerbations, and decreased dependence upon glucocorticoids (Bel et al. 2014; Ortega et al.

Interleukin-5, Table 1 Characteristics of biologics involved in the inhibition of IL-5 pathway signaling. IL-5 associated biologics

	Mechanism of action	Biomarkers	FDA approved	Dosing frequency	Route
Mepolizumab	Directly binds to IL-5	Peripheral eosinophilia (>150 cells/mcL) within 6 weeks of treatment initiation	Eosinophilic asthma (6 years or older) and eosinophilic granulomatosis with polyangiitis (18 years and older)	*Eosinophilic asthma*: 6–11 years old, 40 mg for 4 weeks; 12+ years, 100 mg for 4 weeks *EGPA*: 300 mg every 4 weeks	Subcutaneous
Reslizumab	Directly binds to IL-5	Sputum eosinophilia (>3%)	Eosinophilic asthma (18 years or older)	3 mg/kg every 4 weeks via IV infusion for 20–50 min	Intravenous
Benralizumab	Directly binds to IL-Rα leading to antibody-mediated cytotoxicity	Peripheral eosinophilia (>300 cells/mcL)	Eosinophilic asthma (12 years or older) and eosinophilic esophagitis (approved under Orphan Drug Designation)	30 mg (single dosed pre-filled syringe) every 4 weeks (for three doses) then every 8 weeks	Subcutaneous

2014). Cumulative findings demonstrated that treatment with mepolizumab was most beneficial for patients with a baseline eosinophilia of ≥150 cells/mcL obtained within 6 weeks of treatment initiation.

Current dosing guidelines suggest 100 mg mepolizumab given subcutaneously every 4 weeks. In 2017, the FDA approved mepolizumab for the treatment of adult patients with eosinophilic granulomatosis with polyangiitis at a slightly higher dose than that considered in eosinophilic asthma (300 mg every 4 weeks) (Pelaia et al. 2017). Within the same year, mepolizumab was also approved for the treatment of severe eosinophilic asthma in patients as young as 6 years old. Dosing for eosinophilic asthmatics receiving mepolizumab is 40 mg for 4 weeks and 100 mg for 4 weeks for patients 6–11 years old and 12 years and older, respectively (Lexicomp; Pelaia et al. 2017).

Reslizumab

Developed by Teva Pharmaceuticals, reslizumab was approved for use in March 2016 under its trade name, Cinqair (Varricchi et al. 2016). Reslizumab is indicated for eosinophilic asthma in patients above 18 years of age that have failed empiric treatments. At its core, reslizumab is an IgG4-kappa immunoglobulin that selectively binds IL-5 in order to decrease eosinophilic proliferation by blocking interactions with IL-5Rα (Bagnasco et al. 2017).

The first clinical trial for reslizumab was performed in 2003 when Kips et al. showed significantly decreased blood eosinophil levels in patients with severe persistent asthma (Kips et al. 2003). Then, in 2011, Castro et al. published a 271-patient phase III clinical trial demonstrating improved FEV1, asthma symptom profile, and frequency of exacerbations when compared to placebo among patients with evidence of sputum eosinophilia (≥3%) (Castro et al. 2011). Building on the work of Castro et al., Bjermer et al. demonstrated similar findings with patients having blood (vs sputum) eosinophilia (≥ 400 cells/μL) (Bjermer et al. 2016). Numerous trials have repeated the work of Castro and Bjermer et al., highlighting the clinically significant impact reslizumab has in patients with active eosinophilic airway inflammation. Currently, reslizumab is available in 100 mg/10 mL (10 mg/mL) single-use vials for IV infusion only. Dosing is determined based on patient weight at 3 mg/kg every 4 weeks and require 20–50-minute infusion times (Lexicomp).

Benralizumab

Benralizumab (US trade name Fasenra) was developed by AstraZeneca (formerly MedImmune) and approved by the FDA in November 2017 and August 2019 for eosinophilic asthma and eosinophilic esophagitis, respectively. This humanized monoclonal antibody of the IgG1-kappa subclass differs from mepolizumab and reslizumab in that it selectively binds IL-5Rα on eosinophils and basophils (Yanagibashi et al. 2017). Besides its ability to block IL-5 signaling through the receptor, benralizumab is also uniquely afucosylated during production, which confers a high affinity for the FcγRIIIα in natural killer cells, macrophages, and neutrophils (Dhanji et al. 2005; Yanagibashi et al. 2017). This unique approach results in depletion of eosinophils via antibody-dependent cell-mediated cytotoxicity. Benralizumab has been shown to substantially reduce or eliminate *tissue* eosinophilia associated with specific eosinophilic diseases (Matucci et al. 2019).

Three placebo-controlled clinical trials provide evidence for clinical effectiveness (in order of date performed): SIROCCO, CALIMA, and ZONDA. SIROCCO examined the safety and efficacy of benralizumab, specifically, its effect on the frequency of asthma exacerbations over 48 weeks (Bleecker et al. 2016). CALIMA focused on the efficacy and safety of benralizumab as add-on therapy for those with severe, uncontrolled asthma and elevated blood eosinophils (FitzGerald et al. 2016). Both SIROCCO and CALIMA demonstrated a

significant decrease in exacerbation frequency while improving FEV1 when compared to placebo in those with blood eosinophils ≥300 cells/μL. In ZONDA, researchers questioned whether administration would decrease the need for oral corticosteroids over 28 weeks. Outcomes from this trial confirmed a 75% reduction in oral corticosteroid dosage for those on benralizumab as compared to 25% reduction for the placebo group (Nair et al. 2017). In regard to dosing and administration, benralizumab is given subcutaneously via a single-dose pre-filled syringe with a recommended dose of 30 mg. Initial injections are given every 4 weeks for three doses and then every 8 weeks (Lexicomp).

Conclusions

Further investigation is underway for use of mepolizumab, reslizumab, and benralizumab in the treatment of other diseases attributed to eosinophilic inflammation. As collective understanding of IL-5 expands, new disease phenotypes and endotypes will emerge that may benefit from IL-5 antagonism. However, ongoing research is needed to establish clear biomarkers that will identify the particular patients that will most benefit from these interventions. In reflection, IL-5 uniquely represents an exciting step for the scientific community as it illustrates the cumulative effort from the research bench to clinical practice.

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Interleukin-6

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Synonyms

B-cell stimulating factor 2; Hepatocyte stimulating factor; Hybridoma-plasmacytoma growth factor, 26 kDa protein

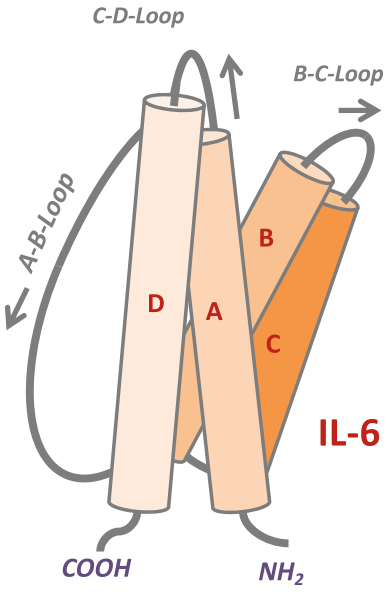
Definition

Interleukin-6 (IL-6) together with IL-1 and tumor necrosis factor alpha (TNFα) are regarded as the most important inflammatory cytokines, which are involved in many if not all inflammatory states of the human body including many autoimmune diseases such as rheumatoid arthritis and inflammatory bowel disease (Scheller et al. 2011b).

The cytokine IL-6 is a four-helical protein (Fig. 1), which is synthesized by many cells during immunological stress situations (Hunter and Jones 2015; Jones and Jenkins 2018). Such immunological stress situations include the detection of so-called pathogen-associated molecular patterns or PAMPs by Toll-like receptors on neutrophils, monocytes, macrophages, and other cells of the innate immune system (Akira et al. 2006). Structurally, IL-6 belongs to a large family of mediators, which show the same four-helical topology and bind to a family of receptor molecules, which are called type I cytokine receptors (Rose-John 2012; Rose-John et al. 2015).

Basic Characteristics

On target cells, IL-6 first binds to a type I membrane protein called IL-6 receptor (IL-6R). The complex of IL-6 and IL-6R associates with a second type I membrane protein called gp130, which consequently dimerizes and initiates intracellular signal transduction (Rose-John 2012). As



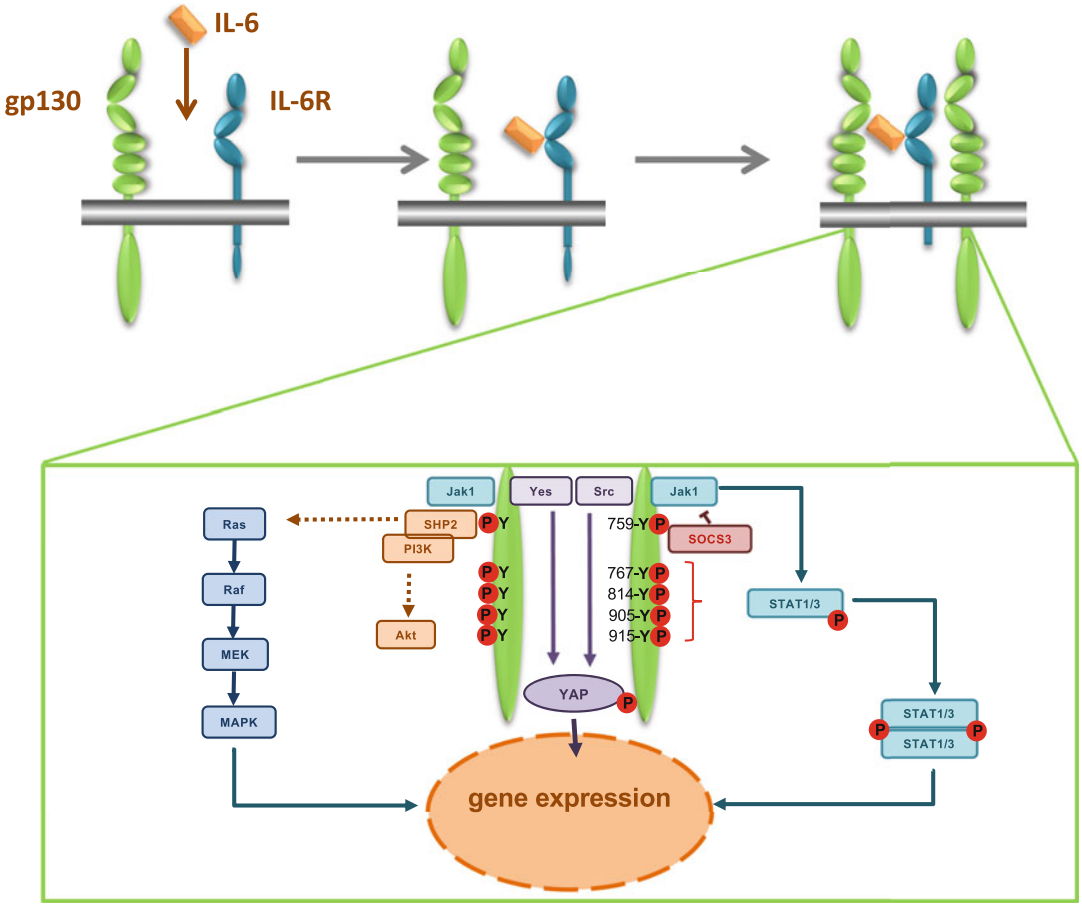
Interleukin-6, Fig. 1 Schematic view of the four-helical protein Interleukin-6 (IL-6). Note that as a consequence of the long loop between helix A and helix B, followed by the short loop between helix B and helix C and followed by the long loop between helix C and helix D, helix A and helix B are oriented upwards whereas helix C and helix D are oriented downwards. This topology is common to all four helical cytokines

shown in Fig. 2, the dimerization of gp130 leads to activation of the cytoplasmic tyrosine kinase called Janus kinase 1 or JAK1, which is constitutively associated with the cytoplasmic portion of gp130 (Schaper and Rose-John 2015). Upon tyrosine phosphorylation of the gp130 cytoplasmic domain, STAT factors (STAT3 and to some extent STAT1) become recruited via their SH2 domains and are phosphorylated by the JAK1 whereupon they dimerize, translocate into the nucleus, and activate STAT1/3 target genes. The membrane-proximal tyrosine of the gp130 cytoplasmic domain recruits the phosphatase SHP2, which leads to the activation of the MAP kinase cascade (Fig. 2). STAT3 activation additionally leads to the transcriptional activation of the negative feedback inhibitor SOCS3, which targets the membrane-proximal membrane-proximal tyrosine residue of the gp130 cytoplasmic domain thereby blocking the activity of JAK1 and leading to feedback inhibition of gp130 signaling. Furthermore, activation of src family kinases leads to activation of the YAP pathway and to the transcription of YAP target genes (Fig. 2).

Whereas the gp130 receptor is expressed on all cells of the human body, the IL-6R is only expressed on some cells including hepatocytes and some leukocytes (Rose-John 2012). Since IL-6 only exhibits a measurable affinity to the IL-6R but not to gp130, it follows that only cells, which express the IL-6R can respond to the cytokine IL-6. It was, however, discovered that the IL-6R from the cell membrane can be proteolytically cleaved by the membrane-bound metalloprotease ADAM17 leading to the generation of a soluble IL-6R (sIL-6R) (Mullberg et al. 1993). In human cells the sIL-6R can also be generated by translation from an differentially spliced mRNA (Lust et al. 1992), although this mechanism leads only to a small fraction of the total sIL-6R moiety in the circulation (Riethmueller et al. 2017). Interestingly, IL-6 can bind not only to the membrane-bound IL-6R but also to the sIL-6R. The complex of IL-6 and sIL-6R can bind to gp130 on cells, which do not express IL-6R. In such a way, these cells, which in the absence of sIL-6R would be completely refractory to IL-6, can now respond to the cytokine. Consequently the range of target cells of IL-6 is dramatically enlarged by the IL-6 trans-signaling mechanism. This paradigm of IL-6 signaling has been named IL-6 trans-signaling (Rose-John and Heinrich 1994) whereas IL-6 signaling via the membrane-bound IL-6R is referred to as classic signaling (Fig. 3a).

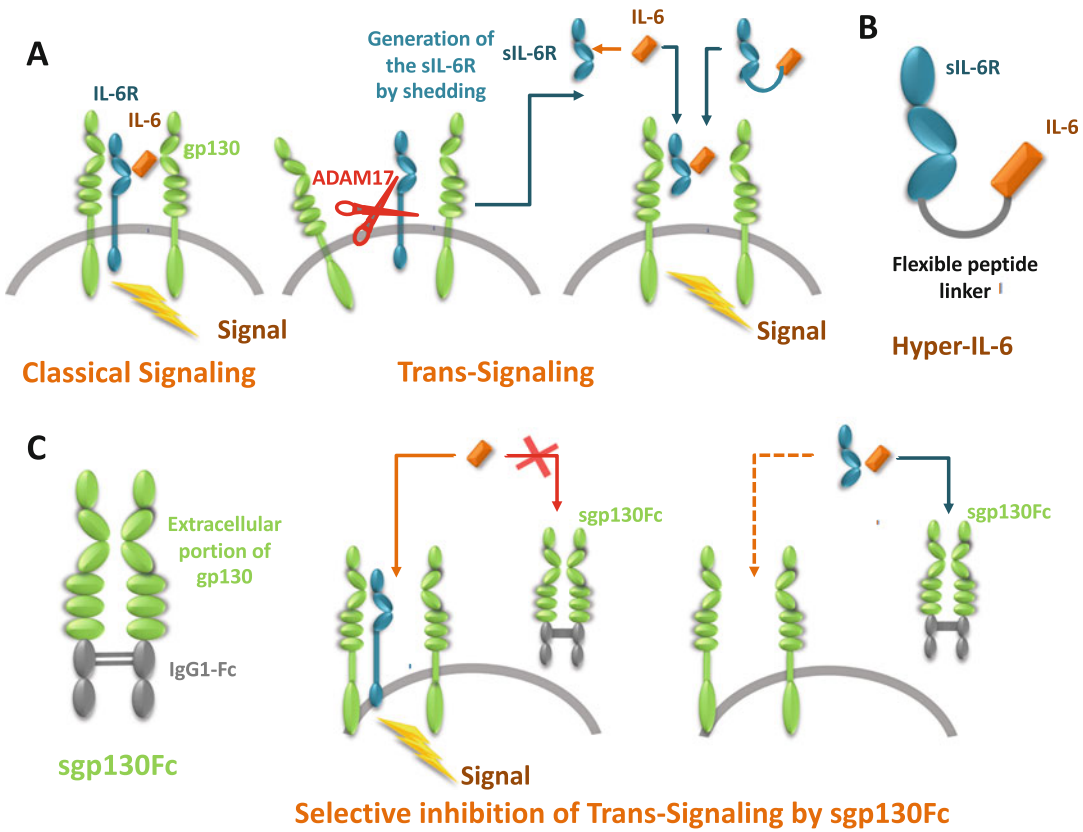
Molecular Tools to Study Interleukin-6

To analyze the physiologic role of IL-6 trans-signaling in vitro and in vivo, we generated two designer proteins, which could be used as molecular tools. Hyper-IL-6 is a fusion protein of IL-6 and sIL-6R in which the two proteins are covalently linked by a flexible peptide linker (Fischer et al. 1997) (Fig. 3b). With Hyper-IL-6, cells can be stimulated regardless of their IL-6R expression. A comparative stimulation with IL-6 and Hyper-IL-6 can be used in vitro and in vivo to



Interleukin-6, Fig. 2 Binding of IL-6 to the IL-6R leads to the assembly of the signaling IL-6 receptor complex. Top, IL-6 binds to IL-6R and subsequently assembles two molecules of the signaling receptor gp130. Bottom, dimerization of gp130 leads to the activation of the JAK1 kinase, which phosphorylates the cytoplasmic portion of gp130. The phosphorylated tyrosine residues recruit signaling molecules such as SHP2, which leads to an activation of the PI3K, Akt, Ras, and MAPK pathways. Furthermore, STAT1 and STAT3 molecules are recruited, become phosphorylated, homo- or heterodimerize and translocate into the nucleus, where they act as transcription factors. Additionally, non-receptor tyrosine kinases such as Src or Yes activate the YAP pathways, which also leads to changes in gene expression. SOCS3 is a feedback inhibitor, which is induced transcriptionally by STAT3 and which binds to the membrane-proximal phosphorylated tyrosine residue leading to inhibition of JAK1 activity. Akt, serine/threonine kinase involved in many cellular processes; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; PI3K, phosphoinositide 3-kinase; Raf, serine/threonine kinase involved in many cellular processes; RAS, small G-protein; SOCS, suppressor of cytokine signaling; Src, proto-oncogene tyrosine-protein kinase; STAT, signal transducer and activator of transcription; YAP, proto-oncogene yes associated protein; Yes, cellular homolog of the Yamaguchi sarcoma virus oncogene

define cellular reactions, which depend on the presence of the sIL-6R. Using Hyper-IL-6, we could demonstrate that regeneration of the liver and survival of neurons is largely accelerated via the IL-6 trans-signaling mechanism (Scheller et al. 2011b). The soluble gp130Fc protein (sgp130Fc) is a fusion of the entire extracellular portion of gp130 with the Fc portion of human IgG1 antibody (Fig. 3c). This protein has the interesting property that it exclusively blocks IL-6 trans-signaling without affecting classic signaling via the membrane-bound IL-6R (Jostock et al. 2001). Thus, the sgp130Fc protein can be used as a molecular tool to dissect between IL-6



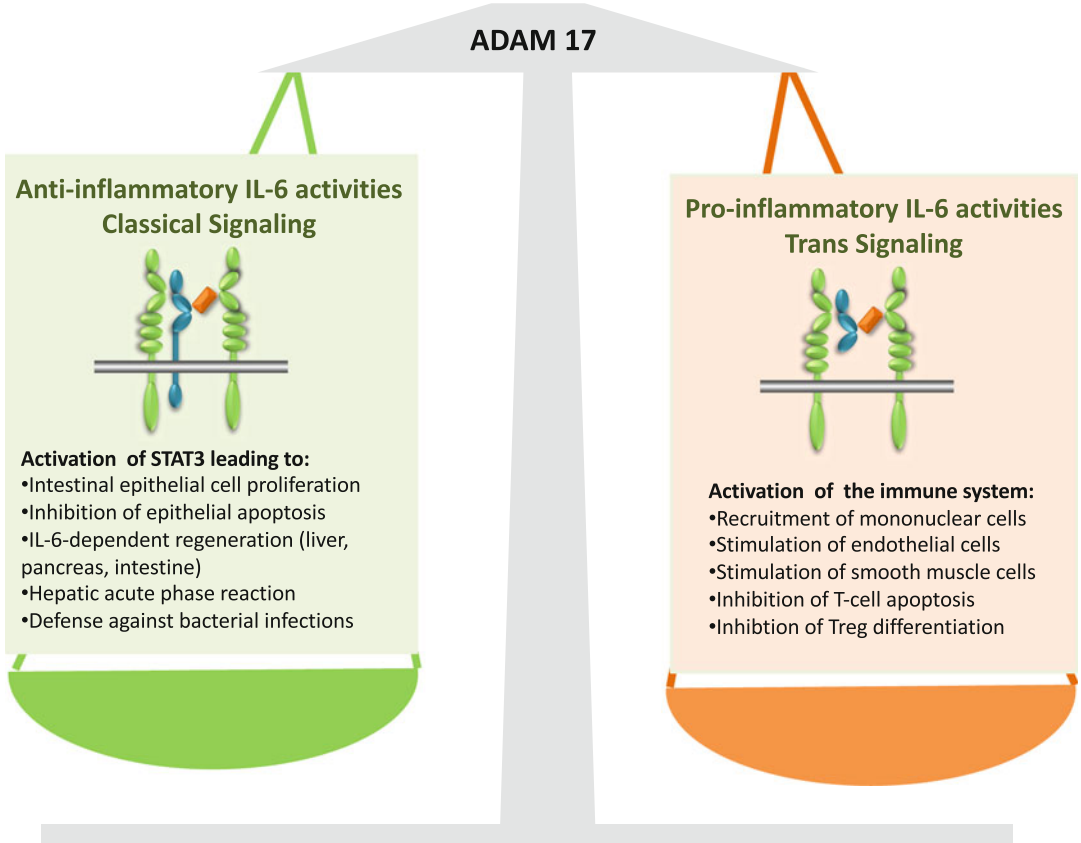
Interleukin-6, Fig. 3 Classic signaling and trans-signaling of IL-6. (**a**) Left, in classic signaling, IL-6 binds to the IL-6R which subsequently associates with gp130 that initiates signaling. Right, the protease ADAM17 cleaves the IL-6R close to the cell membrane, thereby releasing sIL-6R. The sIL-6R can still bind IL-6 and the complex of IL-6/sIL-6R complex can bind to and activate gp130 on cells, which do not express IL-6R leading to gp130 signaling. (**b**) The designer cytokine Hyper-IL-6 is a fusion protein of IL-6 and sIL-6R, which can mimic IL-6 trans-signaling in vitro and in vivo. (**c**) The sgp130Fc protein consists of the extracellular portion of gp130 fused to the constant region of human IgG1. The sgp130Fc protein specifically blocks IL-6 trans-signaling without affecting IL-6 classic signaling. ADAM, a disintegrin and metalloprotease; sIL-6R, soluble IL-6R

classic- and trans-signaling (Scheller et al. 2011b).

When the IL-6 cDNA was cloned in the group of Kishimoto it became clear that several biologic activities, which were analyzed by groups around the world, including B-cell stimulatory factor, hepatocyte stimulatory factor, hybridoma-plasmacytoma growth factor, and Interferon β2 were identical with IL-6, underlining the pleiotrophic activity of this cytokine (Rose-John 2018). In the meantime it was found that many physiologic functions in the body and within the immune system are regulated by IL-6 and that the coordination of the innate and acquired immune system is mediated by this cytokine (see reviews (Hunter and Jones 2015; Garbers et al. 2018; Jones and Jenkins 2018; Kang et al. 2019)).

Pharmacological Relevance

Neutralizing antibodies directed against IL-6 or IL-6R block both classic- and trans-signaling whereas the sgp130Fc protein only blocks trans-signaling. A systematic comparison of IL-6 or IL-6R neutralizing antibodies and sgp130Fc protein was performed in mouse models of human diseases to define the nature of classic- and trans-



Interleukin-6, Fig. 4 Pro- and anti-inflammatory activities of IL-6. Anti-inflammatory activities of IL-6 are mediated by classic signaling via the membrane-bound IL-6R (left) whereas pro-inflammatory activities of IL-6 are mediated by IL-6 trans-signaling via the soluble IL-6R (right). The balance between classic- and trans-signaling is orchestrated by the membrane-bound metalloprotease ADAM17

signaling in vivo. As depicted in Fig. 4, it turned out that IL-6 classic signaling mainly consists of protective, regenerative, and anti-inflammatory responses whereas IL-6 trans-signaling is rather pro-inflammatory (Garbers et al. 2018; Kang et al. 2019).

IL-6 plasma levels in normal, healthy individuals are in the 1–5 pg/ml range. In contrast, levels of sIL-6R are about 40–80 ng/ml. Moreover, levels of sgp130 in normal individuals are in the range of 400 ng/ml. Since the affinity of IL-6 to the IL-6R is in the range of 1 nM whereas the affinity of the IL-6/IL-6R to gp130 is about 100 times higher, that is, in the range of 10 pM, IL-6 secreted into the plasma will bind to sIL-6R and the complex will immediately bind to sgp130 and be neutralized. Therefore, we have hypothesized that sIL-6R and sgp130 in the plasma form a biologic buffer for IL-6 and that the capacity of the buffer is determined by the concentration of the sIL-6R (Garbers et al. 2015). Interestingly, a single nucleotide polymorphism (rs7529229) has been identified, which leads to the change of aspartate at position 358 to alanine. It was demonstrated that the IL-6R protein with alanine at position 358 is cleaved more efficiently by the protease ADAM17, and therefore, individuals with this mutation show about twofold higher sIL-6R levels in the blood as compared to individuals with IL-6R with aspartate at position 358 (Garbers et al. 2014).

Interestingly, it has been shown that that individuals carrying the SNP rs7529229 in the IL-6R gene are protected from coronary heart disease, rheumatoid arthritis, atrial fibrillation, and abdominal aortic aneurysm (Interleukin-6 receptor pathways in coronary heart disease: a collaborative meta-analysis of 82 studies, 2012; The interleukin-6 receptor as a target for prevention of coronary heart disease: a Mendelian randomization analysis, 2012; Ferreira et al. 2013). These data demonstrated that the steady-state concentration of sIL-6R and hence the capacity of the biologic buffer for IL-6 is important for the susceptibility of inflammatory diseases (Garbers et al. 2018).

Pathophysiological and Therapeutic Relevance

We systematically compared the neutralizing activity of antibodies directed to IL-6 or IL-6R with the more specific activity of sgp130Fc, which only blocks IL-6 trans-signaling but not IL-6 classic signaling via the membrane-bound IL-6R (Garbers et al. 2018). These analyses revealed that IL-6 activities via the membrane-bound and via the soluble IL-6R were fundamentally different. IL-6 responses via the membrane-bound were protective or regenerative. We could demonstrate that the regeneration of intestinal epithelial cells was dependent on classic IL-6 signaling (Grivennikov et al. 2009). Furthermore, global blockade of IL-6 activities with the help of neutralizing IL-6 antibodies or the use of IL-$6^{-/-}$ mice led to a decline in the defense against bacteria such as *Listeria monocytogenes* or *Mycobacteria tuberculosis* (Sodenkamp et al. 2012; Hoge et al. 2013). In contrast, specific blockade of IL-6 trans-signaling by the sgp130Fc protein or in sgp130Fc expressing transgenic mice did compromise the control of bacterial infection. Likewise, in a mouse model of abdominal aortic aneurysm, global IL-6 blockade aggravated whereas specific blockade of IL-6 trans-signaling improved the condition of the animals (Paige et al. 2019). On the other hand, selective blockade of IL-6 trans-signaling by the sgp130Fc protein led to significant improvement in many mouse models of human inflammatory diseases and cancer (Table 1). The different activities of IL-6 are depicted in Fig. 4, where it is shown that IL-6 responses via the membrane-bound IL-6R are anti-inflammatory whereas IL-6 activities executed by trans-signaling via the sIL-6R are rather pro-inflammatory and might also reflect stress situations of the immune system (Garbers et al. 2018; Kang et al. 2019). As depicted in Fig. 4, the result of our systematic analysis was that IL-6 classic signaling is predominantly anti-inflammatory and protective whereas IL-6 trans-signaling is rather pro-inflammatory and plays a prominent role in the induction of many neoplastic diseases (Garbers et al. 2018). Interestingly, the balance between these two IL-6 activities is

Interleukin-6, Table 1 Efficacy of specific blockade of IL-6 trans-signaling by the sgp130Fc protein in animal models of inflammation

Disease model	Study outcome	Reference
Intestinal inflammation	Suppression of inflammatory bowel disease	(Atreya et al. 2000)
Atherosclerosis	Regression of atherosclerosis plaques	(Schuett et al. 2012)
Rheumatoid arthritis	Improvement of established arthritis	(Nowell et al. 2009)
Sepsis	Up to 100% survival in sepsis models	(Barkhausen et al. 2011)
Pancreatitis-lung failure	100% survival of severe, acute, experimental pancreatitis	(Zhang et al. 2013)
Lung emphysema	Improvement of disease by blockade of alveolar cellular apoptosis	(Ruwanpura et al. 2016)
Abdominal aortic aneurism	Improved survival in two animal models	(Paige et al. 2019)

apparently governed by the activity of the membrane-bound metalloprotease ADAM17, which is responsible for the generation of the sIL-6R (Scheller et al. 2011a). Importantly, it turned out that ADAM17 not only orchestrates the IL-6 pathway but also signaling via TNFα and ligands of the EGF-R (Zunke and Rose-John 2017). TNFα and all ligands of the EGF-R are transmembrane proteins, which need to be cleaved in order to be systemically active (Scheller et al. 2011a). It has for instance been shown that in the absence of ADAM17, activation of the EGF-R and subsequent milk duct formation in female mice is severely compromised (Chalaris et al. 2010).

In diagnostic terms, the acute phase response of the liver in response to microbial stimuli such as bacteria or viruses leading to the strong induction of C-reactive-protein (CRP) was a hallmark of the biologic activity of IL-6 in inflammatory situations (Baumann and Gauldie 1994). In the last decade, the scientific interest in IL-6 biology was strongly rekindled by two novel insights.

First, about 10 years ago it was demonstrated by three groups that IL-6-induced STAT3 activation in combination with TGFβ was essential for the differentiation of so-called TH_{17} cells whereas TGFβ alone induced the differentiation of regulatory T cells or T_{regs}. These results clearly demonstrated that besides its important role in the innate immune response, IL-6 plays a crucial role in the orchestration of the innate and acquired immune system (reviewed in (Garbers et al. 2018)).

Secondly, TNFα neutralization of autoimmune diseases such as rheumatoid arthritis and inflammatory bowel disease in the 1990s was a historical breakthrough in the treatment of these debilitating disorders (Aggarwal et al. 2012). In the 2010s, a neutralizing monoclonal antibody (Tocilizumab) directed against the human IL-6R for treatment of rheumatoid arthritis and other autoimmune diseases such as systemic juvenile idiopathic arthritis was approved by the regulatory agencies in Japan, the USA, and Europe. This was followed by the approval of an additional neutralizing antibody (Sarilumab) against the human IL-6R in 2017 (Garbers et al. 2018; Kang et al. 2019). Remarkably, these IL-6R neutralizing antibodies outperformed the TNFα neutralizing antibody adalimumab when given as monotherapy (Gabay et al. 2013; Burmester et al. 2017), indicating that blockade of IL-6 biologic activity is an extremely promising strategy in the treatment of autoimmune diseases (Jones et al. 2011; Garbers et al. 2018; Kang et al. 2019).

Outlook

The sgp130Fc protein under the name Olamkicept is currently in phase II clinical trials in Germany and Asia in patients with inflammatory bowel disease. This can be seen as a proof of the concept that the specific blockade of IL-6 trans-signaling without affecting classic signaling via the membrane-bound IL-6R not only efficiently inhibits the progression of the inflammatory state in an autoimmune disease but that the IL-6 activities mediated by classic IL-6 signaling indeed govern regenerative and protective functions, which are important for the functioning of the human immune system and which are not blocked when IL-6 trans-signaling is specifically blocked (Hunter and Jones 2015; Garbers et al. 2018; Jones and Jenkins 2018).

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Interleukins

► Cytokines

Inward Rectifier Potassium Channels

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Synonyms

Anomalous rectifiers; Inward rectifiers; Inwardly rectifying potassium channels; Kir channels

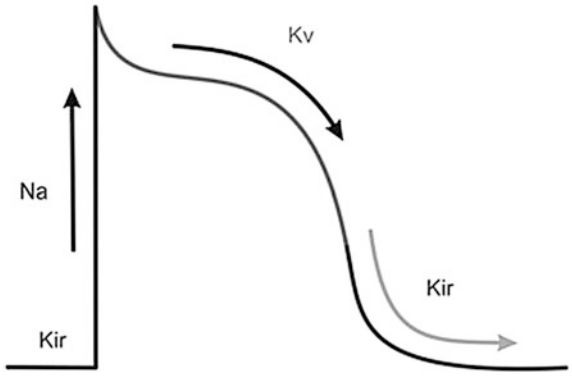
Definitions

Inward Rectifier Potassium Channels or Kir Channels: a class of potassium channels generated by tetrameric arrangement of one-pore/two-transmembrane helix (1P/2TM) protein subunits, often associated with additional beta subunits. Kir channels modulate cell excitability, being involved in repolarization of action potentials (Fig. 1), setting the resting potential (Fig. 1) of the cell, and contributing to potassium homeostasis.

Inward Rectification: decreased conductance upon depolarization. In classical inward rectifiers, rectification is "strong," and currents decline steeply at voltages positive to the equilibrium potential for K^+ (Fig. 2). In other Kir channels, rectification is "weak," and currents decline only gradually at voltages positive to the equilibrium potential for K^+(Fig. 2).

Molecular Basis and Functional Diversity of Kir Channels

Seven subfamilies of eukaryotic Kir channels (Fig. 3), each sharing ~60% amino acid identity between individual members within each subfamily and ~ 40% identity between subfamilies, are



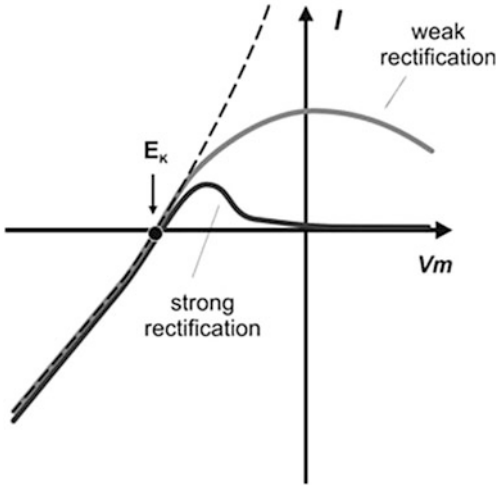
Inward Rectifier Potassium Channels, Fig. 1 The role of inward rectifier (Kir) channels in cardiac action potentials. Depolarization is generated and maintained by Na and Ca currents (I_{Na}, I_{Ca}). Voltage-gated K currents (K_v) and currents through Kir channels contribute to repolarization and maintenance of a negative resting potential

known (Hibino et al. 2010). In addition, multiple prokaryotic Kir channels (Kirbac 1.x-8.x) have been identified in bacterial genomes. We will focus on the eukaryotic channels.

Kir1 subfamily. Kir1.1 (ROMK1, gene *KCNJ1*) (Hibino et al. 2010) encodes a "weak" inward rectifier and is expressed predominantly in the kidney. Alternate splicing at the 5′ end also generates multiple Kir1.1 splice variants, Kir1.1a (ROMK2) through Kir1.1f (ROMK6), expressed in various tissues, including the kidney, brain, heart, liver, pancreas, and skeletal muscle. In the kidney the renal Kir1.1 channels control salt reabsorption.

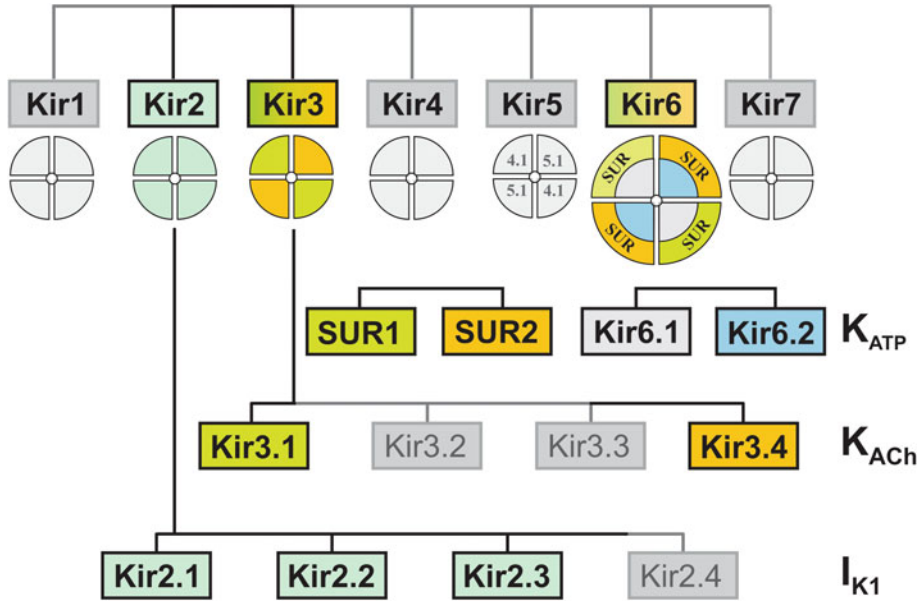
Kir2 subfamily. Six distinct Kir2 subfamily members (Kir2.1–Kir2.6; *KCNJ2*, *KCNJ12*, *KCNJ4*, *KCNJ14, KCNJ17, KCNJ18*) (Hibino et al. 2010), all encoding classical "strong" inward rectifiers, differ in single-channel conductance and in sensitivity to phosphorylation and other second messengers. Members of the Kir2 subfamily are highly expressed in muscles of all types but are also found in a number of other tissues (e.g., nervous system). Kir2 subunits are the key players in the cardiac inward rectifier current I_{K1} in atrial and ventricular myocytes. Kir2.1 is a predominant isoform in all species, while Kir2.2 and Kir2.3 may also contribute. Kir2.4 is believed to be restricted to neuronal cells within cardiac tissue.

Kir3 subfamily. Four members of the Kir3 subfamily (Kir3.1–Kir3.4: *KCNJ3*, *KCNJ6*, *KCNJ9*, *KCNJ5*) express G-protein-activated



Inward Rectifier Potassium Channels, Fig. 2 High $[K^+]$ inside cells relative to outside results in "normal" (outward-going) rectification (dashed line), whereby outward (positive by convention) potassium currents (I), when cells are depolarized (V_m is positive relative to equilibrium potential for K^+, E_K), are bigger than inward (negative) currents at membrane potentials (V_m) more negative than E_K. Conversely, inward (or "anomalous") rectifiers show "strong" or "weak" inward rectification whereby outward currents are smaller than inward currents

"strong" inward rectifier K^+ channels (GIRK channels) underlying G-protein-coupled receptor-activated currents in heart, brain, and endocrine tissues. Functional channels typically require co-assembly of two different subunits (e.g., Kir3.1 and Kir3.4 in atrial channels). Several studies have provided evidence for a



Inward Rectifier Potassium Channels, Fig. 3 The family of inward rectifier potassium channels. Kir channels may be homo- or hetero-tetrameric complexes of individual subunits, in some cases in tight association with beta-subunits (e.g., as in the K_{ATP} channel). SUR – sulfonylurea receptor. Modified from Anumonwo and Lopatin (2010)

promiscuous assembly between the various members of the Kir3 subfamily.

Kir4 and Kir5 subfamilies. These subfamilies of Kir channels (Kir4.1 and Kir4.2, *KCNJ10*, *KCNJ15*, Kir5.1, *KCNJ16*) are abundantly expressed in the brain, glia, and kidney. Kir4.1 forms "weak" inward rectifier K^+ channels when expressed alone. Kir5.1 does not form homomeric channels, but co-expression of Kir4.1 and Kir5.1 subunits results in formation of channels with novel properties: Kir5.1/Kir4.1 heteromeric channels are very sensitive to intracellular pH, and this property is conferred predominantly by the Kir5.1 subunits.

Kir6 subfamily. Two members (Kir6.1, Kir6.2, *KCNJ8*, *KCNJ11*) of this subfamily encode the pore-forming subunits of ATP-sensitive K^+ channels (K_{ATP}) that are found in most excitable tissues. Functional expression of channels that are inhibited by intracellular ATP and activated by ADP, thereby coupling cell metabolism to excitability (Nichols 2006), requires octameric assembly of four Kir6.x subunits with four sulfonylurea receptor subunits (SUR1 or SUR2) (Martin et al. 2017). K_{ATP} channels display "weak" rectification, allowing substantial outward current to flow at positive potentials, thus causing action potential shortening and reduced excitability when activated, for example, during metabolic inhibition.

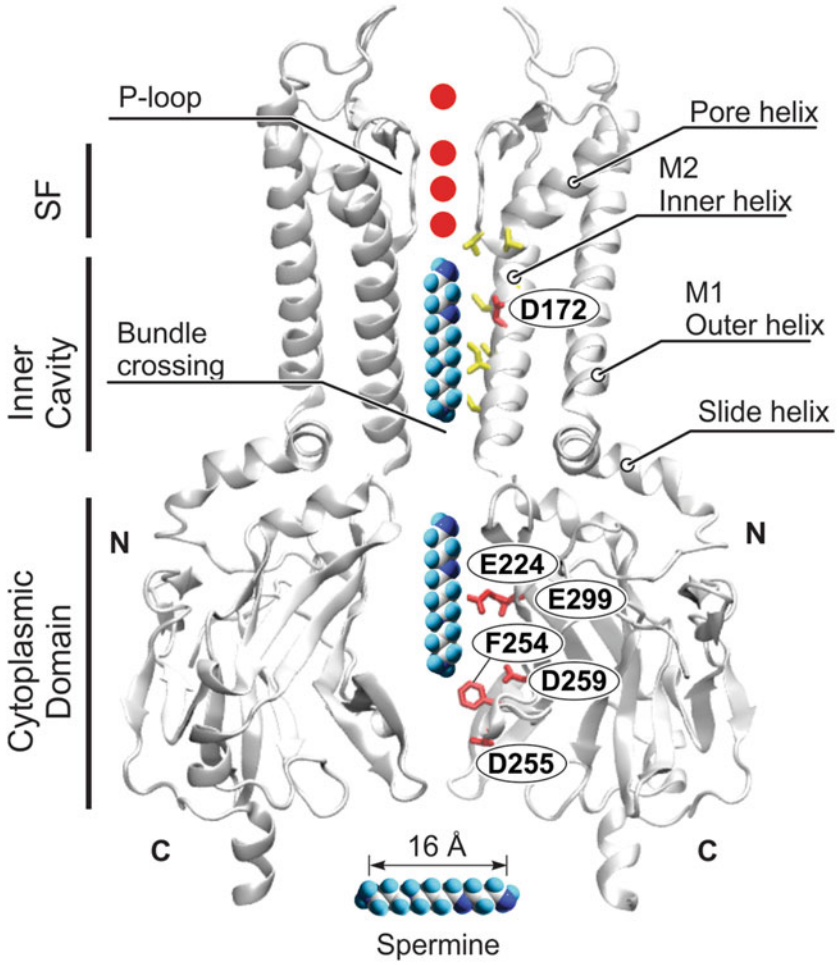
Kir7 subfamily. The only known member (Kir7.1, *KCNJ13*) is primarily expressed in the retinal pigment epithelium in the brain but is also found in a variety of other tissues, including the kidney and intestine. This weakly rectifying channel has an apparently very low single-channel conductance and very shallow dependence on external K^+ and is virtually insensitive to intracellular Mg^{2+}. The physiological role of this channel remains unclear.

Molecular Structure of Kir Channels

All Kir channels are tetrameric proteins (Fig. 3) of one-pore/two-transmembrane (1P/2TM) domain subunits which generate highly selective K^+ channels. Most Kir channels can be assembled as functional homotetramers, while some require heteromeric assembly (Fig. 3). For example,

functional GIRK channels underlying I_{KAch} (acetylcholine-activated) current in atria are obligate heteromers of two members of Kir3 subfamily: Kir3.1 and Kir3.4.

A feature of all K^+-selective channels is the signature G(or F)YG sequence within the P-loop (Fig. 4) that acts as a filter, to confer high selectivity to K^+ ions and to control single-channel conductance and kinetics. Two transmembrane domains with N- and C-termini facing the cytoplasm flank the P-loop (Fig. 4). The assembly of the Kir2.1 channel is supported by the presence of intrasubunit disulfide bonds between highly conserved cysteine residues at the outer surface of the channel which are absolutely required for proper channel folding. Scanning cysteine mutagenesis studies of relatively large stretches of N- and C-termini of Kir2.1 reveal that nearly half of them



Inward Rectifier Potassium Channels, Fig. 4 Kir channel subunits consist of two transmembrane helices (M1, M2), separated by a pore loop (P-loop) that contains the signature K^+-selectivity sequence (-GYG-), as well as a characteristic "Kir domain" formed by the extended cytoplasmic N- and C-termini that generates a long cytoplasmic extension to the pore. Only two (opposing) subunits are shown for clarity (transmembrane regions from a and c subunits, cytoplasmic Kir domains from b and d subunits). Several residues implicated in causing rectification are indicated (see text). Spermine may block the channel in the inner cavity and entrance to the selectivity filter (upper), and in the wider cytoplasmic vestibule (lower), potentially occupying both sites simultaneously. (●) K^+ ions. Structural data: Protein Data Bank code 5KUM; Kir2.2 channel at 2.8 Å resolution. Modified from Nichols and Lee (2018)

are water accessible, and potentially facing the pore, in addition to residues in the second transmembrane region. Crystal structures of eukaryotic Kir2.2 and Kir3.2 channels have been resolved, revealing the canonical K^+ channel pore structure (Hansen et al. 2011) in which the cytoplasmic "Kir" domain generates an additional wide cytoplasmic intracellular vestibule, large enough to accept multiple permeating and blocking ions. Multiple studies suggest that the Kir domains undergo subtle structural changes to open or close the channel via "gate" at the M2 helix bundle crossing point which generates the entrance to the inner cavity from the cytoplasmic vestibule.

The Mechanism of Strong Inward Rectification

Inward, or "anomalous," rectification of potassium permeability refers to increased potassium conductance under hyperpolarization and decrease under depolarization (Fig. 2), the effect being opposite to that of "normal" outward rectification resulting from physiological intracellular and extracellular [K^+]. It should be noted that not all Kir channels rectify strongly enough to fit the definition of classical rectification, such as observed in cardiac I_{K1} channels. In this regard, classical inward rectification is so strong that only small currents can be measured in the outward direction at voltages positive to the K^+ reversal potential (E_K), while large inward currents can be easily observed negative to it. This "strong" voltage-dependent rectification also depends on the concentration of external K^+ ([K_{OUT}]): increasing K_{OUT} relieves the rectification, so that the midpoint voltage of rectification shifts nearly perfectly with corresponding change in E_K. It is now established that strong inward rectification results primarily from voltage-dependent block by naturally occurring intracellular organic cations called polyamines (Hibino et al. 2010). Of the polyamines, spermine (Fig. 4) and spermidine are the most potent inducers of rectification, and micromolar concentrations of free spermine and spermidine are sufficient to reproduce the degree of rectification seen in native cells (Hibino et al. 2010), although putrescine and Mg^{2+} ions are also relevant. Importantly, total polyamine levels in cells are typically quite high (up to 10 mM), although most polyamines are bound to various intracellular targets such as RNA, DNA, ATP, and other, primarily phosphate-containing, molecules.

The varying degree of rectification between members of the Kir superfamily is fundamental to their respective functional roles. Kir2 and Kir3 encode classical "strong" inward rectifier channels, while other members encode channels with variably "weaker" rectification (Fig. 2). For example, because of weak rectification of the K_{ATP} channel, its activation can cause considerable shortening of the cardiac action potential, reducing entry of Ca^{2+} through voltage-dependent Ca^{2+} channels and hence conserving ATP under conditions of metabolic stress. Conversely, the strong inward rectification of Kir2.1 channels underlying the cardiac I_{K1} current results in very small currents flowing through these channels during the long depolarization phase of the action potential, while increased conductance once the cell hyperpolarizes makes the resting potential very stable, a combination that is essential to the normal pump cycle of the heart.

Structural Elements of Inward Rectification

Each Kir subunit consists of two transmembrane helices (M1, M2), with a pore-forming selectivity filter linking them, and cytoplasmic N- and C-termini (Hansen et al. 2011) (Fig. 4). The channel is formed as a tetrameric arrangement of these subunits, surrounding an external selectivity filter, an inner vestibule, and a cytoplasmic entrance to the pore (Fig. 4). Aspartate 172 located in the M2 region of Kir2.1 was the first residue implicated in the classical rectification of these channels and is sometimes referred to as the "rectification controller." A number of other residues in the C-terminus of Kir2.1 (e.g., E224, F254, D255, D259, and E299) also contribute to polyamine-induced rectification; neutralization of a few negatively

charged amino acids (D172, E224, and E299) can transform the strongly rectifying Kir2.1 channel into one that is nearly insensitive to blockage by polyamines and Mg^{2+}. The exact location(s) of the binding sites for polyamines remains controversial. Probably one spermine molecule is required to maximally block the channel, within the inner vestibule, or perhaps within the selectivity filter itself, and the charge movement through the electric field that underlies the voltage dependence of the block may arise from K^+ ions displaced from within the channel pore to the outside of the cell, or from movement of the spermine itself into the field within the selectivity filter, or a combination of both. Despite the relatively large size of the spermine molecule (~16 Å long) (Fig. 4), its diameter is close to that of a dehydrated K^+ ion, thus potentially allowing its head amine group to "squeeze" into the selectivity filter and block ion flow. Polyamines may also permeate Kir channels and can clearly permeate, as well as block, other nonselective cationic channels, consistent with such an interpretation.

Pharmacology of Kir Channels

Voltage-dependent block by external Ba^{2+} and Cs^+ ions, yet insensitivity to the Kv channel blocker tetraethylammonium (TEA), have been the classical tools to examine Kir channel activity. The honeybee venom tertiapin has been found to be an effective blocker of certain (Kir1, Kir3) inward rectifier subfamily members, and a spider toxin SpTx-1 is a potent blocker of Kir6.2 channels. Tight association of Kir6 family members with SUR subunits endows K_{ATP} channels with a rich pharmacology: channel activity is very specifically inhibited by sulfonylurea drugs such as tolbutamide and glibenclamide and is enhanced by a broad class of "potassium channel opening" (KCO) drugs such as pinacidil and diazoxide, all of which interact with the SUR subunits.

A potent antiarrhythmic drug RP58866 and its active enantiomer RP62719 (known as Terikalant) have been shown to block I_{KACh} (encoded by Kir3.x members) and I_{K1} (encoded by Kir2.x members) currents in the heart in the low micromolar range. Unfortunately, neither agent discriminates well between Kir and other K channels (e.g., underlying I_{Kr}) and thus has not received much attention as a selective blocker for Kir channels.

Kir channel-specific pharmacological tools also include chloroquine, mefloquine, tamoxifen, and quinacrine which inhibit Kir channels underlying cardiac I_{K1}, I_{KATP}, I_{KACh} currents. Chloroquine and quinacrine as well as pentamidine were also shown to effectively inhibit Kir4.1 channels with a number of serotonin reuptake inhibitors such as sertraline, fluoxetine, and fluvoxamine adding more to relatively rich pharmacology of this channel. An excellent review of Kir channel modulators can be found in Bhave et al. (2010).

Diseases Resulting from Kir Channel Mutations

Multiple channelopathies resulting from mutations in Kir channels are now recognized.

Kir1.1 Bartter syndrome. Several mutations in the core region as well as in the N- and C- termini of Kir1.1 are found in patients with hyperprostaglandin E syndrome (HPS; renal disorder resulting from impairment of tubular reabsorption), an antenatal form of Bartter syndrome. Some of these mutations result in the loss of function of Kir1.1 channels causing impaired renal K^+ secretion and NaCl reabsorption.

Kir2.1 Channelopathies. Dominantly inherited long QT syndrome (LQT), a disorder of cardiac action potential repolarization, is most commonly associated with mutations in cardiac Na^+ or voltage-gated K^+ channels, but multiple mutations in *KCNJ2* cause Andersen's syndrome (LQT7), a rare disease characterized by periodic paralysis, cardiac arrhythmias, and dysmorphic features. Mutations in *KCNJ2* associated with Andersen's syndrome were found to cause dominant negative suppression of the wild-type Kir2.1 channels when expressed in *Xenopus* oocytes or cultured cell lines, thus mimicking the effects of the *KCNJ2* gene knockout which is characterized

by prolonged QT interval. Alternatively, a gain-of-function mutation in the "rectification controller" of Kir2.1 channel (D172N mutation) causes a form of short QT syndrome (SQT3), which is characterized by a unique short QT interval on the ECG. Another gain-of-function mutation in human *Kir2.1* gene (V93I) was also found to underlie a familial atrial fibrillation. A number of mutations in *KCNJ2* gene linked to catecholaminergic polymorphic ventricular tachycardia (CPVT) have also been identified.

Kir3.2 Keppen-Lubinsky syndrome. A mutant mouse (the Weaver mouse) with cerebellar degeneration and motor dysfunction resulting from a serine for glycine substitution in the -GYG- sequence of the K^+ selectivity filter of Kir3.2 leads to loss of the K-selectivity of cerebellar G-protein-activated K^+ channels, a consequent Ca^{2+} overload, and cell death. Mutations in the same region of human Kir3.2, resulting in loss of K^+ selectivity, have been identified in Keppen-Lubinsky syndrome, a rare disease characterized by developmental delay and intellectual disability, facial dysmorphology, and severe movement disorder.

Kir4.1 SeSAME/EAST syndrome. Kir4.1 is critical for glial function, control of neuronal excitability, and systemic K^+ homeostasis. Homozygous or compound heterozygous loss-of-function Kir4.1 mutations are associated with EAST/SeSAME syndrome, characterized by mental retardation, epilepsy, ataxia, seizures, hearing loss, and renal salt wasting (Sala-Rabanal et al. 2010).

Kir6.2/SUR1 Persistent hyperinsulinemic hypoglycemia of infancy (PHHI) and neonatal diabetes. Lowered blood glucose normally results in decreased ATP/ADP ratios in the pancreatic islet β-cells, triggering the opening of K_{ATP} channels, hyperpolarization, inhibition of Ca^{2+} entry, and cessation of insulin secretion. In PHHI, K_{ATP} channel mutations lead to abolition of activity and hence maintained depolarization and maintained Ca^{2+} entry and insulin secretion. Many mutations in the SUR subunit abolish ADP activation of channels, but point mutations in Kir6.2 are implicated in abolition of channel activity in some cases. Conversely, K_{ATP} channel mutations that lead to increased activity and hence maintained hyperpolarization and failure of Ca^{2+} entry block insulin secretion and cause neonatal diabetes. Many mutations in the Kir6.2 and SUR subunits have now been identified, and this has led to a change in therapy for the disease, from injected insulin to sulfonylurea pills (Nichols 2006).

Kir6.1/SUR2 Cantu syndrome. Kir6.1 and SUR2 subunits make up the K_{ATP} channels of smooth muscle. Cantu syndrome results from gain-of-function mutations that generally reduce the sensitivity to inhibition by ATP, in either subunit. This causes reduced smooth muscle electrical activity and reduced Ca^{2+} entry and hence vessel dilation and in turn leads to lymphedema and vasodilation, with secondary consequences of enlarged hearts, and persistent fetal blood flow. In addition, all Cantu syndrome patients have excess hair growth, explaining the hair growth-promoting effects of the K_{ATP} channel openers diazoxide and minoxidil (sold commercially as Rogaine©) (Nichols et al. 2013).

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Inward Rectifiers

► Inward Rectifier Potassium Channels

Inwardly Rectifying Potassium Channels

► Inward Rectifier Potassium Channels

Islet Amyloid Polypeptide

► Amylin

Ixazomib (Ninlaro)

► Proteasome Inhibitors

J

JAK Inhibitors

► Rheumatoid Arthritis

JAK-STAT Pathway

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Definition

The *Ja*nus *k*inase-*s*ignal *t*ransducer and *a*ctivator of *t*ranscription (JAK-STAT) signaling pathway is activated in response to more than 50 cytokines, hormones, and growth factors. As their name implies, the STAT proteins exhibit the dual function of transducing signals from the cell surface into the nucleus as well as activating transcription of target genes, thus converting extracellular stimuli into a wide range of cellular responses. STATs have been identified as important regulators of a multitude of cellular processes, such as immune response, antiviral protection, and proliferation.

Basic Characteristics

The JAK-STAT pathway is widely used by members of the cytokine receptor superfamily. Upon ligand binding and oligomerization of the cognate receptor chains, the receptor-associated JAKs themselves become tyrosine phosphorylated and consecutively phosphorylate critical tyrosine residues on the cytoplasmic domain of the receptors, thereby generating docking sites for STAT proteins and other intracellular signaling molecules (Levy and Darnell 2002). The STATs generally are recruited to specific phosphotyrosine-containing motifs located in the cytoplasmic part of the receptor through their Src-homology-2 (SH2) domain and in turn are phosphorylated by the activated JAKs at a single tyrosine residue in their C-terminus (Stark and Darnell 2012). Tyrosine phosphorylation, also called "STAT activation," induces a conformational change in the STAT molecules, which exist as preformed dimers (M_r ~ 180 kDa). Crystallographic and biochemical data indicate that the unphosphorylated STAT1 dimer is formed by mutual interactions between N-domains and the coiled-coil domain of one protomer and the DNA-binding domain of the other, a conformation referred to as "antiparallel." By virtue of reciprocal phosphotyrosine SH2 domain interactions, the activated dimer can adopt an equally stable "parallel" conformation. Phosphorylation-dependent conformation switching has important functional consequences. Unphosphorylated STAT dimers can shuttle in

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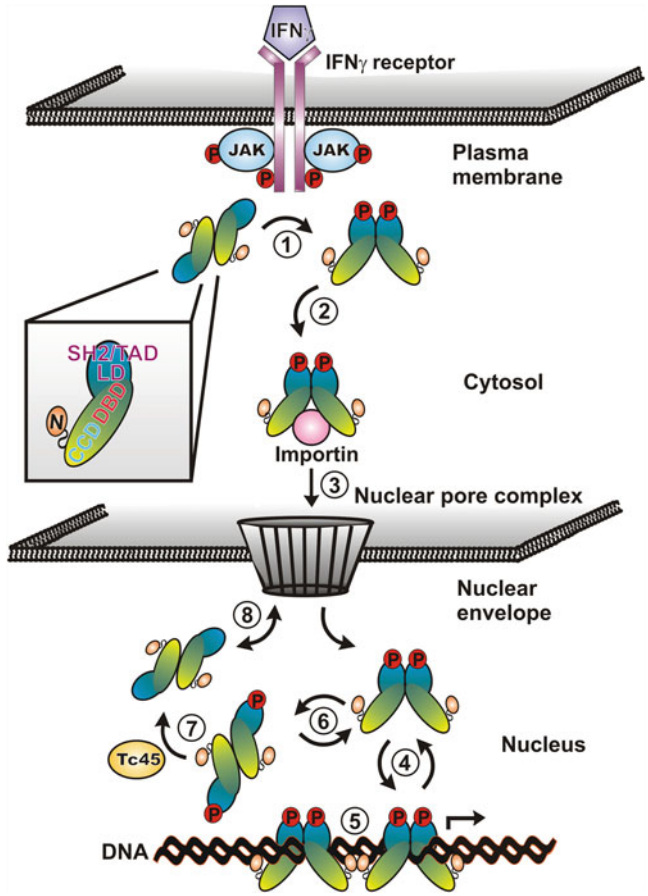
and out of the nucleus without assistance from carrier proteins, while the activated STATs are barred from nuclear export and require the canonical carrier-facilitated mechanism for nuclear import. The phosphorylated STATs are also capable of binding to palindromic gamma-activated site (GAS) elements with the consensus sequence 5′-TTC$(N)_{3-4}$GAA-′3 in the promoter regions of cytokine-responsive genes, where they function as cytokine-regulated transcription factors. STATs are susceptible to enzymatic tyrosine dephosphorylation, which is the main mechanism to inactivate their transcription activity (Fig. 1).

Physiological Functions of JAKs

Named after the two-faced ancient Roman god Janus, the mammalian JAK family of protein tyrosine kinases consists of four members. While



JAK-STAT Pathway, Fig. 1 Model of the JAK-STAT pathway, as exemplified by the founding member of the family of the STAT transcription factors, STAT1. The cartoon depicts the following steps in the activation/inactivation cycle of STAT1: JAK-induced STAT1 tyrosine phosphorylation and formation of parallel phospho-dimers (1), formation of an import-capable complex of phospho-STAT1 with importin (2) including its nuclear import (3), sequence-specific binding to a GAS site on DNA (4), polymerization on DNA through interdimeric amino-terminal interactions resulting in cooperative DNA binding as a requirement for full-fledged gene induction (5), tyrosine dephosphorylation of STAT1 catalyzed by the Tc45 phosphatase after its dissociation from DNA and switching to an antiparallel dimer conformation (6), followed by nuclear translocation of the dephosphorylated STAT1 (7) through the nuclear pore complex (8). The inlet depicts the domain structure of a STAT1 monomer with the amino-terminal domain (N) and the core domain which are connected by a flexible linker. The core domain is comprised of the coiled-coil domain (CCD), the DNA-binding domain (DBD), the linker domain (LD), and the SH2 domain, followed carboxy-terminally by the transactivation domain (TAD)

JAK1, JAK2, and TYK2 are being ubiquitously expressed, the expression of JAK3 is mostly restricted to cells of hematopoietic origin (Babon et al. 2014). These multi-domain proteins have a molecular mass of approximately 130 kDa and consist of an N-terminal FERM-domain, a SH2-like domain, a pseudokinase domain (JAK homology 2, JH2), and, the catalytically active, signaling PTK domain (JH1). The essential role of JAKs in mediating signal transduction via members of the cytokine receptor superfamily became apparent from studies of knockout mice. Targeted disruption of the mouse JAK1 gene results in perinatal lethality, obviously caused by defective neural function, and altered lymphoid development. JAK2-deficient mice exhibited an embryonic lethal phenotype caused by a block in definite erythropoiesis but showed intact lymphoid development, demonstrating the obligatory and non-redundant roles of JAKs in cytokine-induced biological responses. Mutant mice lacking JAK3 are viable but display severe defects in both cellular and humoral immune responses with profound reduction in mature B and T cells, resembling the clinical symptoms of patients suffering from an autosomal recessive form of severe combined immunodeficiency, in which inactivating mutations in the JAK3 gene have been identified (Hammarén et al. 2019).

Roles of STAT Proteins in Gene Induction

In Mammalia, seven different members have been identified, all of which are activated by a distinct set of cytokines. Diversity in signaling is provided by variants of STAT proteins derived from either alternative splicing of RNA transcripts or proteolytic processing (e.g., STATs 1, 3, 4, and 5) and the ability of certain STATs to form both homodimers and heterodimers with each other. For example, STAT1 homodimers mediate type 2 interferon responses, while type 1 interferons require STAT1:STAT2 heterodimers. Major structural features in STAT proteins are the N-terminal region involved in cooperative DNA binding of multiple STAT dimers, the central DNA-binding domain, the dimerization region containing the SH2 domain and the site of tyrosine phosphorylation, and the C-terminal transcriptional transactivation domain. Phosphorylation of a critical serine residue within the transactivation domain is necessary for maximal transcriptional activity of some STAT family members.

STAT1 knockout mice exhibit selective signaling defects in their response to interferons, including an impaired expression of MHC class II, complement protein C3, the MHC class II transactivating protein CIITA, interferon regulatory factor 1, and guanylate-binding protein 1. In humans, loss-of-function mutations in the gene encoding STAT1 have been identified causing susceptibility to a range of infections, including severe viral and bacterial infections, such as non-tuberculous mycobacterial diseases (Mendelian susceptibility to mycobacterial disease, MSMD) (Olbrich and Freeman 2018). Autosomal dominant gain-of-function mutations in the *STAT1* gene characteristically result in chronic mucocutaneous candidiasis (CMC), likely caused by impaired IL-17 immunity (van de Verdonk et al. 2011). Almost all monogenic point mutations conferring CMC have been identified in regions of the *STAT1* gene coding for the coiled-coil domain and the DNA-binding domain, which are both engaged in the formation of the antiparallel dimer (Toubiana et al. 2016).

STAT3, activated through binding of IL-6, leptin, EGF, PDGF, LIF, or other ligands to their cognate receptors, appears to have important roles in preventing apoptosis and promoting proliferative processes. A deficiency in STAT3 causes embryonic lethality in mice, indicating the essential role of STAT3 in growth regulation, embryonic development, and organogenesis. Patients with STAT3 gain-of-function mutations have been identified, too (Fabre et al. 2019). They present with early-onset autoimmune and lymphoproliferative disease. Besides the role as a cytokine-inducible transcription factor in the nucleus, STAT3 elicits nonclassical functions in mitochondria by augmenting the activities of complex I and II of the electron transport chain. Furthermore, STAT3 directs self-renewal of pluripotent embryonic stem cells and induces pluripotent stem cells downstream of the LIF-receptor/gp130 complex.

STAT4 is activated in T cells in response to IL-12 and stimulates the development of TH1 cells. The two ubiquitously expressed STAT5 proteins (STAT5a and 5b) are encoded by distinct genes and share more than 90% sequence identity. They are activated by many growth stimulatory cytokines, including interleukins, GM-CSF, GH, prolactin, EGF, as well as erythropoietin, and exert critical roles in anti-apoptosis and proliferation. STAT6 functions in response to IL-4 and IL-13 signaling to induce TH2 cell development, CD23 and MHC class II expression, immunoglobulin class switching, and B- and T-cell proliferation.

Originally discovered as a DNA-binding protein that mediates interferon signaling, recent data demonstrated that STAT1 can also exert constitutive functions in the nucleus, which do not require STAT activation with tyrosine phosphorylation. Cells lacking STAT1 are resistant to apoptotic cell death induced by tumor necrosis factor α (TNFα) due to an inefficient expression of caspase genes, while reintroduction of STAT1 in these cells restores protease expression and sensitivity to apoptosis. For the transcription of certain target genes, a phosphorylation of the critical tyrosine residue 701 is not necessary, suggesting that unphosphorylated STAT1 can also bind to DNA. Recent data indicate that unphosphorylated STAT1 can either positively or negatively regulate the constitutive expression of a wide range of different genes.

Besides the cytokine receptors that lack intrinsic kinase activity but have associated JAK kinases, STAT proteins can be activated by a variety of G-protein-coupled receptors and growth factor receptors with intrinsic tyrosine kinase activity (e.g., EGF, PDGF, CSF-1, and angiotensin receptor). Increasing evidence suggests a critical role for STAT family members in oncogenesis and aberrant cell proliferation. Constitutively activated STATs have been found in many transformed cell lines and a wide variety of human tumor entities. Numerous non-receptor tyrosine kinases and viral oncoproteins, such as v-Src, v-Abl, v-Sis, and v-Eyk, have been identified to induce DNA-binding activity of STAT proteins.

Several mechanisms that negatively regulate the JAK-STAT pathway are known. The SOCS proteins (suppressor of cytokine signaling), also named as CIS (cytokine-inducible src homology 2-domain containing protein), JAB (JAK-binding protein), or SSI (STAT-induced STAT inhibitor), are induced by cytokine signaling and act in a negative feedback loop to inhibit JAK kinase activity. Another important negative regulatory mechanism involves the recruitment of tyrosine phosphatases containing tandem SH2 domains (SHP-1 and SHP-2) to the intracytoplasmic portion of receptor complexes, where they dephosphorylate and thus inhibit JAK activity. It was demonstrated that the activity of STAT1 is inhibited by conjugation of the small ubiquitin-like modifier (SUMO) to the lysine residue in position 703. Although only a disproportionately small fraction of the intracellular, unphosphorylated STAT1 pool is SUMOylated, the modification by SUMO interferes with the formation of paracrystalline arrays in the nucleus which sequester activated STAT1 molecules and protect them from being dephosphorylated (Droescher et al. 2011). Naturally occurring STAT variants lacking the transactivation domain can also suppress STAT signaling, but their role may be more complex. Likewise, PIAS proteins (protein inhibitor of activated STAT) have been shown to interact with STATs and to inhibit their DNA-binding activity, but they appear to play a limited role as physiological STAT regulators.

Drugs

Given the crucial role of the JAK-STAT signaling in autoimmune diseases and malignancies, it is not surprising that components of this pathway have become attractive therapeutic targets for pharmacological interventions (O'Shea et al. 2015). Small-molecule JAK inhibitors (i.e., jakinibs) that competitively bind to the ATP-binding site in the JAK kinases and thereby block their activity have been identified (Changelian et al. 2003). Such orally active JAK inhibitors have been marketed for rheumatoid arthritis and other immune system disorders, and these drugs have shown promising results in phase 2 and phase 3 clinical trials for other inflammatory conditions

and beyond. Approved compounds include ruxolitinib against JAK1/JAK2 for psoriasis, myelofibrosis, rheumatoid arthritis, and polycythemia vera and tofacitinib against JAK3 for psoriasis and rheumatoid arthritis. In addition, long-term treatment in CMC patients with ruxolitinib also showed good clinical responses in some patients.

In contrast, STAT protein inhibitors have not reached the clinic yet. However, given the high level of STAT activation in tumor cells and their contribution to malignancy, drugs specifically blocking STAT molecules could be highly beneficial in the treatment of cancer and autoimmune diseases. In contrast to the JAKs possessing a kinase domain, STATs do not have intrinsic catalytic activity, and their pharmacological targeting therefore is more challenging. Current approaches focus on STAT gene expression, SH2-mediated STAT dimerization, and DNA binding using a variety of chemical entities including small interfering RNAs, natural pharmacological inhibitors, peptides and peptidomimetics, non-peptidic small molecules, and decoy oligonucleotides.

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Janus Kinase

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Synonyms

Just another kinase; Tyrosine-protein kinase JAK

Definition

Janus kinases transduce signals emanating from dozens of extracellular cytokines inside the cell by activating their downstream effectors such as STAT transcription factors. JAKs regulate critical cellular functions such as survival, proliferation, homeostasis, and immune and inflammatory responses. On the other hand, JAKs participate in the pathogenesis of several immunological and inflammatory diseases. Activating JAK mutations cause hematological diseases and cancers, and inactivating JAK mutations lead to immunodeficiencies. Currently, JAK inhibitors (JAKi) are used for the treatment of myeloproliferative neoplasms, rheumatoid or psoriatic arthritis, and inflammatory bowel disease, and clinical trials

are ongoing for a wide range of other autoimmune and malignant diseases.

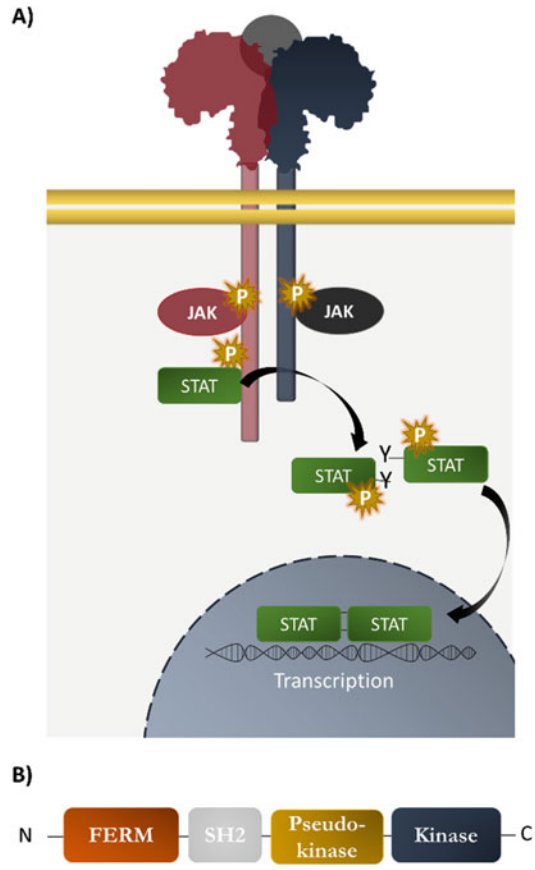
Basic Characteristics

Janus Kinases and the Cytokine Signaling

Janus kinases (JAKs) are nonreceptor tyrosine kinases that catalyze the transfer of ATP γ-phosphate within their active site to the substrate. JAKs mediate the signaling for approximately 60 small extracellular cytokines, particularly interleukins, interferons, hematopoietic growth factors, and some hormones by binding and activating their transmembrane receptors (Silvennoinen and Hubbard 2015).

Mammalian cells express four JAKs: JAK1-3 and TYK2 (tyrosine kinase 2). The JAK-family proteins are large cytoplasmic proteins (120–140 kDa) that constitute of N-terminal FERM (the band-4.1, ezrin, radixin, and moesin-domain) that forms the receptor-binding moiety with the adjacent Src homology 2 (SH2) domain. The kinase-domain resides in the C-terminus and shows the typical kinase characteristics with the nucleotide (ATP)-binding pocket and the activation loop with tandem tyrosine residues that become phosphorylated during activation. Between the kinase domain and SH2 resides the characteristic feature of JAKs, namely pseudokinase domain that shows structural homology to the kinase domain but lacks some of the conserved residues required for kinase transferase activity. However, the pseudokinase domain is a critical regulator of JAK activation.

Cytokine binding to the extracellular part of the receptors reorients or oligomerizes the receptors leading to conformational changes that bring the cytoplasmic JAKs in close proximity that allows the adjacent kinase domains to transphosphorylate each other and become activated (Fig. 1). Activated JAKs phosphorylate the specific tyrosine residues in the receptors that create docking sites for STATs. The seven STAT transcription factors (STAT1-6 including 5a and 5b homologs) are important downstream effectors of JAKs. Once phosphorylated by JAKs, STATs dimerize via their SH2 domains and translocate to nucleus, where they bind to their cognate promoter elements in cytokine-responsive genes to regulate the transcription of the cytokine-specific gene products.



Janus Kinase, Fig. 1 (**a**) Schematic presentation of the JAK-STAT signaling (illustrated here as heterodimeric receptor complex). Cytokine-binding induces conformational changes in the receptors, which draws the JAKs into proximity and allows their transphosphorylation and activation of downstream effectors such as STATs. Phosphorylated STATs translocate to the nucleus to regulate transcription. (**b**) The domain structure of JAKs

JAK1 associates with several cytokine receptors and is involved in several of cytokine-signaling pathways (see Table 1). JAK1 can form signaling complexes with all the other JAK family members. JAK1 is ubiquitously expressed throughout the tissues but is especially crucial for the development and function of the immune system. JAK1-deficient mice die perinatally

caused by severe defects in lymphoid development and neurogenesis.

JAK2 is critical for myeloid blood formation, although it also plays an important role in immune system together with other JAKs. JAK2 deficiency in mice leads to embryonic lethality due to impaired hematopoiesis. JAK2 is the only family member that signals as homodimeric complexes in, e.g., EPOR, TPOR, and GHR systems. JAK2 is ubiquitously expressed and also forms complexes with JAK1 and TYK2.

JAK3 has restricted expression pattern predominantly in the lymphoid cells. JAK3 binds specifically only to the common gamma chain (γc) receptor and plays a crucial role in the function of the lymphoid cell department in the immune system. JAK3 deficiency causes autosomal recessive form of severe combined immune deficiency (SCID).

TYK2 forms a complex with JAK1 and/or JAK2 to transduce signals emanating from the IFN type I family and gp130 family cytokines (Table 1). Consequently, TYK2 is important for immune function via its role in IL-12 and IL-23 signaling that regulates T and NK cells. Loss of TYK2 signaling is not lethal but causes immunodeficiency.

Function and Regulation of JAKs

Downstream Signaling

Cytokine receptors use different combinations of JAK and STAT family members to mediate signaling, which is based on binding specificity between a JAK FERM-SH2 domain with a cytokine receptor membrane proximal region (Table 1). The activated STATs form predominantly homodimers, but in some cases they form heterodimers (e.g., STAT1:STAT2). The dimeric STATs translocate from the cytoplasm to the nucleus with the help of importins and the Ran-GTP nuclear import pathway where they directly bind to the specific regulatory sequences in DNA. The specificity of a STAT for the promotor is a key determinant of the cytokine specificity and transcriptional outcome from a JAK kinase pathway. The canonical JAK-STAT-signaling pathway is relatively simple, but JAKs are triggering kinases for all downstream signaling and phosphorylate and activate substrates from several signaling pathways including MAPK and PI-3K pathways.

Intramolecular Regulation of JAK Kinases

JAK activation is tightly regulated at several levels to ascertain to suppression of signaling in the absence of ligand and to ensure rapid and transient signaling activation upon ligand binding to its receptor. Prior to stimulation, the JAK is maintained in an inactive state by a combination of inter-domain interactions within the JAK kinases as well as by the cell surface organization of receptor-JAK complex. The different JAK domains need to be correctly positioned to form a functional unit that allows inter-domain interactions and intricate regulation of activity. The interaction of the FERM domain with the receptor and membrane positions JAKs in an optimal orientation for interactions (Wilmes et al. 2020). The linkers connecting the domains are also critical for the regulation of the JAK domains. The key inter-domain regulation of JAK kinase is mediated by kinase-pseudokinase domain auto-inhibitory interaction where pseudokinase domain maintains kinase domain in an inactive state in the absence of ligand stimulation. Importantly, majority of the activating JAK mutations localize in this auto-inhibitory interphase (Babon et al. 2014; Silvennoinen and Hubbard 2015). Activation of JAK catalytic activity is initiated by the disruption of the auto-inhibitory interaction resulting in kinase domain trans/auto-phosphorylation. In addition to the negative regulatory role, the pseudokinase domain also has a positive regulatory role in JAK activation. This function is less well defined than the inhibitory role, but mutations in pseudokinase domain (of JAK3 and TYK2) can impair the catalytic activity as in SCID mutations. Furthermore, deletion of the pseudokinase domain in JAK2/3 abolishes cytokine-induced signaling. Thus, the pseudokinase domain serves as a key regulatory domain with dual functions and maintains JAK kinase in inactive state (or low basal activity) in the absence of cytokine and to stabilize the active conformation essential for kinase activity during cytokine

Janus Kinase, Table 1 Activation of JAKs and the primary STATs (nontypical STATs in parenthesis) in cytokine receptor families

Receptor family	Cytokine	JAK kinases				STATs					
		JAK1	JAK2	JAK3	TYK2	STAT1	STAT2	STAT3	STAT4	STAT5	STAT6
Single chain	EpoR, GHR, PRL, and TpoR		**X**							**X**	
γc cytokines	IL-2, IL7, IL9, and IL-15	**X**		**X**				**(X)**		**X**	
	IL-4	**X**		**X**							**X**
	IL-13	**X**	**X**		**X**			**(X)**			**X**
	IL-21	**X**		**X**		**(X)**		**X**		**(X)**	
IL-3/ βc	IL3	**X**	**X**					**(X)**		**X**	
	IL-5		**X**			**(X)**		**(X)**		**X**	
	GM-CSF		**X**							**X**	
gp130	IL-6, IL-11, and LIF	**X**	**X**		**X**	**(X)**		**X**			
	G-CSF	**X**	**X**					**X**			
	IL-12		**X**		**X**				**X**		
	IL-23		**X**		**X**	**(X)**		**X**	**X**		
	IL-27	**X**	**X**		**X**	**(X)**		**X**	**(X)**		
	IL-35	**X**	**X**			**X**			**X**		
IFN	IFNα, IFNβ	**X**			**X**	**X**	**X**	**(X)**	**(X)**		
	IFNγ	**X**	**X**			**X**					
	IL-28	**X**			**X**	**X**	**X**	**(X)**			
	IL-10	**X**			**X**	**(X)**		**X**			
	IL-19	**X**	**X**			**(X)**		**X**			
	IL-22, IL26	**X**			**X**	**(X)**		**X**			

stimulation (Saharinen and Silvennoinen 2002; Ungureanu et al. 2011).

Intermolecular Regulation of JAK Kinases

It is important that JAK activation is transient, and this is achieved by negative regulator proteins at different levels: dephosphorylation, inhibition of transcription, and by internalization and degradation of the receptors (Rawlings et al. 2004; Babon et al. 2014). Initially, the activation is controlled by phosphatases that dephosphorylate JAKs as well as other phosphorylated effector proteins. The main phosphatases for JAK kinases are SHP1, SHP2, CD45, PTP1B, and TCPTP. Other main negative regulators which can bind to JAKs through their SH2 domains are SOCS and LNK family proteins. The SOCS family is composed of SOCS1-SOCS7 and CIS-1, and their transcription is activated by STATs thus creating a negative regulatory loop in cytokine signaling. Different SOCS proteins regulate JAK-STAT signaling by different mechanisms which can involve inducing ubiquitination and targeting JAKs for proteasomal degradation, block JAK catalytic activity or compete with the receptor interactions, and physically block STAT binding. The internalization of receptor complex and the Ubiquitin-proteasome pathway-mediated degradation terminates the signaling from JAKs. An additional negative regulation of JAK-STAT signaling is mediated by PIAS proteins which binds to the dimeric STATs and inhibits its binding to the DNA.

JAK-STAT Signaling in Disease

Cytokines play a central role in the pathogenesis of immunological and inflammatory diseases, and thereby JAK kinases and their regulation are clinically relevant in these diseases. Failure in regulation of JAK–STAT pathway is a common cause of myeloid and lymphoid disorders (Hammaren et al. 2019; Virtanen et al. 2019). Numerous JAK mutations have been identified in leukemia, myeloproliferative neoplasm (MPN), solid cancers, and SCID. Gain of function mutations in JAK1 and JAK3 is commonly found in lymphoid leukemias, while activating JAK2 mutations are more recurrent in myeloid leukemias and MPN. The best characterized example is somatic JAK2 V617F mutation that is highly prevalent in MPN and causes approximately 95% of polycythemia vera (PV), 50–60% essential thrombocythemia (ET), and primary myelofibrosis (PMF) cases. Although the pathogenic TYK2 mutations are infrequent, activating mutations have been identified in acute lymphocytic and myeloid leukemia patients, and defective mutants in a few primary immunodeficiency cases. Loss-of-function germline mutations in JAK3 cause autosomal recessive type of SCID: a disease resulting in complete lack of T- and NK-cells and reduced B-cell function. Approximately half of all JAK mutations locate in the pseudokinase domain, especially clustering in the auto-inhibitory interface that highlights the critical regulatory role of the domain.

The pathogenic JAK-STAT regulation failure may also be driven by mutations in other components or regulatory elements of the pathway (Hammaren et al. 2019). For example, the majority of the inherited SCID cases are covered by loss-of-function mutations either in JAK3 (10–18%) or in JAK3-associated receptors IL-7R (<10%) and IL-2RG (25–46%). Similarly, JAK2 V617F-negative MPN patients often carry mutations in either the thrombopoietin receptor (TPOR) gene MPL (1–5% of all PMF and ET cases), in a multifunctional endoplasmic reticulum protein calreticulin (CALR; ~13% of MPNs), or in the inhibitory adaptor protein LNK (5–7% of MPNs), which result in ligand-independent activation of JAK2 signaling (MPL and CALR mutants) or TPO hyperresponsiveness (LNK mutants). SOCS as inducible negative feedback regulators of cytokine signaling may also participate in various diseases, and decreased SOCS levels due to DNA hypermethylation have been observed in cancers, while elevated SOCS levels have been found in autoimmune diseases. Activating mutations also in STATs have been found in leukemia.

Drugs

JAKs as tyrosine kinases are traditionally targeted by ATP-competitive inhibitors. Currently, six JAK inhibitors have obtained a global approval

for clinical use: ruxolitinib and fedratinib for MPNs, tofacitinib for rheumatoid arthritis (RA), psoriatic arthritis (PsA) and ulcerative colitis (UC), and baricitinib, upadacitinib, and peficitinib (Japan only) for RA as listed in Table 2. The first JAK inhibitors, i.e., ruxolitinib, tofacitinib, and baricitinib, are so-called nonselective first-generation inhibitors that target at least two JAKs at high affinity. During the past decade, the research has focused more on so-called second-generation inhibitors with higher level of selectivity on particular JAK (Virtanen et al. 2019). All the approved JAK inhibitor drugs and vast majority of the ones currently in clinical trials are type-I inhibitors that target the active site of kinase domain. Few examples of non-type-I JAK inhibitors exist: A preclinical JAK inhibitor CHZ868 is a type-II inhibitor, which stabilizes the inactive kinase conformation, and clinical (Phase III) inhibitor BMS-986165 is an allosteric type-IV inhibitor, which targets TYK2 JH2 domain.

Approved JAKinibs

Ruxolitinib

Ruxolitinib (trade names Jakafi, Jakavi) was the first JAK inhibitor drug to enter the clinic. The potent JAK1/JAK2 inhibitor (IC50 2.8/3.3 nM for JAK2/JAK1; >115-fold selectivity over JAK3, and >5-fold over TYK2) was approved (11/2011 FDA; 8/2012 EMA) for treatment of intermediate or high-risk myelofibrosis (Table 2). Ruxolitinib also was the first drug to be approved for treatment of MF and, since, has been considered as the first-line therapy option. Phase III clinical trials with ruxolitinib (COMFORT-I and -II) have demonstrated reductions in splenomegaly and disease-related symptoms as well as improvements in quality of life measures and overall survival in patients with intermediate-2 or high-risk myelofibrosis despite the JAK2 mutational status (Vainchenker and Constantinescu 2013; Plosker 2014). Ruxolitinib is generally well tolerated. Anemia and cytopenias (thrombocytopenia, neutropenia), the most common hematologic adverse events, are dose-dependent and generally manageable with dosage modifications and/or red blood cell transfusions. The most common nonhematologic side-effects are generally mild and include bruising, dizziness, and headache. Ruxolitinib has also been approved for polycythemia vera (PV) patients when there has been an inadequate response to or intolerance of hydroxyurea, the best available therapy in PV. Currently, ruxolitinib is in clinical trials for coronavirus disease 2019 (COVID-19), AD, psoriasis, RA, cancers, and several other autoimmune and inflammatory diseases.

Tofacitinib

Pan-JAK inhibitor (IC50 1.6/3.2/4.1 nM for JAK3/JAK1/JAK2; >20-fold selectivity for JAK3 over TYK2) tofacitinib (trade names Xeljanz, Jakvinus, and Tofacinix) as the second JAK inhibitor drug was approved (FDA 11/2012, EMA 3/2017) for the treatment of moderate to severe active RA in patients who had an inadequate response to the first-line-therapy methotrexate (MTX) (Table 2). Efficacy and safety of tofacitinib monotherapy and combination therapy with MTX was demonstrated for RA in Phase III ORAL trials (Salgado and Gómez-Reino 2013). Using dosages applied in clinics approximately 50–65% of the inadequate responders or patients intolerant to MTX or other disease-modifying antirheumatic drugs obtained 20% enhancement of the symptoms (ACR20). The effects were generally sustained for at least 72 months. Tofacitinib treatment, although generally well tolerated, may cause infections that are typical also for biologics, as well as, decreases in CD4+ T cell count, elevated cholesterol levels, headache, and slight reversible increase in serum creatinine levels. In patient with increased risk for thrombosis, tofacitinib is contraindicated. Tofacitinib has also been approved for moderate or severe active UC (FDA 5/2018, EMA 8/2018) and active psoriatic arthritis (FDA 12/2017; EMA 6/2018) and is in clinical trials for ankylosing spondylitis (AS), psoriasis, alopecia areata (AA), and various other inflammatory and autoimmune diseases.

Baricitinib

Baricitinib (trade names Olumiant, Baricinix) is a JAK1/JAK2 inhibitor (IC50 5.7/5.9 nM for JAK2/JAK1; >70-fold selectivity over JAK3,

Janus Kinase, Table 2 Clinical JAKinibs, target JAKs, and main indications (Phase II and higher) (ClinicalTrials.gov 2020; Biggioggero et al. 2019; Virtanen et al. 2019)

Inhibitor	Target(s)	Disease	Clinical status
Ruxolitinib (Jakavi, Jakafi)	JAK1/2	MF, PV AD, COVID-19, vitiligo, GVHD, HLH AA, RA, psoriasis, leukemia, multiple myeloma, lymphoma, solid cancers, bone marrow fibrosis, HPS, HS, and HIV	Approved Phase III Phase II
Tofacitinib (Xeljanz, Jakvinus, Tofacinix, and others)	JAK1–3	RA, PsA, UC AA, AS, Takayasu arteritis psoriasis, SLE, JIA DLE, dermatomyositis, CD, and COVID-19	Approved Phase IV Phase III Phase II
Baricitinib (Olumiant, Baricinix)	JAK1/2	RA COVID-19 AD, SLE, JIA, uveitis psoriasis, giant cell arteritis, primary biliary cholangitis, AGS, IIM, and diabetic kidney disease	Approved Phase IV Phase III Phase II
Fedratinib (Inrebic)	JAK2	MF	Approved
Upadacitinib (Rinvoq)	JAK1	RA PsA, AS, CD, UC, giant cell arteritis, AD, Takayasu arteritis, and HS SLE	Approved Phase III Phase II
Peficitinib (Smyraf)	pan-JAK	RA UC, psoriasis	Approved (Japan) Phase II
Filgotinib	JAK1	RA, UC, CD, PsA AS, Sjögren syndrome, cutaneous lupus, lupus nephropathy, and uveitis	Phase III Phase II
Abrocitinib (PF-04965842)	JAK1	AD	Phase III
Momelotinib	JAK1/2	MF	Phase III
Pacritinib	JAK2	MF, COVID-19	Phase III
NS-018	JAK2	MF	Phase I/II
Gandotinib (LY-2784544)	JAK2	MPNs	Phase II
Cerdulatinib	JAK2	Vitiligo Lymphomas	Phase II Phase I/II
Decernotinib (VX-509)	JAK3	RA	Phase II/III
PF-06651600	JAK3	AA RA, UC, CD, vitiligo	Phase III Phase II
BMS-986165	TYK2	Psoriasis SLE, lupus nephritis, UC, and PsA	Phase III Phase II
PF-06826647	TYK2	UC, psoriasis, and HS	Phase II

MF myelofibrosis, *PV* polycythemia vera, *AD* atopic dermatitis, *COVID-19* coronavirus disease 2019, *GVHD* graft-versus-host disease, *HLH* hemophagocytic lymphohistiocytosis, *AA* alopecia areata, *RA* rheumatoid arthritis, *HPS* hemophagocytic syndrome, *HS* hidradenitis suppurativa, *HIV* human immunodeficiency virus, *PsA* psoriatic arthritis, *UC* ulcerative colitis, *AS* ankylosing spondylitis, *SLE* systemic lupus erythematosus, *JIA* juvenile arthritis, *DLE* discoid lupus erythematosus, *CD* Crohn's disease, *AGS* Aicardi Goutieres syndrome, *IIM* idiopathic inflammatory myopathies, *MPN* myeloproliferative neoplasm

and >10-fold over TYK2) structurally analogous to ruxolitinib. Baricitinib was approved for treatment of moderate-to-severe active RA in patients who have responded inadequately to, or who are intolerant to, one or more disease-modifying antirheumatic drugs (EMA 02/2017) or in patients

who have had an inadequate response to one or more tumor necrosis factor (TNF) antagonists (FDA 05/2018). Baricitinib has proven to be superior to placebo and TNF-blocker adalimumab in MTX background for RA patients with inappropriate response to MTX (Taylor et al. 2017). The improvements in baricitinib arm were statistically significant at week 1 compared to placebo and at week 2–4 compared to adalimumab, and the measures of efficacy maintained or improved through week 52. Adverse events were more frequent in baricitinib and adalimumab arms compared to placebo, and similar between baricitinib and adalimumab arms. Very common ($\geq$1/10) side effects for baricitinib are upper respiratory tract infections, hypercholesterolemia, whereas common ($\geq$1/100 to $<$1/10) side effects include other infections (herpes zoster, herpes simplex, urinary tract infections, and gastroenteritis), thrombocytosis, nausea, and hepatobiliary disorders. Anemia is a dose-dependent adverse event of baricitinib but has not been reported being an issue in RA patients at approved dosages (2 mg and 4 mg once daily). Baricitinib is in Phase IV clinical trials for COVID-19, and in Phase III trials for atopic dermatitis (AD), systemic lupus erythematosus (SLE), juvenile arthritis (JIA), and uveitis (Table 2).

Fedratinib

Fedratinib (trade name Inrebic) is a selective JAK2 inhibitor (IC50 of 3 nM for JAK2; 35-fold selectivity over JAK1, 135-fold over TYK2, and 334-fold over JAK3) (Wernig et al. 2008; Bewersdorf et al. 2019). Other primary targets of the inhibitor are kinases FLT3 (IC50 15 nM) and RET (IC50 48 nM). Fedratinib was recently (FDA 8/2019) approved for treatment of intermediate-2 or high-risk primary or secondary MF (Table 2). In Phase III JAKARTA trials, fedratinib was demonstrated significantly to reduce splenomegaly and symptom burden compared to placebo. More importantly, fedratinib was shown to be clinically effective in some ruxolitinib-resistant or intolerant patients. Three-fourth of common grade side effects include anemia (58%), fatigue (13%), gastrointestinal events, and nausea. Rare cases of Wernicke's encephalopathy resulted in a boxed warning for the drug.

Upadacitinib

Upadacitinib (trade name Rinvoq) is a so-called next-generation JAK inhibitor selective for JAK1 (IC50 45 nM for JAK1; 74-fold over JAK2, and 58-fold over JAK3). Upadacinib is a type-I inhibitor, the selective nature of which arises from utilization of differences in the nonconserved interactions outside the active ATP-pocket of JAK1 and JAK2 (Virtanen et al. 2019). Upadacitinib was recently approved (FDA 8/2019, EMA 12/2019) for the treatment of moderate to severe active RA, which is not controllable with methotrexate. In clinical trials, upadacitinib demonstrated fast response and efficacy shown by significant differences in ACR20 response rates compared to placebo already at week 2 after the start of the treatment. Upadacitinib treatment led to dose-dependent elevation of LDL and HDL cholesterol levels, while the LDL:HDL ratio remained unchanged. Dose-dependent reduction in hemoglobin levels also was observed, suggesting the possibility of JAK2 inhibition, especially at higher doses. Currently, Phase 2 and 3 trials are ongoing with upadacitinib for several indications, such as, AD, UC, CD, PsA, and SLE (Table 2).

Peficitinib

Peficitinib (trade name Smyraf) is pan-JAK inhibitor displaying moderate selectivity towards JAK3 (IC50 3.9/5.0/0.71/4.8 for JAK1/JAK2/JAK3/TYK2) (Markham and Keam 2019). Peficitinib was recently (3/2019) approved in Japan for treatment of moderate to severe active RA in patients who have an inadequate response to conventional therapies. In clinical trials, peficitinib showed statistically significant reduction of RA symptoms with similar responses as seen with nonselective JAKinibs. The adverse events also were similar to those displayed by nonselective JAKinibs, e.g., neutropenia, but increase rather than decrease in hemoglobin was observed. In a recent Phase 2b study for UC, peficitinib failed to show dose-response although at higher doses it displayed a trend for increased rates of clinical response and remission as well as mucosal healing. Peficitinib is currently assessed in Phase 2 for psoriasis and UC (Table 2).

JAKinibs in Clinical Trials

JAK1-Selective Inhibitors

JAK1 selective inhibitors target the broadest cytokine profile among the JAKs (Table 1). JAK1 has been suggested to dominate in IL-2-induced JAK1/JAK3 and IL-6-induced JAK1/JAK2/TYK2 signaling pathways, and either or both have been implicated in pathogenesis of most inflammatory or autoimmune diseases, implying that selective JAK1 inhibition provides efficacy in treatment of various inflammatory diseases. Although the JAK1-selective inhibitors target a broad cytokine spectrum and therefore the safety might be questionable, the inhibitors still spare JAK2-dependent EPO and TPO pathways responsible for side effects such as anemia, neutropenia, and thrombocytopenia. Upadacitinib was the first JAK1-selective inhibitor approved for the clinics, while filgotinib was the first JAK1-selective JAKinib to enter clinical trials (Virtanen et al. 2019). Filgotinib displays roughly 30-fold selectivity over JAK2 in whole blood assays, was efficacious in preclinical mouse model for arthritis, and was found not to interfere with JAK2 signaling in a Phase 1 study. In Phase II studies for RA, filgotinib demonstrated dose-dependent clinical efficacy with an early onset of action and was well tolerated with infections being the most frequent adverse event. Increase in hemoglobin was observed and attributed to reduced inflammation and lack of JAK2 inhibition. Currently, filgotinib is in Phase 3 trials for RA, UC, CD, and PsA. Other JAK1-selective inhibitors currently in clinical trials include abrocitinib, itacitinib, and solcitinib (Table 2).

JAK2-Selective Inhibitors

JAK2 signaling has critical roles in hematopoiesis, and therefore targeting JAK2 by inhibitors generally leads to dose-dependent hematological adverse events of anemia and cytopenias (Bewersdorf et al. 2019). However, hyperactive JAK2 signaling is in central role in pathogenesis of MPNs, which is the rationale for JAK2-selective inhibitors. Currently, JAK2-selective fedratinib has been approved for treatment of myelofibrosis, while pacritinib (56-fold selectivity over JAK1) is in Phase III clinical trials (Table 2). In addition to JAK2, pacritinib targets FLT3, IRAK1, and CSF1R. It has demonstrated significant improvements in efficacy compared to ruxolitinib, the best available therapy, as well as efficacy in some patients who previously had failed or were intolerant for ruxolitinib treatment. Thrombocytopenia, anemia, and gastrointestinal adverse events were the most common adverse events. Development of pacritinib was shortly placed on hold in 2016 due to few deaths caused by heart failure and intracranial hemorrhage. The hold was lifted after close review of the cases.

JAK3-Selective Inhibitors

JAK3 together with the signaling partner JAK1 has a central role in the regulation of immune responses, and therefore it is considered as a relevant target for immunosuppression. The expression of JAK3 is confined to hematopoietic cells, which implies that selective targeting of JAK3 in autoimmune diseases could escape the non-immunological side effects, e.g., neutropenia and anemia (Virtanen et al. 2019). Peficitinib, the approved JAKinib in Japan, and decernotinib assessed in Phase II/III clinical trials for RA have moderate JAK3 selectivity, whereas PF-06651600 currently in Phase III for AA targets JAK3 with high specificity (Table 2). PF-06651600 was developed by structure-guided design to covalently target JAK3. It is the only covalent, irreversible JAK inhibitor in clinical trials and acts through a nonconserved cysteine (Cys909) in the ATP-pocket of JAK3 kinase domain. PF-06651600 is potent and highly selective inhibitor with negligible potency toward other JAKs allowing a selective inhibition of signaling through γc cytokines. Kinome scans have revealed the only off-targets being TEC family of kinases, which share the cysteine in the same position in the active site as JAK3. PF-06651600 has demonstrated remarkable efficacy, in Phase II trials for AA, and was granted (Pfizer 2019) Breakthrough Therapy designation from US FDA. The most common side effects in the AA clinical study were the infections, gastrointestinal and skin/subcutaneous tissue-related. Herpes zoster reactivation was not observed.

TYK2-Selective Inhibitors

Biologics targeting TYK2 cytokine signaling pathways (IL-12/IL-23, IFNα, and IL-6) have proven efficacious in treatment of various autoimmune diseases including RA, UC, psoriasis, PsA, and AS, which gives rationale for development of TYK2-selective JAKinibs (Virtanen et al. 2019). BMS-986165 is an allosteric TYK2 pseudokinase-domain-binding inhibitor, which, despite strong binding to JAK1, via unique and mostly unknown mechanisms inhibits selectively TYK2-mediated cytokine signaling. The allosteric inhibitor currently is in Phase III for psoriasis, and in Phase II trials for SLE, lupus nephritis, UC, and PsA (Table 2). In Phase 2 study of 267 patients with moderate-to-severe psoriasis, BMS-986165 demonstrated greater clearance of psoriasis and improvements in quality of life compared to placebo. Most common treatment-related adverse events included nasopharyngitis, headache, diarrhea, nausea, and upper respiratory tract infections.

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Just Another Kinase

▶ Janus Kinase

K

Kallidin (Lysyl-Bradykinin)

► Kinins

Kinins

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Synonyms

Bradykinin; DesArg^{10}-kallidin (Lys^{0}, desArg^{9}-bradykinin); DesArg^{9}-bradykinin; Kallidin (lysyl-bradykinin); T-kinin (Ile-Ser-bradykinin)

Definition

Kinins are a group of short-lived proinflammatory peptide hormones that bind to G-protein-coupled receptors and trigger Ca^{2+}-dependent nitric oxide and/or prostaglandin-dependent signaling pathways.

Inflammation
Nociception
Blood pressure
Edema

Basic Characteristics

Liberation of Kinins

Kinin hormones are locally produced on cell surfaces by limited proteolysis from their precursors, the kininogens (Fig. 1).

In humans, two types of kininogens are circulating in plasma, namely high-molecular-weight kininogen (H-kininogen, HK) and low-molecular-weight kininogen (L-kininogen, LK). The human kininogens result from alternative splicing of a single *KGN1*gene. A third type of kininogen, T-kininogen, is exclusively found in rats but not in other mammalians. Kininogens are predominantly expressed by the liver and secreted by hepatocytes into the circulation. The kidney and some secretory glands also express kininogens.

Kininogens are processed by kinin-liberating enzymes named kallikreins. Kallikreins are a group of serine proteases that are expressed by hepatocytes and glandular cells, neutrophils in their zymogen forms. Kallikreins are found in biological fluids such as plasma and urine. In humans, two types of kallikreins exist: tissue kallikrein and plasma kallikrein. Despite their similarities in name, the serine proteases differ in their structure, function, and expression. Tissue kallikrein (TK) mostly utilizes L-kininogen as the substrate to produce the kinin kallidin, whereas plasma kallikrein (PK) acts mostly if not exclusively on H-kininogen to generate the bradykinin (BK) hormone (Table 1). The substrate specificity of kallikrein for H-kininogen is due to the fact that

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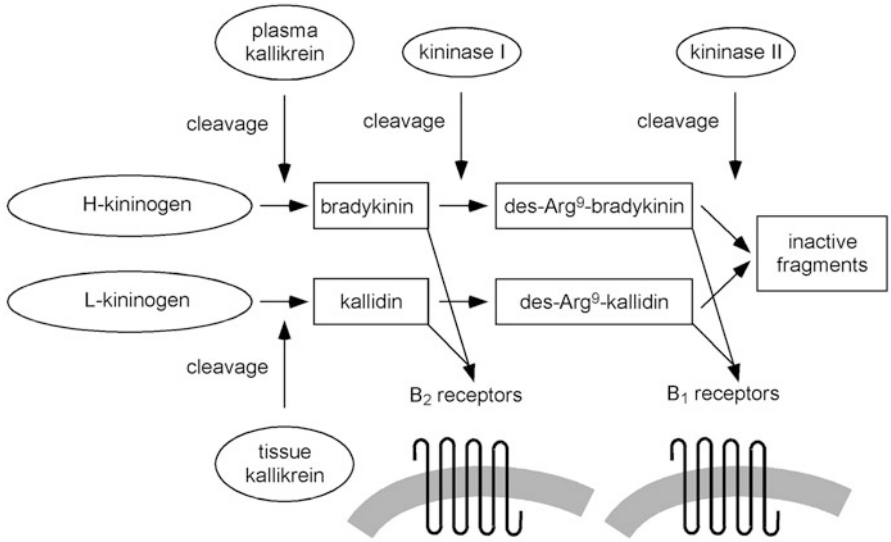


Kinins, Fig. 1 Scheme of kinin liberation, turnover, and action

Kinins, Table 1 Enzymes involved in the generation and degradation of kinins

Processing enzyme	Substrate	Proteolysis product	Signaling receptor
Plasma kallikrein Tissue kallikrein	H-kininogen	Bradykinin (RPPGFSPFR) Kallidin	B_2 receptor
Tissue kallikrein Plasma kallikrein	L-kininogen	Kallidin (KRPPGFSPFR) Bradykinin	B_2 receptor
Carboxypeptidase N Carboxypeptidase M Neutral endopeptidase (NEP) Angiotensin-converting enzyme (ACE)	Bradykinin	$DesArg^9$-bradykinin (RPPGFSPF) Bradykinin 1–7 (RPPGFSP) Bradykinin 1–7 (RPPGFSP) 1–5 (RPPGF)	B_1 receptor Inactive Inactive Inactive
Carboxypeptidase N Carboxypeptidase M Neutral endopeptidase (NEP) Angiotensin-converting enzyme (ACE)	Kallidin	$DesArg^{10}$-kallidin (KRPPGFSPF) Kallidin 1–8 (KRPPGFSP) Kallidin 1–8 (KRPPGFSP) 1–6 (KRPPGF)	B_1 receptor Inactive Inactive Inactive
Neutral endopeptidase (NEP) Angiotensin-converting enzyme (ACE)	$DesArg^9$-bradykinin (RPPGFSPF)	Bradykinin 1–7 (RPPGFS), 1–4 (RPPG) Bradykinin 1–5 (RPPGF)	Inactive Inactive
Neutral endopeptidase (NEP) Angiotensin-converting enzyme (ACE)	$DesArg^{10}$-kallidin (KRPPGFSPF)	Kallidin 1–5 (KRPPG) Kallidin 1–6 (KRPPGF)	Inactive Inactive

kallikrein is tightly bound to a substrate. The bimolecular complex circulates in the blood and attaches to cell surfaces (Weidmann et al. 2017). In addition to kallikreins, kinins can also be released by other proteases such as trypsin, elastase, or cathepsin D; however, the biological significance of these kininogenases has remained unknown. Apart from mammalian kininogenases, a large number of proteases derived from microorganisms have the capacity for liberating kinins

from host kininogens with implications for bacteria- and parasite-driven inflammatory and infectious reactions.

Kinins are short-lived peptide hormones with plasma half-lives in the second range that are rapidly proteolytically converted and/or inactivated. Active hormones bradykinin and kallidin are chopped at their carboxyterminal ends by carboxypeptidases of the N and M type, collectively referred to as kininases type I. Processing leads to desArg^9-bradykinin and desArg^{10}-kallidin, respectively (Table 1). Bradykinin and kallidin act on B_2 receptors, but desArg^9-bradykinin and desArg^{10}-kallidin activate B_1 receptors. Together, the proteolytic conversion of the kinins "switches" the relative binding affinity of the ligands for the two kinin B_1 and B_2 receptors, respectively (Fig. 1). DesArg^{10}-kallidin and des Arg^9-bradykinin are rapidly degraded by the dipeptidylpeptidase angiotensin-converting enzyme (ACE), also known as kininase type II, and neutral endopeptidase, respectively (Table 1). In humans and some other mammalian species, kininogens are modified by posttranslational hydroxylation of a proline residue within their kinin sequence, that is, position 3 in bradykinin or position 4 in kallidin. Hydroxylation does not affect the specificity, affinity, or intrinsic efficacy of the kinins.

Bradykinin

The best characterized kinin is bradykinin (from the Greek brady-, slow; −kinin, kīn(eîn) to move). The nine-amino acid peptide is mostly if not exclusively produced by the plasma contact system, comprising the proteases factor XII (FXII) and plasma kallikrein. The name "contact system" originates from the unique mode of FXII zymogen activation following binding ("contact") to negatively charged surfaces that induce a conformational shift leading to limited proteolytic activity. Initially, a small amount of active FXII (FXIIa) is formed that in turn converts plasma kallikrein to the active protease PKa. PK then reciprocally activates new FXII zymogens. The feedback loop amplifies FXII activation and initiates the intrinsic pathway of coagulation via FXIIa-mediated activation of Factor XI. Additionally, formed PKa cleaves H-kininogen to release bradykinin (Kallikrein-kinin system, Fig. 3). The bradykinin-forming contact system components bind to endothelial cells, platelets, and neutrophils with H-kininogen serving as a docking site for kallikrein. H-kininogen binds to cells via heparan and chondroitin sulfate glycosaminoglycans (GAG) of cell surface proteoglycan. FXII may also bind to GAGs, facilitating contact system protein assembly on endothelial cells. GAGs do not only assemble contact system proteins on cellular surfaces, the polysaccharide also regulates kinin generation. H-kininogen binding to GAGs interferes with contact system-driven bradykinin generation, indicating that H-kininogen needs to dissociate from GAGs to allow for efficient plasma kallikrein-mediated kinin formation (Maas and Renne 2018).

An array of synthetic polymers has the capacity for initiating FXII contact activation leading to bradykinin formation. Within the last decade, natural FXII contact activators have been identified including misfolded protein aggregates, DNA/RNA, collagens, and the inorganic polymer polyphosphate (polyP). Platelet- and bacteria-derived polyphosphate form insoluble Ca^{2+}-rich nanoparticles that potently initiate bradykinin formation in an FXII- dependent manner, and targeting polyphosphate ablates their proinflammatory activity, for example, in edema models. Furthermore, the FXII activator platelet polyphosphate has potent procoagulant activities with implication for thrombosis (Mailer et al. 2019).

Diagnostics of Kinins

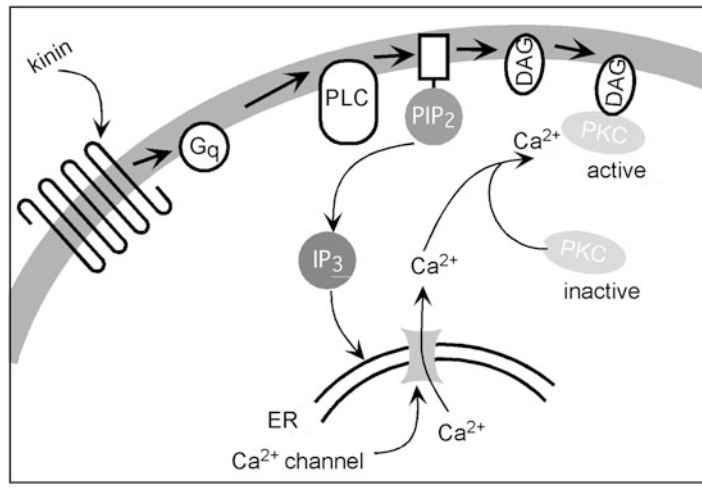
Analysis of kinins has remained challenging due to their short half-life and artificial contact-mediated production during sampling and storage. HPLC-based and RIA/capture-EIA measurements are established to determine kinins in human plasma, liquor, or urine in experimental studies. Most accurate kinin measurements are mass spectrometry-based methods that either determine the kinin hormones directly or indirectly via measuring H-kininogen/L-kininogen breakdown products. Furthermore, cleaved H-kininogen (cHK, that is devoid of the kinin moiety) has been established as a biomarker for

kinin generation with implications for clinical trials. Serine protease inhibitors targeting active FXII and plasma kallikrein need to be added to prevent artificial generation of kinins in vitro during sample preparation. Kinins and their degradation products have been studied in various biological milieus such as plasma/serum, urine, joint fluids, kidney, lung, and skeletal muscles. Under physiological conditions, the concentration of kinins in these compartments is low in the femtomolar and lower picomolar range. In contrast, plasma levels of kininogens are in the micromolar range, that is, the ratio of hormone to precursor is low and kinin release is tightly controlled. Kinin levels can rise considerably (>100-fold) in patients with diseases such as hereditary angioedema, rheumatoid arthritis, or sepsis and septic shock. Kinin levels correlate with the severity of the disease state (van der Poll and Herwald 2014).

Chromogenic substrates including H-D-prolyl-L-phenylalanyl-L-arginyl-*p*-nitroanilide and *N*-benzoyl-L-propyl-L-phenylalanyl-L-arginyl-*p*-nitroanilide are available for measuring the proteolytic activity of plasma kallikrein. Synthetic kallikrein inhibitors such as diisopropyl fluorophosphate (DFP), L-phenyl-L-phenyl-L-arginyl-chlormethylketone, or *p*-amidino-phenylmethylsulfonyl-fluoride (PMSF) covalently bind to the catalytic triad serine and thus completely block the proteolytic activity of the enzyme. The major inhibitor of kallikrein in human plasma is the C1 esterase inhibitor (C1INH), which belongs to the large family of serine protease inhibitors (serpins). In addition, α2-macroglobulin (α2M), antithrombin III, plasminogen activator inhibitor-1, and protein C inhibitor interfere with plasma kallikrein activity in plasma and on cell surfaces with implications for kinin formation.

Kinin Receptors

Cellular kinin functions are mediated by their interaction with cell surface receptors (Fig. 2). In humans, two types of kinin receptors have been identified, namely kinin B_1 and B_2 receptors. The two receptors are coded by two distinct genes on closely apposed loci in human chromosome 14q32. *Bdkr1* and *Bdkr2* genes likely arose from a common progenitor. Kinin B_1 and B_2 receptors belong to the large family of G-protein-coupled



Kinins, Fig. 2 Intracellular signaling cascades triggered by kinins. Following kinin binding the receptor, the associated heterotrimeric G protein complex dissociates. The α subunit stimulates phospholipase C (PLC), which in turn catalyzes the breakdown of phosphatidylinositol-4,5-bisphosphate (PIP_2) into 1,2-diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). IP_3reacts with Ca^{2+} channels in the endoplasmic reticulum (ER) releasing Ca^{2+} into the cytosol. The increase in intracellular Ca^{2+} levels activates protein kinase C (PKC), which translocates to the plasma membrane, anchoring to DAG and phosphatidylserine

receptors characterized by seven transmembrane-spanning helices. Unlike B_2 receptors that are constitutively expressed in many cell types and tissues, the expression levels of B_1 receptors are very low under physiological conditions. However, inflammatory or noxious stimuli boost expression of the B_1 receptor. Several cytokines such as interleukin IL-1β, as well as growth factors and bacterial lipopolysaccharides, upregulate transcription of *Bdkr1* (Qadri and Bader 2018). B_1 and B_2 receptors are predominantly coupled to the pertussis toxin-insensitive G_q type of G protein leading to phospholipase C activation, mobilization of intracellular calcium by inositol-1,4,5-trisphosphate (IP_3), and activation of protein kinase C. Kinin receptors are stimulators of the biosynthesis of potent downstream effectors such as prostaglandins and leukotrienes due to phospholipase A_2 activation, and of nitric oxide via stimulation of the endothelial isoform of nitric oxide synthase (eNOS).

Kinin Receptor-Deficient Mice

Homozygous B_1 or B_2 receptor-deficient mice are healthy, fertile, and normotensive. In B_1-deficient mice, lipopolysaccharide-induced hypotension is diminished, recruitment of polymorphonuclear leukocytes to the sites of tissue injury is impaired, and the animals show signs of hypoalgesia. Deletion of the B_2 gene in mice leads to salt-sensitive hypertension and altered nociception. B_2 receptor-deficient embryos subjected to salt stress in utero show suppressed renin expression, an abnormal kidney phenotype, and develop early postnatal hypertension. Consistently, although basal bradykinin formation is reduced, tissue kallikrein-null mice have normal blood pressure and however suffer from cardiovascular abnormalities, which support a function of kinins during development. Mice deficient in both kinin receptors have been generated that are normotensive but protected from endotoxin-induced hypotension supporting a function for kinins in infectious and inflammatory disease states.

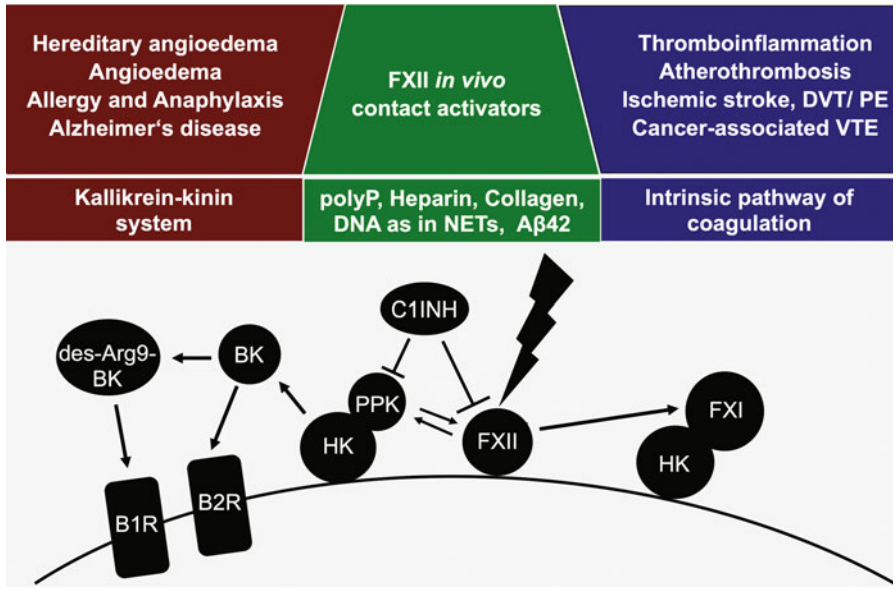
Biological Functions and Clinical Implications

Kinins are implicated in many physiological and pathological processes including the induction of pain and hypotension, contraction of smooth muscles, regulation of local blood flow, stimulation of electrolyte fluxes, activation of sensory neurons, and increase of vascular permeability. Many of these effects are triggered at least in part via the major downstream effectors prostaglandins, leukotrienes, the small GTPase Cdc42, and nitric oxide.

Bradykinin shows a variety of physiological and pathological effects. The hormone induces contraction of nonvascular smooth muscle in the bronchus and gut and relaxes vascular smooth muscle cells leading to hypotension, natriuresis and diuresis, and decrease in blood glucose level. It is a mediator of inflammation and causes an increase in vascular permeability, stimulation of nociceptors, and release of other mediators of inflammation (e.g., prostaglandins). It has a cardioprotective effect (directly via bradykinin action and indirectly via endothelium-derived relaxing factor action).

Uncontrolled activation of the FXII-driven contact system (Fig. 3) triggers massive formation of kinins under certain pathological conditions. The best-studied kinin-mediated disease is hereditary angioedema (HAE), a life-threatening swelling disorder. Three different types of HAE exist that are either due to deficiency in C1 esterase inhibitor (C1INH, the major inhibitor of PKa and FXIIa; Type I), defective C1INH function (Type II), or an FXII point mutation at Thr309 that causes loss of a single glycosylation site leading to excessive FXII contact activatability (Type III).

Angioedema (AE) has been associated with angiotensin-converting enzyme (ACE) inhibitor intake. ACE degrades kinins (Table 1) and interferes with kinin metabolism leading to kinin accumulation and kinin-mediated swellings and coughing. Furthermore, excess bradykinin formation is seen in patients with a systemic inflammatory response syndrome (SIRS) due to sepsis or trauma. SIRS progression is accompanied by depletion of contact system factors, and low levels of H-kininogen and plasma kallikrein are indicative of a fatal outcome. Other diseases associated with a massive, often systemic release of kinins are pancreatitis, Alzheimer disease (AD), allergic



Kinins, Fig. 3 The factor XII (FXII)-driven contact system and its roles in diseases. Polyphosphate (polyP), mast cell-derived heparin, collagen, nucleic acids (DNA and RNA), and misfolded protein aggregates such as Aβ42 induce FXII contact activation in vivo. Activated FXII (FXIIa) triggers fibrin formation through the factor XI (FXI)-mediated intrinsic coagulation pathway, contributing to thrombo-inflammation, atherothrombosis, ischemic stroke, venous thromboembolism such as deep vein thrombosis (DVT), pulmonary embolism (PE), and cancer-driven VTE. FXIIa also activates plasma kallikrein (PK) leading to release of bradykinin (BK) by active plasma kallikrein (PKa)-mediated cleavage of high-molecular-weight kininogen (HK). Both FXI and PK are bound indirectly to cells via HK. Bradykinin activates kinin B2 receptors (B2R), while the BK metabolite des-Arg9 BK binds to kinin B1 receptor (B1R), both of which activate proinflammatory signaling cascades that contribute to increased vascular leak in HAE and others such as angiotensin-converting enzyme (ACE) inhibitor-induced angioedema, allergic and anaphylactic reactions, and vascular dysfunction in patients with Alzheimer disease. Serpin C1 esterase inhibitor (C1INH) is the major inhibitor of both activated FXII and PK. (Adapted from (Nickel et al. 2017))

and nonallergic rhinitis, and asthma (Fig. 3). Notably, kinins are important endogenous mediators exerting acute protective effects in the ischemic myocardium via nitric oxide-dependent mechanisms.

Drugs

Aprotinin (Trasylol®) was the first drug to be used in the clinic to prevent the formation of kinins. However, due to aprotinin's anaphylactic potential, the use of Trasylol has raised serious concern worldwide and has been withdrawn from the market. To target plasma kallikrein activities, a specific inhibitor (Ecalantide ®) and neutralizing antibody (Takhzyro®) were introduced for acute and prophylactic treatment of hereditary angioedema of attacks. Furthermore, a variety of plasma-derived and recombinant C1INH are approved for interference with kinin formation in HAE types I and II. Alternatively, a peptide-based inhibitor of B2 receptors (Icatibant®) is used to prevent swellings of HAE patients and possible other kinin-driven alterations of vascular leak such as burn injuries.

In addition to this inhibitor, a large number of specific B_1 and B_2 receptor antagonists and agonists have been developed, and data from clinical trials suggest potential future use of these agents in SIRS, sepsis, ischemic and traumatic brain injury, hyperalgesia, and angioedema.

Angiotensin-converting enzyme (ACE) inhibitors represent a class of drugs that have proven

anti-hypertensive and anti-proteinuric effects. They delay the progression of renal disease in conjunction with the ability to reduce systemic blood pressure. Furthermore, they have been shown to reduce mortality and morbidity in myocardial infarction associated with chronic heart failure. The cardioprotective effect of ACE inhibitors is a combined result of the diminished conversion of angiotensin I and of the attenuated kinin breakdown leading to kinin accumulation in ischemic myocardia. Given their pleiotropic effects, ACE inhibitors may well use alternative mechanism(s) to exert their beneficial roles, for example, through the resensitization of kinin receptors.

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Kir Channels

► Inward Rectifier Potassium Channels

Kv-Channels

► Voltage-Gated K^+ Channels

L

Laboratory Medicine

► Ca^{2+}-Binding Proteins
► S100 Proteins

Lactam β Antibiotics

► β-Lactam Antibiotics

β-Lactam Antibiotics

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Synonyms

Wall peptidoglycan inhibitors; Lactam β antibiotics

Definition

β-Lactam antibiotics are bicyclic or monocyclic azetidinone ring-containing compounds (Fig. 1). They kill bacteria by preventing peptide cross-linking in the assembly of peptidoglycan. This covalently closed net-like polymer forms the matrix of the cell wall by which the bacteria can grow and divide despite their high internal osmotic pressure.

Mechanism of Action

Peptidoglycan (Fig. 2) is comprised of glycan chains made of alternating β-1,4-linked *N*-acetylglucosamine and *N*-acetylmuramic acid residues (Vollmer et al. 2008). The D-lactyl groups on carbon C_3 of the muramic acids are substituted by L-alanyl-γ-D-glutamyl-L-diaminoacyl-D-alanine stem tetrapeptides. The peptides borne by adjacent glycan chains are cross-linked by the DD-transpeptidases (DD-TPases) through direct linkages or cross bridges (comprising one or several intervening amino acid residues) that extend from the D-alanine residue at position 4 of a stem peptide to the ω-amino group at position 3 of another stem peptide ((4–3) peptidoglycan). Lipid II (Fig. 3) is the immediate biosynthetic precursor. A disaccharide bearing an L-alanyl-γ-D-glutamyl-L-diaminoacyl-D-alanyl- D-alanine stem pentapeptide (the diamino acid residue of which can be either free, i.e., unsubstituted, or substituted by one or several amino acid residues, i.e., branched) is synthesized in the cytoplasm and transported to the outer face of the plasma membrane, linked to a C_{55}-undecaprenyl via a pyrophosphate. From this precursor, the formation of polymeric peptidoglycan relies on glycosyl transferases (GTases) ensuring glycan chain elongation and acyl transferases (DD-Tpases) ensuring peptide cross linking.

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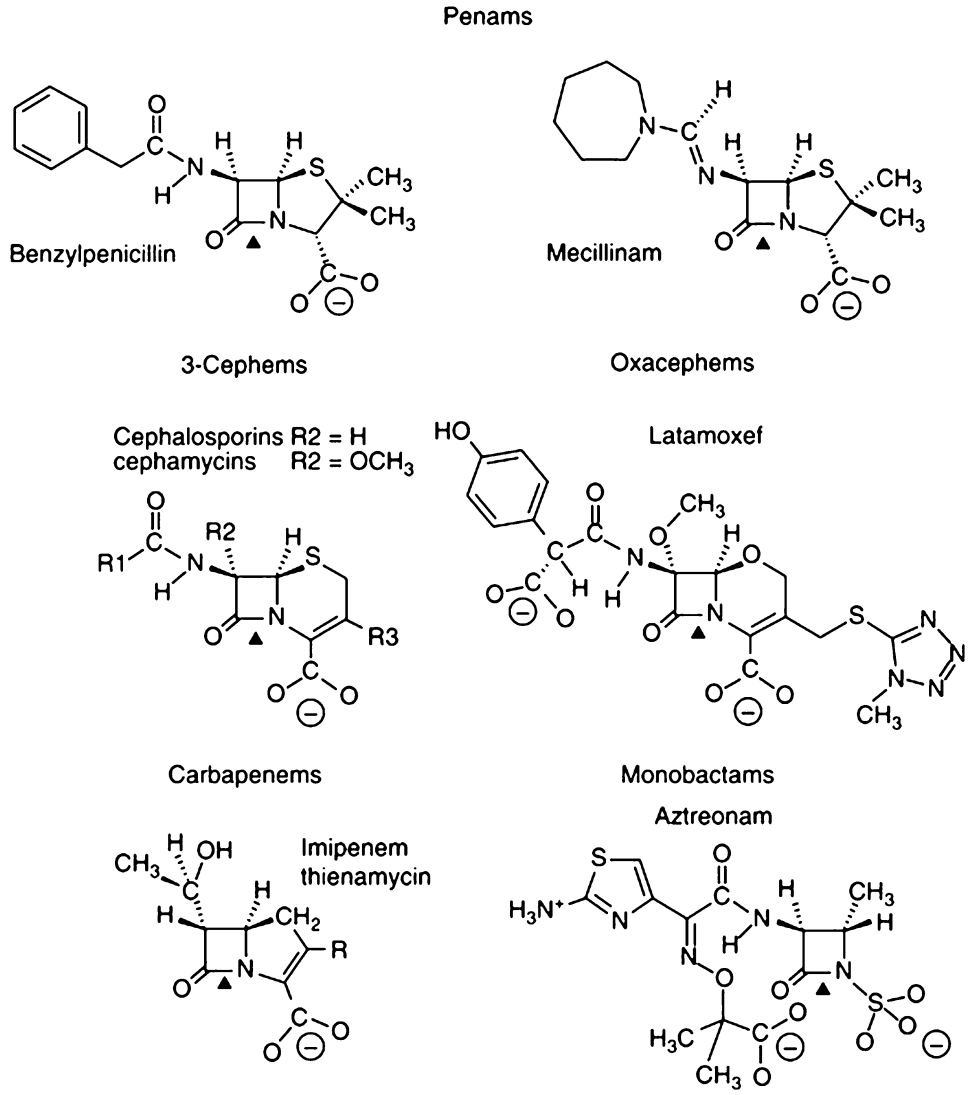
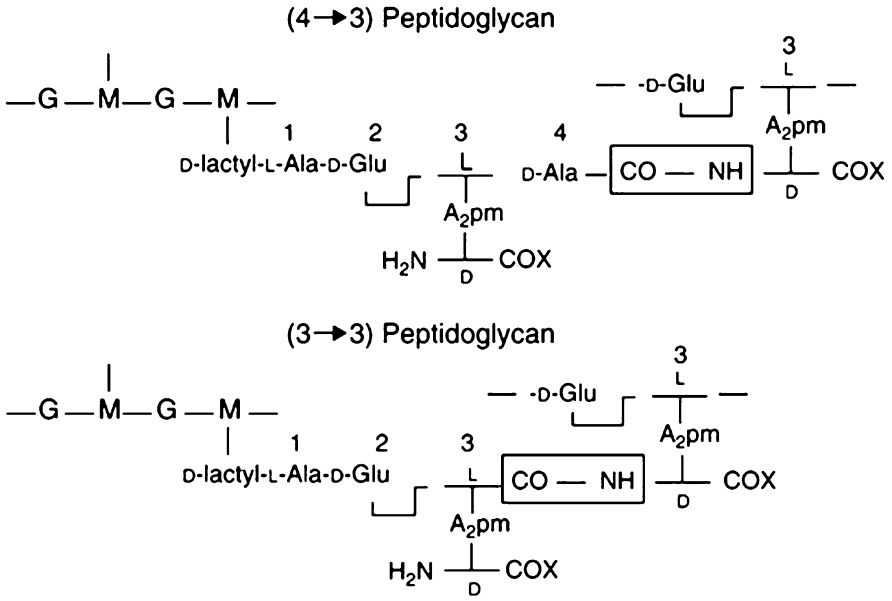


β-Lactam Antibiotics, Fig. 1 Bicyclic (penams, 3-cephems, oxacephems, and carbapenems) and monocyclic (monobactams, e.g., aztreonam) β-lactam antibiotics. In carbapenems, the methylene in the 5-membered ring can be substituted by a methyl group. Rupture of the scissile amide bond of the azetidinone ring (arrow) by the SXXK acyl transferases implicated in (4–3) peptidoglycan synthesis results in the formation of long-lived, serine-linked acyl ester derivatives. Thus, these enzymes behave as penicillin-binding proteins or PBPs

Acyl transferases of the SXXK superfamily (SXXK Acyl Transferases, also called DD-transpeptidases, with X denoting a variable amino acid residue) are implicated in cross-linking and they exhibit a specific bar code in the form of three motifs, SXXK, SXN (or analogue), and KTG (or analogue), occurring at equivalent distances along the polypeptide chains (Sauvage et al. 2008). As a result of the polypeptide folding, the motifs are brought close to each other at the immediate boundary of the catalytic center between an all-α and an α/β domains. SXXK acyl transferases identify *N*-acyl-D-alanyl-D-alanine sequences as carbonyl donors, produce an *N*-acyl-D-alanyl moiety linked as an ester to the serine residue of the invariant SXXK motif, and transfer the peptidyl moiety onto an amino group (DD-transpeptidation) or a water molecule (DD-carboxypeptidation). SXXK



β-Lactam Antibiotics, Fig. 2 (4–3) Peptidoglycan (the cross-linking of which, catalyzed by PBPs, is susceptible to β-lactam antibiotics) and (3–3) peptidoglycan (the cross-linking of which, catalyzed by LD-transpeptidase or LDTs, is resistant to most β-lactam antibiotics with the exception of carbapenems, see below, PBP-mediated resistance) in *Escherichia coli* and *Mycobacterium tuberculosis*. G: *N*-acetylglucosamine. M: *N*-acetylmuramic acid (i.e., *N*-acetylglucosamine with a D-lactyl substituent on carbon C_3). A_2pm: *meso*-diaminopimelic acid. In *E. coli* and *M. tuberculosis*, the stem peptides are unbranched. COX: COOH (in *E. coli*) or $CONH_2$ (in *M. tuberculosis*)

acyl transferases recognise penicillin (used as a generic term for β-lactam antibiotics) as a suicide carbonyl donor. Because the serine-ester linked penicilloyl enzymes formed by SXXK acyl transferases generally exhibit long half-lives, they remain immobilized in the form of penicillin-bound proteins, hence the DD-transpeptidases (and DD-carboxypeptidases) are usually called PBPs for Penicillin Binding Proteins (Sauvage et al. 2008). The inactivation reaction involves the formation of an acylenzyme as shown in Fig. 4 where E-OH is the enzyme with -OH the hydroxyl group of its active-site serine residue.

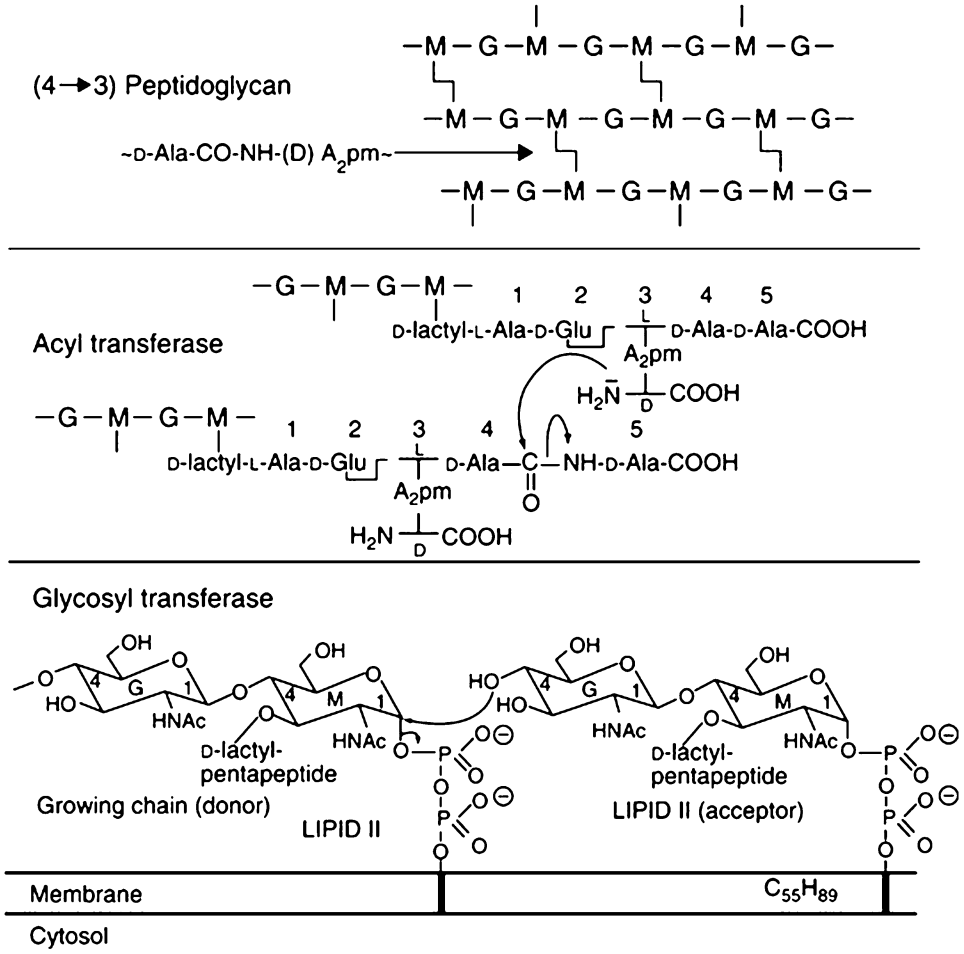
Kinetically, the interaction is described by a three-step model (Fig. 5) where E is the enzyme, I the β-lactam and P(s) the product(s) resulting from the very slow decay of EI*.

In all cases, k_3 has been found to be very small and K is generally large so that the sensitivity of a PBP to a β-lactam is best characterized by the k_2/K ratio (Frère et al. 1975). This ratio varies from 100,000 to 1,000,000 $M^{-1}s^{-1}$ for the most sensitive PBP to less than 1 for the most resistant.

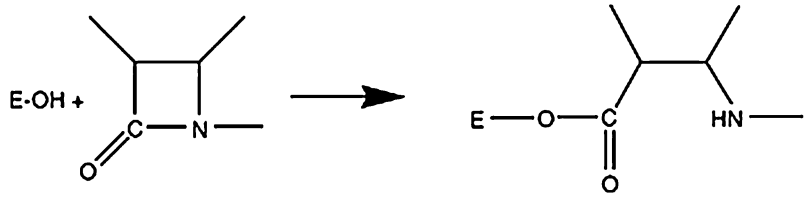
Lethal Target Proteins

A constellation of genes code for PBPs of varying amino acid sequences and functionalities. The PBP functional domain can be associated with additional domains whose exact function is not always clear. This combinatorial system of structural modules results in a large degree of diversity.

Penicillin-binding proteins are historically divided based on their molecular mass into two group known as high and low molecular mass (HMM, LMM) PBPs (Sauvage et al. 2008). HMW-PBPs are involved in the late stages of peptidoglycan polymerization while LMW-PBPs, also referred to as class C PBPs, are mainly peptidoglycan hydrolases (carboxypeptidases, endopeptidases) involved in the remodeling and recycling of peptidoglycan. Loss of these auxiliary proteins causes varying morphological aberrations but is not fatal, at least in the laboratory environment. As a result, HMM-PBPs are the primary targets of β-lactam antibiotics. HMM-PBPs are mostly multidomain enzymes divided

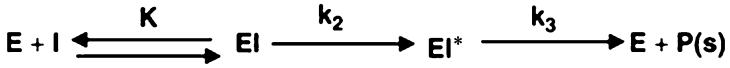


β-Lactam Antibiotics, Fig. 3 Lipid II precursor (bottom) and polymeric (4–3) peptidoglycan of *Escherichia coli* and *Mycobacterium tuberculosis*. Glycosyl transferase and acyl transferase-catalyzed reactions. G: *N*-acetylglucosamine. M: *N*-acetylmuramic acid. A $_2$pm: *meso*-diaminopimelic acid. (3–3) Peptidoglycan cross-linking (Fig. 2) may proceed via the formation, in a penicillin-resistant (but not carbapenem-resistant) manner, of an *N*-acyl-L-diaminoacyl moiety linked as an ester to the cysteine residue and the transfer of the peptidyl moiety to the ω-amino group of the diamino acid residue of another peptide



β-Lactam Antibiotics, Fig. 4 Inactivation reaction of PBPs (E-OH) by β-lactams. The covalent adduct (the acyl enzyme) is referred to as EI* in Fig. 5

in two classes A and B (aPBP and bPBP, respectively) based on their domain organization and function (Sauvage et al. 2008; Sauvage and Terrak 2016). In aPBPs the DD-TPase domain is invariably linked to an N-terminal GTase domain. The latter converts the disaccharide–pentapeptide units borne by lipid II precursor molecules into uncross-linked glycan chains. Peptide



β-Lactam Antibiotics, Fig. 5 Three-step kinetic model describing the interaction of a PBP enzyme (E) and a β-lactam (I). EI and EI* are respectively a non-covalent complex (dissociation constant: K) and a covalent adduct. EI* is the ester depicted in Fig. 4. k_2 and k_3 are first-order rate constants

cross-linking between elongated glycan chains is then carried out by the associated SXXK acyl transferase module. The PBPs of class B are comprised of an SXXK acyl transferase module, bound to the carboxy end of an N-terminal module devoid of enzymatic activity. These proteins are components of large morphogenetic complexes that control wall expansion, ensure cell-shape maintenance, and carry out septum formation (Derouaux et al. 2014; Typas et al. 2011). The GTase and TPase activities are coupled within the same aPBPs. The TPase activity of bPBPs act in concert with the GTase activity of aPBPs and SEDS (shape, elongation, division, and sporulation) protein partners (Egan et al. 2015; Taguchi et al. 2019).

Escherichia coli is killed in a number of ways (Sauvage and Terrak 2016); via cell lysis as a result of the selective inactivation of the class A PBP1a and PBP1b (which can substitute for each other) by cephaloridine and cefsulodin; via transformation of the cells into round bodies as a result of the selective inactivation of the cell-cycle subclass B2 PBP2 by mecillinam and thienamycin; via cell filamentation as a result of the selective inactivation of the cell-cycle subclass B3 PBP3 by mezlocillin, cefaperazone, cefotaxime, cefuroxime, cephalothin, and aztreonam; or via different combinations of these morphological alterations by ampicillin, benzylpenicillin, carbenicillin, and cefoxitin. In the end, all these defects result in cell lysis.

In addition to the catalytic GTase and TPase domains, some PBPs, such as the class A PBP1a and PBP1b of *E. coli*, exhibit small domains called ODD and UB2H, respectively, located between the GTase and the TPase domains (Typas et al. 2011). They have been shown to function as the binding sites of regulatory lipoproteins LpoA and LpoB which stimulate the activities of PBP1a and PBP1b, respectively (Typas et al. 2011). The bPBPs can also contain additional domains. The subclass B4-PBPs (e.g., PBP2x of *Streptococcus pneumoniae*), for example, have two additional C-terminal PASTA domains (PBP and Serine/Threonine kinase Associated). These domains have been found to play a role in β-lactam binding, protein stability, and bacterial morphogenesis (Sauvage et al. 2008; Sauvage and Terrak 2016).

Generally, two HMW-PBPs (one aPBP and one bPBP) are necessary for viability in most bacteria, but this paradigm has been challenged in *Bacillus subtilis* and enterococcus as the deletion of all aPBPs was tolerated (Arbeloa et al. 2004; McPherson and Popham 2003). This led to the discovery of new class of peptidoglycan transglycosylases, the SEDS protein family (RodA, FtsW). They require to be activated by a bPBP partner and their coupled activities were shown to produce cross-linked peptidoglycan (Meeske et al. 2016; Taguchi et al. 2019).

Bacterial cell wall growth and division is highly coordinated and regulated within specialized multiprotein complexes, the elongasome and divisome. Several protein-protein interactions between PBPs and between PBPs and the proteins in these complexes have been identified and shown to regulate the activities of the PBPs. In *E. coli*, the divisome proteins PBP3 and FtsW interact with each other and were shown to form a ternary complex with PBP1b (Leclercq et al. 2017). PBP3 regulates the interaction between FtsW and the lipid II substrate and its availability for the PBP1b synthase (Leclercq et al. 2017). Recently, the divisome proteins FtsBLQ were shown to directly interact with PBP1b and inhibit (via FtsL) its GTase activity (Boes et al. 2019). PBP1b also interacts with FtsN which stimulates its activities (Müller et al. 2007). The addition of the PBP1b activators, FtsN or LpoB, was found to suppress this inhibition by FtsBLQ and restore PBP1b activity (Boes et al.

2019). In addition, FtsBLQ (via FtsQ) were shown to inhibit the TPase domain of PBP3 but not of PBP1b (Boes et al. 2019).

The periplasmic steps of peptidoglycan synthesis and remodeling exhibit high level of redundancy (over 36 enzymes for 9 steps) (Pazos et al. 2017). Recent results show that bacteria use their redundant PBPs repertoire to cope with the variable environmental challenges. A compelling example is the presence in the pathogenic *Salmonella enterica* serovar Typhimurium of a second specialized penicillin-binding protein (PBP3sal) that is absent in nonpathogenic bacteria, in addition to the classical cell division specific PBP3. PBP3sal displays PG biosynthetic activity restricted to acidic conditions (it is inactive at neutral pH) and promotes cell division inside the host cells. In contrast, PBP3 that displays optimal activity at neutral pH is inactive at acidic pH (Castanheira et al. 2017).

Another example is the finding that the pH value can influence the fitness of *E. coli* cells with defects in PBP1a or PBP1b. Defects in these PBPs affect growth in alkaline and acidic condition, respectively. These results show that the virtually redundant PG synthases are required for optimal fitness across pH environments. Moreover, synthase specialization has consequences for intrinsic resistance to β-lactam antibiotics in nonstandard growth conditions (Mueller et al. 2019).

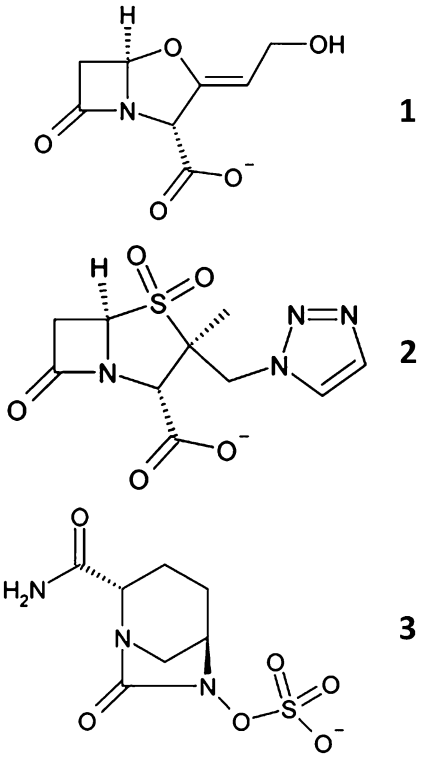
β-Lactamase-Mediated Resistance

The SXXK β- *lactamases* possibly result from the conversion of free-standing PBPs into β-lactam antibiotic-hydrolyzing enzymes, with loss of peptidase activity and conservation of the polypeptide fold. There are three classes of SXXK β-lactamases A, C, and D, which are easily distinguished on the basis of their sequences, although the tertiary structures are clearly similar and also related to those of the acyl transferase modules of PBPs. In particular, in addition to the characteristic SXXK, motifs reminiscent of the bar code SXN and KTG groups are found in nearly superimposable positions. The catalytic pathway of β-lactamases follows the three-step model shown above but with good substrates, the k_3 value can be higher than 1000 s^{-1} and the k_2/K value can be close to that of a diffusion-limited reaction (Matagne et al. 1999).

The class A enzymes have M_r values around 30,000. Their substrate specificities are quite variable and a large number of enzymes have emerged in response to the selective pressure exerted by the sometimes abusive utilization of antibiotics. Some of these "new enzymes" are variants of previously known enzymes, with only a limited number of mutations (1–4) but a significantly broadened substrate spectrum while others exhibit significantly different sequences. The first category is exemplified by the numerous TEM and SHV variants whose activity can be extended to third- and fourth-generation cephalosporins and the second by the NMCA, SME, and KPC enzymes which, in contrast to all other SXXK β-lactamases, hydrolyze carbapenems with high efficiency. Among the "extended-spectrum" enzymes, some β-lactamases whose genes are plasmid-borne are particularly worrying, for example, the CTX-M and KPC families. The first one hydrolyzes extended spectrum cephalosporins and monobactams and the second one many carbapenems and extended spectrum cephalosporins. Despite these specificity differences, the tertiary structures of all class A β-lactamases are nearly superimposable.

The class C enzymes have M_r values around 39,000 and exhibit more uniform properties. They hydrolyze benzyl- and phenoxymethyl penicillin relatively well (turn-over numbers of 20–70 s^{-1}), ampicillin and amoxicillin 10- to 20-fold less rapidly, and the other penicillins extremely slowly (generally due to low k_3 values). The early cephalosporins (cephalothin, cephalexin, cefazolin) are well hydrolyzed but the later generation compounds also exhibit low to very low k_3 values, as do imipenem and aztreonam. A few mutations are known to extend the spectrum of some enzymes but the most currently utilized bacterial strategy is overproduction by deregulation of the biosynthetic control mechanism, so that the MICs of poor or even very poor substrates can increase significantly (Lakaye et al. 1999).

Class D enzymes (M_r of 27–28,000) usually exhibit a high activity versus isoxazolyl



β-Lactam Antibiotics, Fig. 6 β-lacatamase inactivators: (1): clavulanic acid; (2) tazobactam; (3) avibactam

penicillins, such as oxacillin and are referred to as the OXA-family. Surprisingly, the amino group of the SXXK lysine residues is carboxylated in the most active forms of the enzymes (Vercheval et al. 2010). Penicillins are generally better substrates than cephalosporins but the number of identified OXA enzymes has significantly increased (and continues to increase) over the years and some of them hydrolyze extended spectrum cephalosporins, monobactams, and/or carbapenems.

Inactivators of class A β-lactamases (clavulanate, and sulphones such as sulbactam and tazobactam, Fig. 6) are themselves β-lactams and act as suicide substrates. They can be used in combination with β-lactamase-sensitive compounds (ampicillin, amoxicillin) but are not clinically useful against class C and class D producers. Some TEM mutants have also been selected that exhibit reduced sensitivity to these inactivators.

More recently, diazabiscyclo(3.2.1)octanones have been developed (e.g., avibactam, Fig. 6). They exhibit good inactivation properties against class A, class C, and some class D β-lactamases. Interestingly, with these compounds, the decay of the EI* adduct regenerates the EI complex containing the intact inactivator, but the k_{-2} step is generally too slow to be of physiological relevance (Ehmann et al. 2012).

The class B metallo-β-lactamases have emerged more recently as a clinical problem (e.g., the VIM, IMP, and NDM families whose genes are plasmid-borne). They are particularly dangerous since many of them hydrolyze all known β-lactams, with the exception of monobactams. In particular, they hydrolyze, although rather poorly, the suicide substrates mentioned above (to which they are not sensitive), as well as carbapenems that usually escape the activity of most the SXXK enzymes, with the exceptions mentioned above. Their genes also spread rather rapidly and are often associated, on the same plasmids, with genes encoding resistance properties to other classes of antibiotics.

Three subclasses B1, B2, and B3 can be distinguished on the basis of the sequences. B1 and B3 enzymes are optimally active with 2 Zn^{2+} ions, while a second Zn^{2+} ion inhibits the B2 enzymes. These B2 enzymes also exhibit a very narrow activity spectrum and only hydrolyze carbapenems. The 3D structures of representative members of each subfamily have been solved and they highlight a typical αββα fold, completely unrelated to that of the SXXK enzymes (Kerff et al. 2012).

The production of β-lactamases can be inducible or constitutive and the genes carried by the chromosome or plasmids, some genes being parts of integrons. A non-negligible number of clinical strains produce several distinct enzymes.

More information about β-lactamases (more than 1200 of them have been described so far) can be found in recent reviews by (Bush 2018; Bush and Bradford 2019).

PBP-Mediated Resistance

There are at least two modes of intrinsic resistance to β-lactam antibiotics. Determinants conferring a decreased susceptibility to β-lactam antibiotics evolve by the accumulation of point mutations in

genes that code for essential PBP fusions of classes A and/or B. The shuffling and capture of DNA sequences from commensal *Streptococci* having a reduced susceptibility to the drug give rise to *Streptococcus pneumoniae* pathogens in which mosaic genes code for mosaic PBP fusions of classes A and/or B of decreased affinity for the drug. Mosaic and wild-type PBP fusions of the same class (and the same subclass) differ by up to 15% amino acid residues (Calvez et al. 2017; Zapun et al. 2008). Mosaic PBPs occur also in *Neisseria meningitidis* and *Neisseria gonorrhoeae* strains.

Other strategies leading to an increased resistance are the transfer of a complete gene encoding a resistant PBP from a nonpathogenic-related species to yield the methicillin-resistant *Staphylococcus aureus* (MRSA) and the overproduction by *Enterococci* of a preexisting but minor resistant PBP, which can further mutate to even more resistant forms.

Some bacteria can also manufacture a peptidoglycan exhibiting 3–3 peptide cross-links (Fig. 2) with the help of structurally unrelated, penicillin-resistant LD-transpeptidases, which are not SXXK enzymes but active-cysteine enzymes that function according to the same general kinetic scheme as PBPs, with the cysteine -SH replacing the serine -OH group (Lecoq et al. 2013). In mycobacteria and *Clostridium difficile*, the proportion of (3–3) cross-links is large (70–80%). LDTs are also responsible for high levels of resistance to penicillins in laboratory mutants of *E. coli* and *Enterococcus faecium* (Triboulet et al. 2019). However, LDTs can be inactivated by carbapenems (Mainardi et al. 2007), and, in consequence, represent potential targets for the development of carbapenem-based therapies for multidrug-resistant tuberculosis.

However, LD-transpeptidases exclusively use acyl donor containing a tetrapeptide stem except LDT_{fs} from *Enterococcus faecalis,* which also uses a pentapeptide stem (Magnet et al. 2007). Consequently, hydrolysis of the C-terminal D-Ala of pentapeptide stems by a D,D-carboxypeptidase is essential to generate the tetrapeptide-containing substrate of the L,D-transpeptidases (Mainardi et al. 2002). This can be done by a metallo-DD-carboxy peptidase as in *E. faecium*, a resistant class C PBP as in *E. coli* or an "endopeptidase" that cleaves 4–3 cross-links to generate the tetrapeptide in *Mycobacterium smegmatis* (Baranowski et al. 2018).

Outer Membrane Permeability and Active Efflux Systems

In Gram-negative bacteria, diffusion of β-lactam antibiotics into the periplasm (where the activity of PBPs takes place) occurs via the channels that porins create in the outer membrane. The number and properties of the porin molecules are such that diffusion is relatively rapid in *E. coli* but much slower in *Enterobacter* and *Pseudomonas*. Mutants can be selected after the permeability of porin channels or their number has been decreased (Masi et al. 2019). A slow diffusion into the periplasm becomes a particularly important factor when it is combined with the presence of a β-lactamase (even in low concentration or when poorly active) in the same cellular compartment. Note that in *Mycobacteria*, the mycolic acid layer plays a role similar to that of the outer membrane in Gram-negatives.

Finally, β-lactams can be ejected from the periplasm by the active efflux systems of the RND family (e.g., AcrAB-TolC in *E. coli* and *Salmonella typhimurium* and MexAB-OprM in *Pseudomonas*). Compounds bearing a hydrophobic side-chain are particularly good substrates of these machineries whose overexpression can significantly increase the level of resistance but there are also pumps (AcrD and MexY) that can expel hydrophilic ligands (Nikaido 2018).

Clinical Use

Because the SXXK PBPs are specific to the prokaryotes, the β-lactam antibiotics have a high selective toxicity without marked side effects except for possible allergic reactions. Resistance is a problem of great concern. The use of antibiotics fuels the continuing emergence and spreading of novel β-lactamases and intrinsic resistance determinants among bacterial pathogens.

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L-DOPA

► Dopamine System

Lead Discovery by NMR

► SAR-by-NMR

LEAP2

► Ghrelin, Physiological Roles and Clinical Relevance of

Leonardo

► 14-3-3 Proteins

8-Leucine-Vasotocin

► Oxytocin

Leukotrienes

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Synonyms

Eicosanoids; Lipid mediators

Definition

Leukotrienes (LTs) are produced by the enzymatic oxidation of arachidonic acid (AA) released from cellular membrane (Shimizu 2009; Nakamura and Shimizu 2011). The 5-lipoxygenase (5-LO) cascade is involved in the production of these lipid mediators. Structural differences divide leukotrienes into two groups:

(i) leukotriene B_4 (LTB_4), which contains two hydroxy groups, and (ii) cysteinyl-leukotrienes (Cys-LTs), i.e., LTC_4, LTD_4, and LTE_4, based on the cysteine residue in their structures. LTB_4 is synthesized from AA through formation of LTA_4. LTB_4 is well-known as one of the potent chemoattractants and activators of leukocytes and is involved in inflammatory diseases. LTC_4 is also produced via LTA_4 by glutathione conjugation of LTA_4 with opening of the epoxide at the allylic position C-6. Next, LTC_4 is converted to LTD_4 by elimination of glutamic acid. The remaining peptide bond in LTD_4 is further hydrolyzed by a dipeptidase to generate LTE_4. These mediators cause smooth muscle contraction, enhance mucus secretion, recruitment of eosinophils to the airways, and increase vascular permeability, producing edema. They have a relatively short half-life (seconds to minutes) and can be degraded enzymatically.

Basic Mechanisms

LTs play important roles in the regulation of cell proliferation and differentiation, as well as the reproductive, gastrointestinal, and cardiovascular systems. In contrast, the excessive production of these mediators is associated with pathological manifestations of various diseases, such as asthma, allergic inflammation, and innate immunity. These lipid mediators exert their effects by binding to cognate G-protein-coupled receptors (GPCRs): BLT receptors with affinity to LTB_4 and Cys-LTs receptor family for LTC_4, LTD_4, and LTE_4 (Yokomizo et al. 2018).

Pharmacological Relevance

Production of LTB_4

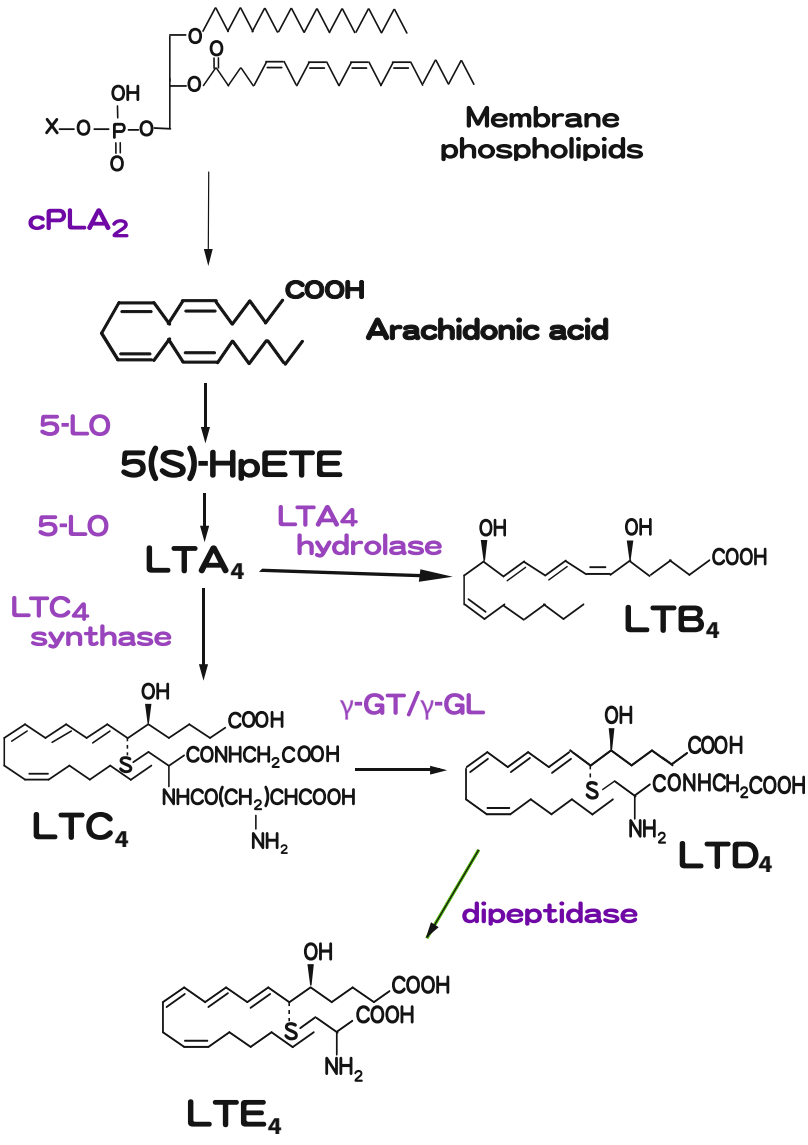
LTB_4 is one of the potent activators of leukocytes and is involved in various immune and inflammatory diseases. LTB_4 is produced from AA released from membrane phospholipids by the action of cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) (Shimizu 2009; Nakamura and Shimizu 2011). Two enzymes, 5-LO and LTA_4 hydrolase, are implicated in the biosynthesis of LTB_4. 5-LO catalyzes the first two steps, i.e., conversion of AA to 5(*S*)-HpETE (hydroperoxyeicosatetraenoic acid) and 5(*S*)-HpETE to LTA_4 (Fig. 1). During the activation of the cells, 5-LO-activating protein (FLAP) serves as a scaffold protein for 5-LO on the nuclear membranes. AA is transferred to 5-LO by FLAP, followed by dehydration to the unstable epoxide LTA_4. Thus, FLAP is a prerequisite protein for the 5-LO cascade. Then, LTA_4 is hydrolyzed by LTA_4 hydrolase to produce LTB_4. Transport of LTA_4 from the synthetic cells into a completely different cell containing either LTC_4 synthase or LTA_4 hydrolase is also involved in the biosynthesis of LTs (Figs. 2 and 3). This is the process called transcellular biosynthesis. Thus, production of LTB_4 is enhanced in both leukocytes and other cells in inflammation. LTA_4 hydrolase is a bifunctional zinc-containing enzyme with epoxide hydrolase and aminopeptidase activities. Although the biological significance of aminopeptidase activity of LTA_4 hydrolase is under debate, one paper suggested that aminopeptidase activity of LTA_4 hydrolase is important in the inactivation of an inflammatory peptide PGP (proline-glycine-proline) derived from collagen (Snelgrove et al. 2010). This enzyme is inactivated by its substrate LTA_4 (suicide inactivation).

Metabolism of LTB_4

LTB_4 has a relatively short half-life and is degraded enzymatically. The metabolic fates of LTB_4 seem to differ among the cells and tissues where LTB_4 is synthesized.

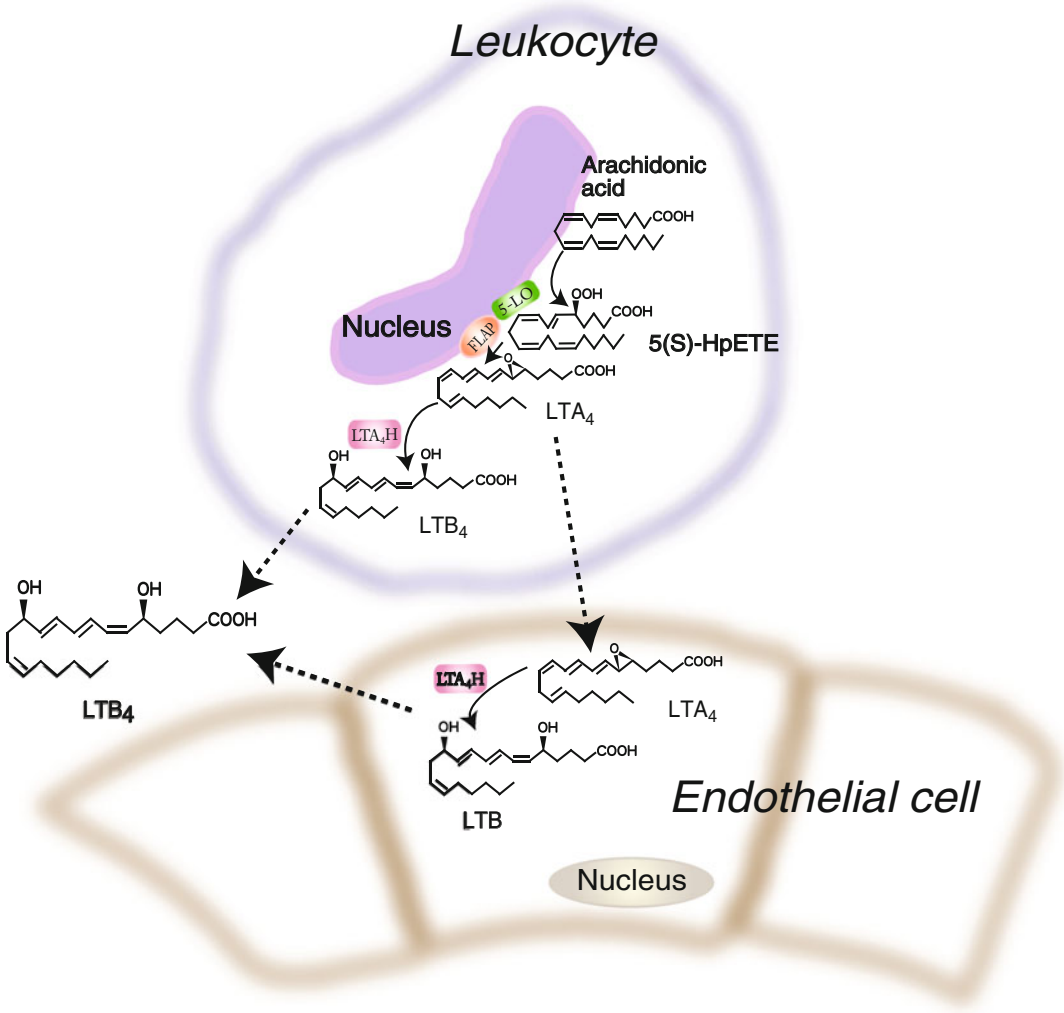
ω-Oxidation of LTB_4: Neutrophils metabolize LTB_4 into 20-hydroxy-LTB_4 (20-OH-LTB_4) by LTB_4 ω-hydroxylases and then 20-carboxy-LTB_4 (20-COOH-LTB_4). In neutrophils, one of the CYP4F isoforms, CYP4F3A, metabolizes LTB_4 with K_m values of 3.9 μM. Because 20-OH-LTB_4 binds to BLT1 with similar affinity to LTB_4, ω-oxidation itself does not inactivate LTB_4. An alternative pathway for subsequent ω-oxidation of 20-OH-LTB_4 was found to involve ADH (alcohol dehydrogenase) and AldDH (aldehyde dehydrogenase) in other tissues, e.g., liver.

β-Oxidation of LTB_4: For this metabolism, LTB_4 requires initial ω-oxidation to form ω-



Leukotrienes, Fig. 1 Biosynthetic pathways of leukotrienes. Enzyme names are also described: 5-LO, 5-lipoxygenase; $cPLA_2$, cytosolic phospholipase A_2

COOH-LTB_4, and CoA ester formation proceeds from the newly formed ω-carboxy group. 20-COOH-LTB_4 is metabolized via the peroxisomal and the mitochondrial β-oxidation pathways. The steps of chemical transformation that take place in peroxisomes start with 20-COOH-CoA esters, which are β-oxidized to a Δ^{18}-intermediate, catalyzed by acyl-CoA oxidase. This is followed by hydration of the C-18 double bond and then oxidation to form the 18-oxo-CoA ester by the peroxisomal bifunctional enzyme having both CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities. The penultimate carbon atoms of LTB_4 are then removed by 3-oxoacyl CoA thiolase, yielding the CoA ester of 18-COOH-LTB_4. This process is repeated for a second round of β-oxidation. The insertion of a new double bond adjacent to the 18-carboxy-CoA ester results in a conjugated diene involving the double bond at carbon atoms 14–15 in LTB_4. The peroxisomal enzyme 2,4-dieneoyl-CoA reductase reduces this diene to a monoene at C-16. The removal of the two terminal carbon atoms from 18-COOH-LTB_4 as acetyl-CoA leads to generation of the metabolites 16-COOH-tetranor-LTB_3.



Leukotrienes, Fig. 2 Biosynthesis pathways of LTB_4. AA is metabolized by 5-LO in the presence of FLAP first to 5(*S*)-HpETE then to LTA_4. LTA_4 is hydrolyzed by LTA_4 hydrolase (LTA_4H) to form LTB_4 in the cytoplasm. LTA_4 is converted to LTB_4 intracellularly and is exported for the transcellular synthesis

Metabolism by 12-hydroxydehydrogenase/15-oxo-prostaglandin-13-reductase: LTB_4 is also metabolized by the action of 12-hydroxydehydrogenase (12-HDH)/15-keto-prostaglandin 13-reductase (15-oPGR) (Yokomizo et al. 1993, 1996). The 12-HDH/15-oPGR cascade for the inactivation of LTB_4 is first discovered in porcine leucocytes, where several metabolites that did not retain the conjugated triene structure of LTs were found. This cascade had three separate structural conversions of the starting substrate. First, 12(*R*)-hydroxyl group of LTB_4 is oxidized by 12-HDH/15-oPGR, resulting in the generation of 12-oxo-LTB_4. The product of 12-hydroxy-oxidation is a conjugated ketone with a structural motif [R-CO-(*trans*)-CH=CH-R′] common to many LTs and PGs and as such is a structural unit that can be reduced in two steps to [R-CH(OH)-CH_2CH_2-R′]. 12-HDH/15-oPGR is known to perform the reduction of 15-oxo-PGs into 15-oxo-13, 14-dihydro-PG metabolites. However, it is still unclear whether this enzyme can reduce

12-oxo-LTB_4 to 12-oxo-10,11-dihydro-LTB_4 or whether a separate reductase carries out this step. Human keratinocytes metabolize LTB_4 to 10-HOTrE (10-hydroxyoctadecatrienoic acid) by the 12-HDH/15-oPGR cascade, suggesting that a C-1 CoA ester can be formed from 10,11-dihydro-LTB_4.

Characteristics of LTB_4 Receptors

Two LTB_4 receptors, BLT1 and BLT2, have been cloned (Table 1) (Yokomizo et al. 2018). BLT1, which is known as a high-affinity LTB_4 receptor, is expressed in various subsets of leukocytes and is responsible for LTB_4-dependent chemotaxis. BLT2 was originally identified as a low-affinity LTB_4 receptor and is now considered as a receptor for 12-hydroxyheptadecatrienoic acid (12-HHT). BLT2 is expressed in epidermal keratinocytes, epithelial cells of intestine, cornea, and lung, and is involved in wound healing and epidermal barrier function.

BLT1: BLT1 shows high affinity for LTB_4 with a *K*d value of 0.15 nM. In Chinese hamster ovary (CHO) cells expressing BLT1, this receptor is able to couple with both Gαi- and Gαq-like (Gα16) G-proteins. BLT1 is mainly expressed in various subsets of leukocytes, e.g., granulocytes, eosinophils, effector-type $CD4^+$ and $CD8^+$ T cells, dendritic cells, and macrophages. This receptor is further expressed in vascular smooth muscle cells and involved in atherogenesis and vascular injury. Docking study with LTB_4 and BLT1 demonstrates that LTB_4 would interact with the residues H96, R158, E187, and S243 of BLT1. These residues were predicted to be involved in the LTB_4 binding by the mutation study. Recently, the crystal structure of BLT1 with an antagonist BIIL260 was reported (Hori et al. 2018). The benzamidine moiety of BIIL260 interacted with the side chains of D66, V69, S106, W236, and S276, which are shared among most GPCRs. These residues bind water molecules as the sodium ion-centered water cluster, stabilizing the inactive form of BLT1. LTB_4 shows potent chemotactic effect on leukocytes because granulocytes and eosinophils from BLT1-null mice do not migrate toward LTB_4. BLT1 activation in leukocytes leads to degranulation through the production of phosphatidylinositol tris-phosphates (IP_3) by phosphatidylinositol 3-OH kinase (PI3 kinase). The receptor for advanced glycation end products (RAGE) was recently identified as a BLT1-binding protein and was determined as a molecular switch for BLT1, inhibits BLT1-dependent NF-κB activation, and stimulates BLT1-dependent chemotaxis. Recent study demonstrated that an LTB_4 gradient around inflammatory sites boosts BLT1 phosphorylation in a stepwise manner to facilitate the precise migration of leukocytes and initiation of local responses, including degranulation.

BLT2: BLT2 shares amino acid identities of 45% with BLT1. This receptor shows low affinity for LTB_4 with a *K*d value of 10–20 nM. Although BLT2 can be activated by 12(S)-HETE, 12(S)-HPETE, and 15(S)-HETE at micromolar concentrations, 12-HHT was identified as a high-affinity agonist for this receptor. 12-HHT activates BLT2 at lower concentrations than LTB_4 followed by the activation of Gαi- and Gαq-type G-proteins. Most classical BLT antagonists inhibit both BLT1 and BLT2, and a synthetic BLT2 specific agonist (CAY10583) is available. BLT2 is expressed in keratinocytes, epithelial cells of intestine and cornea, lung alveolar type 2 cells, and vascular endothelial cells.

Leukotrienes, Table 1 Characteristics of LTB_4 receptors

Receptor	BLT1	BLT2
Ligand	LTB_4 > 20-OH-LTB_4 > 12(*R*)-HETE	12-HHT > LTB_4 > 12(S)-HETE > 12(S)-HpETE > 15(S) HETE
Antagonist	BIIL260, LY255283, ZK158252, CP195543, ONO4057 U75302 (weak agonist)	LY255283, ZK158252 CP195543, LY244283, ONO4057
Expression (human)	Leukocytes > spleen, thymus, smooth muscle, lung, intestine	Intestine, skin > endothelial cells
Coupled G-protein	G_{i2}, G_{16}	G_q-like, G_i-like, G_z-like

LTB_4 in Health and Diseases

The binding of LTB_4 to its specific receptors on immune cells, e.g., neutrophils, macrophages, T cells, and DCs, evokes various cellular responses, such as chemotaxis, adhesion to vascular endothelial cells, release of lysosomal enzymes, and production of reactive oxygen species. For example, exogenous LTB_4 induces production of IL-2 and IFN-γ in $CD4^+$ T cells, and BLT1 antagonists block T-cell proliferation and IFN-γ production in vitro. The importance of LTB_4 in disease development has been determined from the detection of LTB_4 in inflammatory exudates and the efficacy of the receptor antagonists in abolishing or reducing the inflammatory condition. Recently, many studies have associated LTB_4 with various inflammatory events including asthma, atopic dermatitis, rheumatoid arthritis, septic peritonitis, psoriasis, and inflammatory bowel disease. The pathophysiological roles of LTB_4 are summarized in Table 2. In this regard, several blockers for LTB_4 receptors, as well as inhibitors of the LTB_4 biosynthesis, have been developed for clinical use.

Leukotrienes, Table 2 Pathophysiological roles of LTB_4

	Related diseases
Respiratory system	Bronchial asthma (confirmed in BLT1-null mice)
Joints	Rheumatoid arthritis (confirmed in both BLT1-null and BLT1/BLT2-null mice)
Brain and nerve system	Allergic encephalomyelitis Multiple sclerosis (confirmed in BLT1-null mice)
Colon	Inflammatory bowel disease (confirmed in BLT2-null mice)
Skin	Atopic dermatitis Psoriasis
Bone	Osteoporosis (confirmed in BLT1-null mice)

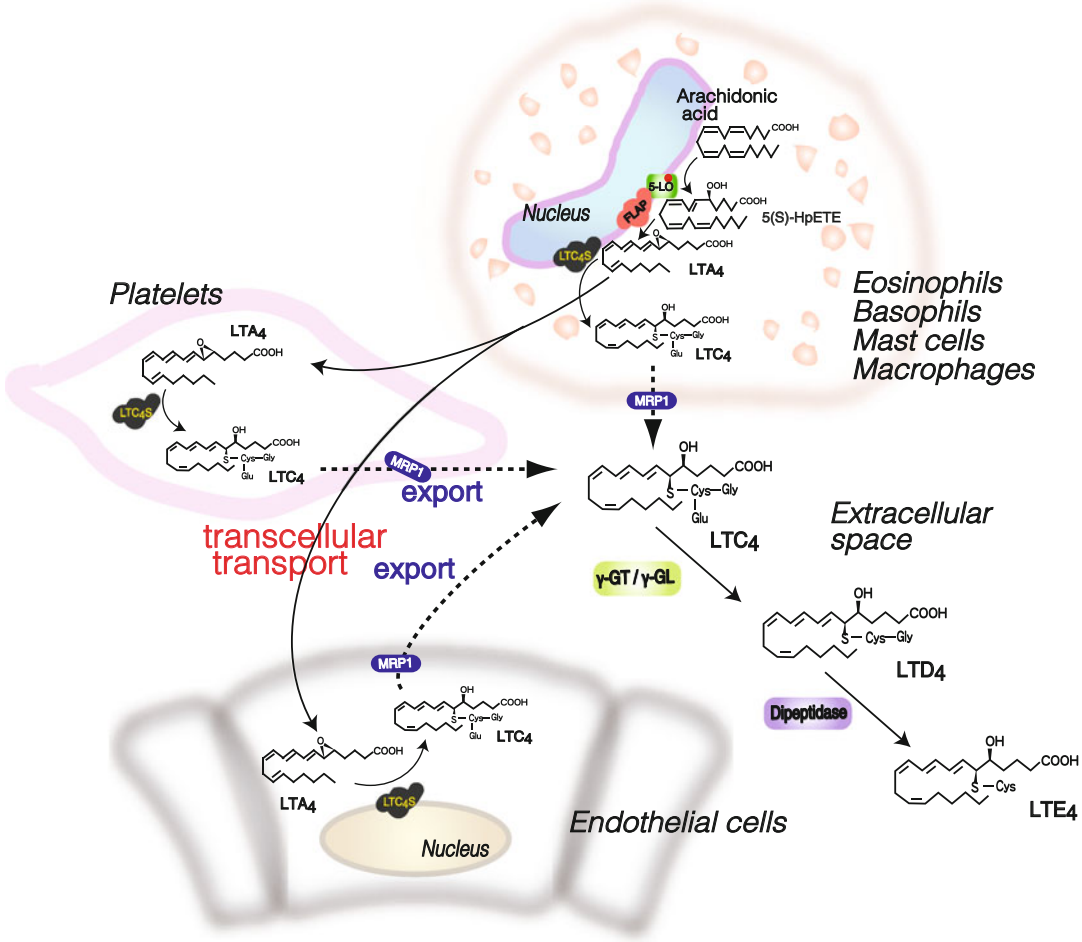
Characteristics of Cys-LTs

Production of Cys-LTs

As mentioned above, AA is converted to the intermediate 5(*S*)-HpETE and quickly to LTA_4 by 5-LO. In neutrophils, LTA_4 is hydrolyzed to LTB_4 by LTA_4 hydrolase, whereas eosinophils, basophils, mast cells, and macrophages preferentially produce LTC_4 through conjugation of a reduced glutathione to LTA_4 by LTC_4 synthase (Fig. 3) (Nakamura and Shimizu 2011). LTC_4 synthase is an integral nuclear membrane protein and is the key enzyme for the biosynthesis of this lipid mediator. Both FLAP and LTC_4 synthase constitutively localize to the perinuclear envelope, where LTC_4 synthesis is thought to occur. LTC_4 synthase is also expressed in platelets and endothelial cells, although these cells cannot synthesize LTA_4 on their own. However, these cells are able to synthesize LTC_4 using LTA_4 released by 5-LO-expressing cells via transcellular transport. The synthesized LTC_4 is exported to the extracellular space by multidrug resistance-associated protein 1 (MRP1). The transcellular metabolism of LTC_4 is associated with formations of LTD_4 and LTE_4. Namely, LTC_4 is converted extracellularly to LTD_4 by a γ-glutamyltranspeptidase (γ-GT) or γ-glutamylleukotrienase (γ-GL) and then to LTE_4 by a dipeptidase. LTE_4 is the most stable Cys-LT and can be measured after excretion into the urine. There were two important reports demonstrating the crystal structure of human LTC_4 synthase in its apo- and glutathione (GSH)-complexed forms. The LTC_4 synthase monomer has four transmembrane segments and forms a homotrimer as a unit with functional domains across each interface. The structure of the enzyme in complex with substrate revealed that the active site enforces a horseshoe-shaped conformation on GSH and effectively positions the thiol group for activation by a nearby Arg at the membrane-enzyme interface. These data provide new structural insights into the mechanism of LTC_4 formation and a structural basis for the development of LTC_4 synthase inhibitors for a pro-inflammatory cascade mediated by Cys-LTs.

Metabolism of LTE_4

LTE_4 is metabolized via similar pathways to LTB_4. After initial ω-oxidation to 20-COOH-LTE_4 and formation of the 20-CoA ester, metabolism proceeds through three steps of β-oxidation exclusively within the peroxisome. In rat hepatocytes, the most abundant metabolite observed is *N*-acetyl-16-COOH-tetranor-LTE_3. *N*-acetylation



Leukotrienes, Fig. 3 Biosynthesis pathway for the conversion of AA into Cys-LTs. AA is metabolized by 5-LO in the presence of FLAP first to 5(*S*)-HpETE then to LTA_4. LTC_4 synthase, which is a nuclear membrane protein, conjugates glutathione (GST) to this intermediate to form LTC_4. In the extracellular space, further generation to LTD_4 and LTE_4 takes place by γ-GT or γ-GL and dipeptidase, respectively

is a major metabolic step for LTE_4 in the rat even before ω/β-oxidation; however, it is not a major metabolic pathway in human tissues. Intact LTE_4 is also excreted into urine as a major metabolite due to its stability. Thus, LTE_4 levels are monitored in the urine, sputum, and exhaled breath condensate as an index of the Cys-LT synthesis in human diseases such as asthma, where its concentrations are markedly elevated.

Characteristics of Cys-LT Receptors

So far, five Cys-LT receptors have been identified: CysLT1, CysLT2, P2Y12, GPR99, and GPR17 (Table 3) (Yokomizo et al. 2018). CysLT1 is widely expressed in the spleen, leukocytes, lung, small intestine, colon, and skeletal muscle. CysLT2 exhibits 37.3% amino acid identity with CysLT1 and is exclusively expressed in the heart, adrenals, leukocytes, spleen, lymph nodes, and brain. CysLT1 is preferentially activated by LTD_4, whereas CysLT2 binds both LTC_4 and LTD_4 with equal affinity. Recently, P2Y12, GPR99, and GPR17 were reported as receptors for LTE_4. Furthermore, GPR17 has been proposed as a putative negative regulator of CysLT1.

CysLT1: CysLT1 is expressed in a variety of inflammatory cells, i.e., neutrophils, mast cells, and monocytes/macrophages, suggesting the

Leukotrienes, Table 3 Characteristics of Cys-LT receptors

Receptor	CysLT1	CysLT2	P2Y12	GPR99	GPR17
Ligand	$LTD_4 > LTC_4 >> LTE_4$	$LTD_4 = LTC_4 >> LTE_4$	$LTE_4 > LTD_4$, ADP	LTE_4, α-ketoglutarate	$LTE_4 > LTD_4$, UDP
Antagonist	Montelukast, zafirlukast, pranlukast, MK571, pobilukast	Zafirlukast, pranlukast, BAY-u9773, gemilukast	Clopidogrel		
Expression (human)	Leukocytes, spleen, smooth muscle > lung, intestine	Leukocytes, spleen, adrenal medulla, lung, heart, brain	Mast cells	Kidney, placenta, trachea, lung, salivary glands, smooth muscle	Brain
Coupled G-protein	G_q,G_{11},G_i,G_o	G_q,G_{11},G_i,G_o	G_i, G_o	G_q, G_{11}	G_i, G_o

clinical effectiveness of the antagonists in asthma. Human CysLT1-expressing cells respond to Cys-LTs with rank order of potency: $LTD_4 > LTC_4 > LTE_4$. Activation of CysLT1 by LTD_4 results in the production of several second intracellular messengers through phospholipase Cβ. Several reports demonstrated that Cys-LTs elicit Ca^{2+} responses via a pertussis toxin (PTX)-sensitive G-protein (Gαi/o) in peripheral blood mononuclear cells or through two distinct G-proteins, PTX-sensitive and insensitive (Gαq/11), in monocyte/macrophage U937 cells, as well as a human epithelial cell line, suggesting the promiscuity of CysLT1 in G-protein coupling.

CysLT2: Although both CysLT1 and CysLT2 are highly expressed in spleen, CysLT2 exclusively existed in the heart and eosinophils. In situ hybridization analyses of human lung demonstrated that CysLT2 is expressed in interstitial macrophages and smooth muscle cells. Moreover, the presence of human CysLT2 mRNA was determined in atrium, ventricle, and intermediate coronary arteries. The potency ranking for the competition with $[^3H]LTD_4$ binding to human CysLT2 is $LTD_4 = LTC_4 >> LTE_4$. The CysLT1 antagonists are either weak (zafirlukast and pranlukast) or inactive (montelukast and pobilukast) at competing for $[^3H]LTD_4$ binding to human CysLT2, whereas full competition was observed with the dual CysLT1/CysLT2 antagonist BAY-u9773. LTC_4 and LTD_4 elicit a dose-dependent activation of Ca^{2+} flux through a PTX-insensitive G-protein (Gαq/11) in human CysLT2-expressing oocytes. In contrast, in human mast cells, the IL-8 production through the CysLT2 activation is completely blocked by PTX, suggesting it occurs via Gαi/o-protein. Recent reports demonstrated that CysLT2 negatively regulates the development of Th2 pulmonary inflammation by inhibiting the CysLT1 functions on dendritic cells. Furthermore, LTC_4, but not LTD_4 and LTE_4, activates mouse platelets via CysLT2, although these cells express both CysLT1 and CysLT2.

Other Receptors for Cys-LTs

Some of LTE_4-dependent responses are resistant in CysLT1/CysLT2-null mice, indicating the presence of other LTE_4 receptor(s). Based on the similarities of CysLT1 and CysLT2 with the nucleotide P2Y receptors, LTE_4 receptor seemed to be P2Y-like GPCRs. Human mast cells express P2Y12, an adenosine diphosphate receptor. Deficiency of this receptor impaired the LTE_4-elicited responses, e.g., productions of MIP-1β and PGD_2 in LAD2 cells without altering their responses to LTD_4. In the CHO cells expressing P2Y12, LTE_4 elicits the ERK1/2 activation which is sensitive to PTX. Furthermore, administration of LTE_4 to the airways of sensitized BALB/c mice induces eosinophilia, goblet cell metaplasia, and IL-13 production in response to low-dose aerosolized OVA. These responses are intact in CysLT1/CysLT2-null mice but are blocked by administration of clopidogrel, a P2Y12-selective antagonist. GPR99 belongs to the P2Y receptor subfamily and was initially identified as a receptor for α-ketoglutarate. Because the α-ketoglutarate-evoked response in the GPR99-expressing cells is insensitive to PTX, GPR99 seems to be coupled with Gαq/11. Recently, GPR99 was shown to be a high-affinity receptor for LTE_4 with a *K*d value of 2.5 nM. This receptor is highly expressed in the kidney, placenta, trachea, salivary glands, lung, and smooth muscle. GPR99 deficiency eliminated vascular leaks in response to Cys-LTs in the CysLT1/CysLT2-null mice. Because LTE_4 elicits airflow obstruction and lung inflammation in asthmatics, blockage of the GPR99 signaling may have therapeutic benefit in asthma. GPR17 also belongs to the P2Y receptor family and is activated by two unrelated ligands: uracil nucleotides and Cys-LTs. Activation of GPR17 leads to intracellular Ca^{2+} increase and inhibition of cAMP synthesis, indicating the coupling with Gαi/o proteins. Recent studies demonstrate that the administration of montelukast, a CysLT1 antagonist, leads to the reduced neuroinflammation, elevation of hippocampal neurogenesis, and improved learning and memory in old rats. These effects are impaired in GPR17-null mice, suggesting the involvement of this receptor in the rejuvenation of the aged brain. Recent reports demonstrated that GPR17 suppresses CysLT1-mediated signaling through heterodimerization. In IgE-dependent passive cutaneous anaphylaxis, vascular permeability is enhanced in GPR17-null mice. This response is blocked by administration of a

Leukotrienes, Table 4 Pathophysiological roles of cys-LTs

	Related diseases
Respiratory system	Bronchial asthma Airway smooth muscle contraction, eosinophil influx, edema, mucus production, smooth muscle hyperplasia/hypertrophy, goblet cell metaplasia, increase in microvascular permeability
	Pulmonary fibrosis Macrophage and neutrophil recruitment, fibroblast accumulation, collagen deposition
Skin	Atopic dermatitis Edema, skin thickening, and fibrosis
Others	Atherosclerosis Allergic rhinitis

CysLT1 antagonist, proposing CPR17 as a negative regulator for CysLT1. This negative regulation was further confirmed in both the antigen presentation and downstream phases of allergic pulmonary inflammation, demonstrating physiological evidence for its negative regulatory role.

Cys-LTs in Health and Diseases

Generations of the mice deficient in Cys-LT receptors and development of receptor antagonists have expanded the scope of functions served by Cys-LTs. Cys-LTs participate in the inflammatory component of asthma by increased microvascular permeability, bronchoconstriction, recruitment of effector cells (especially eosinophils and mast cells), mucus and cytokines secretion, and fibrosis. The pathophysiological roles of Cys-LTs are summarized in Table 4. The importance of Cys-LTs in these diseases has been supported by animal models and awaits validation in humans.

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Lipid Mediators

▶ Leukotrienes

Lipid-Lowering Drugs

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Synonyms

Anti-sense oligonucleotides; Bempedoic acid; Cholesteryl ester transfer protein inhibitors; Fibrates; Microsomal triglyceride transfer protein inhibition; Niacin; Omega-3 fatty acids; PCSK9 inhibition; Statins

Definition and Introduction

Relationships between plasma lipid levels and the risk of having an atherosclerotic cardiovascular

disease (ASCVD) event have been observed consistently in many human population studies. There is also support for a causal relationship between some plasma lipoprotein fractions and ASCVD risk, with overwhelming evidence from large, randomized, double-blind, placebo-controlled, clinical trials that interventions that reduce the level of low-density lipoprotein cholesterol (LDL-C) and its constituent apolipoprotein, apoB, also reduces the risk of having an ASCVD event (Cholesterol Treatment Trialists et al. 2010). This has led to a longstanding, major investment in the development of drugs that lower LDL-C and apoB levels. In recent years, the focus of these efforts has been extended to reducing the cholesterol content of all atherogenic lipoproteins, which is commonly referred to as non-high-density lipoprotein cholesterol (non-HDL-C).

Mechanisms of Action and Clinical Use

LDL-C, Non-HDL-C, and ApoB Lowering Drugs in Current Use

Evidence from epidemiologic studies, genetic studies, and randomized, double-blind, placebo-controlled clinical trials have established that the concentrations of LDL-C, non-HDL-C, and apoB are positively and causally related to the risk of having an ASCVD event (Cholesterol Treatment Trialists et al. 2010). Global guidelines support the use of multiple agents to lower LDL-C, non-HDL-C, and apoB levels for reducing ASCVD risk.

Statins

Statins in current clinical use are atorvastatin, rosuvastatin, pitavastatin, simvastatin, pravastatin, and fluvastatin. All statins inhibit hydroxymethyl-glutaryl-CoA (HMGCoA) reductase, the main rate-limiting enzyme in cellular cholesterol synthesis. The statin-induced inhibition of cholesterol synthesis reduces cell cholesterol concentrations. This activates a series of homeostatic processes that normalize cell cholesterol levels, including activation of sterol regulatory element binding protein-2 (SREBP-2), which increases synthesis of the LDL receptor. Upregulation of the LDL receptor accelerates uptake of LDLs from the circulation, which normalizes cell cholesterol levels and decreases the plasma concentration of LDL-C.

Treatment with a statin can reduce the concentration of plasma LDL-C by up to 50% and has been shown consistently in large, randomized, double-blind, placebo-controlled clinical outcome trials to reduce the risk of having a future ASCVD event in all patient groups (Cholesterol Treatment Trialists et al. 2010).

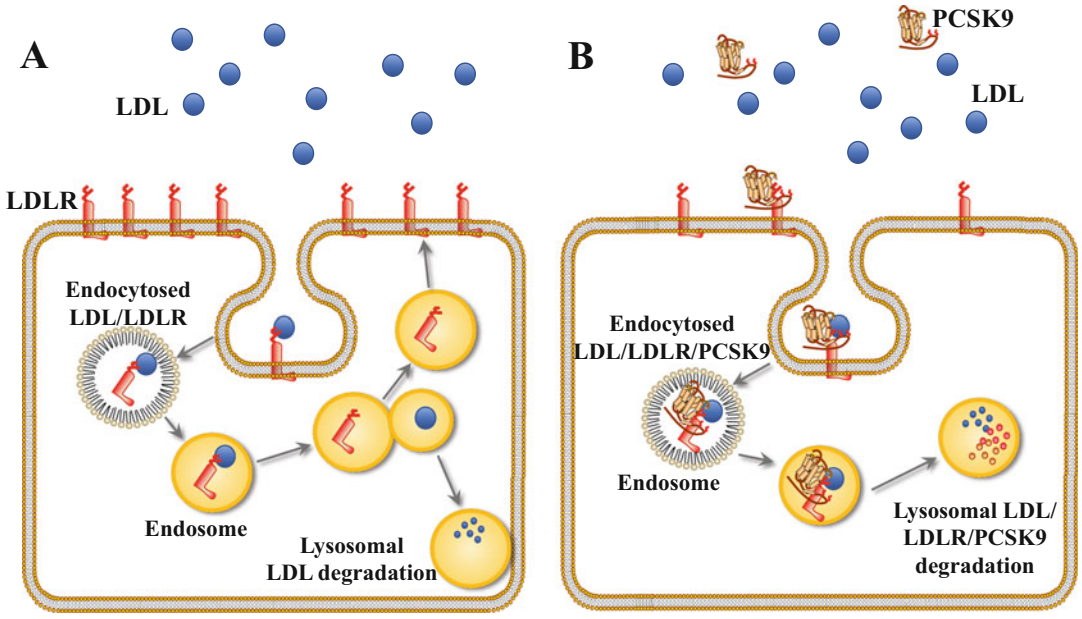
The statin-induced activation of SREBP-2 in response to a fall in cell cholesterol levels not only increases the number of cell surface LDL receptors but also increases the synthesis of proprotein convertase subtilisin/kexin type 9 (PCSK9) (Horton et al. 2007). PCSK9 maintains cholesterol homeostasis by degrading LDL receptors and inhibiting cellular cholesterol accumulation. While this is advantageous under conditions that lead to cellular cholesterol depletion, the ability of PCSK9 to degrade LDL receptors also limits the LDL-C-reducing action of statins.

As a class, when given at recommended doses, statins have few serious adverse effects. Adverse effects of statins that have been reported include myositis and an increase in new onset diabetes in those already at risk of developing type 2-diabetes. However, these adverse effects are greatly outweighed by the cardiovascular benefits of statin treatment.

PCSK9 Inhibitors

PCSK9 is expressed mainly in the liver and secreted into the plasma, where it binds to LDL receptors (Benjannet et al. 2004). In the absence of PCSK9, LDLs that bind to LDL receptors are endocytosed into lysosomes. The low pH within lysosomes causes LDLs to dissociate from the LDL receptor. The LDLs are then degraded, while the LDL receptor recycles back to the cell surface where it binds more LDLs (Fig. 1a). It has been estimated that each LDL receptor can recycle between intracellular compartments and the cell surface up to 150 times (Goldstein et al. 1985).

When a circulating LDL particle interacts with a cell surface LDL receptor to which PCSK9 is



Lipid-Lowering Drugs, Fig. 1 Degradation of the LDL receptor (LDLR) by PCSK9. Panel A: LDL binds to the LDLR on the cell surface and is endocytosed into lysosomes, where it dissociates from the LDLR and is targeted for degradation, freeing up the LDLR for recycling back to the cell surface. **Panel B**: In the presence of PCSK9, LDL and PCSK9 bind to the LDLR on the cell surface, forming an LDL/LDLR/PCSK9 complex that is degraded in lysosomes and inhibits the recycling of the LDLR back to the cell surface

already bound, a ternary complex containing PCSK9, the LDL receptor, and the LDL particle is formed (Fig. 1b). This complex enters the cell, where the PCSK9 prevents the LDL from dissociating from the LDL receptor and the entire complex (including the LDL receptor) is directed towards lysosomes for degradation (Benjannet et al. 2004). This stops LDL receptor recycling, reduces the number of LDL receptors on the cell surface, decreases the removal of LDLs from the circulation, and increases the plasma concentration of LDL-C, non-HDL-C, and apoB. Thus, PCSK9 partly negates the benefits of increasing LDL receptor expression with a statin.

Gain-of-function mutations in the *PCSK9* gene increase the plasma concentration of LDL-C and apoB, while loss-of-function mutations decrease LDL-C levels and are associated with reduced ASCVD risk. Preclinical studies in mice have furthermore established that overexpression of the *PCSK9* gene in mice increases susceptibility to atherosclerotic lesion development, while its inhibition reduces atherosclerotic lesion development (Rashid et al. 2005).

Inhibition of PCSK9 is thus a logical approach to reduce ASCVD risk. Inhibition of PCSK9 has been achieved with: (i) monoclonal antibodies that bind to and inactivate PCSK9 in plasma and (ii) inhibitors of PCSK9 synthesis. Fully human anti-PCSK9 monoclonal antibodies, including alirocumab and evolocumab, have been developed and approved for clinical use. These antibodies reduce the plasma concentration of LDL-C by more than 50%, including when given to people already taking a statin, and no major safety issues have yet been reported (Raal et al. 2012; Stein et al. 2012). Anti-PCSK9 monoclonal antibodies also reduce the risk of having an ASCVD event in large, double-blind, placebo-controlled clinical outcome trials (Sabatine et al. 2017) (Robinson et al. 2015).

Agents that inhibit the synthesis of PCSK9 are under investigation but are not yet available for clinical use. They include a PCSK9 antisense

oligonucleotide (ASO) that reduces plasma cholesterol levels by more than 50% (Graham et al. 2013) and a locked nucleic acid ASO that decreases PCSK9 mRNA levels and increases LDL receptor expression (Gupta et al. 2010). A PCSK9 siRNA that reduces LDL-C levels in nonhuman primates has also been reported (Frank-Kamenetsky et al. 2008). Clinical outcome trials with these agents are underway.

Inhibitors of Intestinal Cholesterol Absorption

Ezetimibe blocks the ability of the protein Niemann-Pick C1-Like 1 (NPC1L1) to promote the intestinal absorption of cholesterol. Inhibition of NPC1L1 by ezetimibe reduces intestinal absorption of cholesterol, thus reducing the hepatic uptake of dietary cholesterol. The resulting decrease in hepatocyte cholesterol levels increases the number of LDL receptors at the hepatocyte surface, lowers the plasma concentration of LDL-C by about 20% and significantly reduces the risk of having an ASCVD event (Cannon et al. 2015).

Bile Acid Sequestering Agents

Bile acid sequestering agents (including cholestyramine) have been used for many years to reduce the plasma concentration of LDL-C. They act by binding to bile acids in the intestine, thus preventing bile acid reabsorption. This increases the synthesis of bile acids from cholesterol in hepatocytes, which transiently reduces hepatocyte cholesterol levels and upregulates the synthesis and cell surface expression of the LDL receptor. Bile acid sequestrants reduce the plasma concentration of LDL-C by ~20% and, in the case of cholestyramine, reduced clinical cardiovascular events in the pre-statin era (Lipid Research Clinics Coronary Primary Prevention Trial 1984).

Inhibitors of ApoB Synthesis

ApoB is the main apolipoprotein component of very low-density lipoproteins (VLDLs) and LDLs. It is synthesized mainly in the liver. Inhibiting apoB synthesis has the potential to reduce the concentration of all apoB-containing lipoproteins. This is especially important in people with homozygous familial hypercholesterolemia (hoFH) who lack functional LDL receptors and do not respond to statins and PCSK9 inhibitors.

Mipomersen, an ASO that inhibits apoB synthesis in the liver, reduces apoB levels by 33–54% and LDL-C levels by 34–52% (Kastelein et al. 2006). While this is encouraging, Mipomersin has a high incidence of adverse effects, including severe injection site reactions in more than 70% of subjects, flu-like symptoms in more than 25% of subjects and headache in more than 15% of subjects.

Inhibitors of Microsomal Triglyceride Transfer Protein (MTP)

Another approach for reducing LDL levels in people who lack functional LDL receptors is to inhibit MTP. This protein is synthesized in the liver and intestine, where it transfers triglycerides to apoB to form apoB-containing lipoproteins.

Lomitapide is a small molecule that binds to MTP and blocks the transfer of triglycerides to apoB, thus preventing the formation of apoB-containing lipoproteins. Treatment with lomitapide reduces the concentration of LDL-C in humans with hoFH (Cuchel et al. 2013). Most patients taking lomitapide experience adverse effects, including gastrointestinal symptoms and elevation of liver transaminases. Lomitapide has been approved in some countries for treatment of people with hoFH.

Triglyceride-Lowering Drugs in Current Use

Evidence that the concentration of plasma triglycerides and triglyceride-rich lipoproteins are causally related to ASCVD risk has recently emerged. This indicates that agents that lower plasma triglycerides may reduce ASCVD risk.

Fibrates

Fibrates are agonists of the hormone-activated nuclear receptor, peroxisome proliferator-activated receptor alpha (PPARα) (Staels et al. 1998). Fibrates increase the oxidation of free fatty acids in the liver and reduce hepatic synthesis of triglycerides. Activation of PPARα also induces expression of lipoprotein lipase (LPL), the enzyme that hydrolyses triglycerides to glycerol and free fatty acids. LPL also hydrolyses the phospholipids in VLDLs and chylomicrons to

lysophospholipids and free fatty acids. Thus, as activators of PPARα, fibrates reduce plasma triglyceride levels by the combined effects of reducing its synthesis and increasing its hydrolysis. Activation of PPARα by fibrates also inhibits the synthesis of apolipoprotein (apo) C-III, an apolipoprotein that delays the catabolism of triglyceride-rich lipoproteins. Moreover, fibrates increase the plasma concentration of high-density lipoprotein cholesterol (HDL-C), possibly by increasing the synthesis of apolipoprotein (apo) A-I and apoA-II, the two main HDL apolipoproteins. The mechanism of this effect is not fully understood.

The results of clinical outcome trials of fibrates have varied, although in all of the human fibrate trials conducted so far, there has been a consistent finding that people with high plasma triglyceride and HDL-C levels have a reduction in cardiovascular events that is greater than what can be explained by the fall in plasma triglyceride levels, or the increase in HDL-C levels (Barter and Rye 2008). The mechanism of this cardiovascular benefit is not known.

Fibrates are relatively weak PPARα agonists and their efficacy is limited by dose-related adverse effects. New PPARα modulators that are more potent than fibrates, and may have fewer adverse effects, are currently under investigation. An insight into whether such agents will eventually play a significant role in the management of ASCVD risk may become more clear when the results of an ongoing human clinical outcome trial are reported.

Omega-3 Fatty Acids

Omega-3 fatty acids are dietary fats that inhibit lipogenesis and increase LPL activity, thus reducing the concentration of plasma triglycerides (Davidson 2006). There is also evidence that omega-3 fatty acids have anti-inflammatory effects and may improve the stability of atherosclerotic plaques (Thies et al. 2003).

Treatment with omega-3 fatty acids reduces the risk of having an ASCVD event (Bhatt et al. 2019), although it is not known whether the reduction in ASCVD risk relates to the fall in triglyceride levels or to some other action of these agents.

Niacin

Niacin (vitamin B3) has been used to modify plasma lipid levels for more than 50 years. When given in pharmacological doses, niacin reduces the level of plasma triglycerides by about 35%, reduces LDL-C levels by 10–15% and increases the concentration of HDL-C by up to 25%. The reduction in plasma triglycerides may be a reflection of the ability of niacin to inhibit the release of free fatty acids from adipose tissue. This decreases the plasma concentration of free fatty acids and the hepatic uptake of free fatty acids, leading to a reduction in hepatic triglyceride synthesis. The mechanism by which niacin decreases LDL-C levels and increases HDL-C levels is not known.

In a trial conducted in the pre-statin era, treatment with niacin significantly reduced ASCVD events. However, two recent large, randomized clinical outcome trials of niacin conducted in people taking statins did not reduce ASCVD events. The outcome of these trials suggest that it is unlikely that niacin will be used in future as a mainstream therapy for reducing ASCVD.

Lipid-Lowering Drugs Not Currently Used Clinically

Inhibitors of Cholesteryl Ester Transfer Protein (CETP)

CETP is a plasma protein that transfers cholesteryl esters from the non-atherogenic HDL fraction where they are synthesized to potentially the pro-atherogenic non-HDL fraction. Inhibition of CETP blocks this transfer, which decreases the concentration of cholesterol in the potentially pro-atherogenic non-HDL fraction and increases the concentration of cholesterol in the non-atherogenic HDL fraction. The mechanism by which CETP inhibitors lower the plasma level of LDL-C and apoB is not known with certainty. It may be the consequence of decreased transfer of cholesteryl esters from the HDL fraction into the LDL fraction. However, there is also evidence that rate of removal of LDLs from plasma is increased (Millar et al. 2015). This implies that the number of LDL receptors in the liver may increase when activity of CETP is inhibited.

The results of human genetic studies support the proposition that CETP is pro-atherogenic and that its inhibition may reduce ASCVD risk (Johannsen et al. 2012). Inhibition of CETP in rabbits protects against development of atherosclerosis (Sugano et al. 1998). Inhibition of CETP as a strategy to reduce ASCVD risk in humans has been disappointing, with no reduction in ASCVD events in trials with three inhibitors (torcetrapib, dalcetrapib, evacetrapib) but a significant reduction in events with a fourth inhibitor (anacetrapib) (Group et al. 2017).

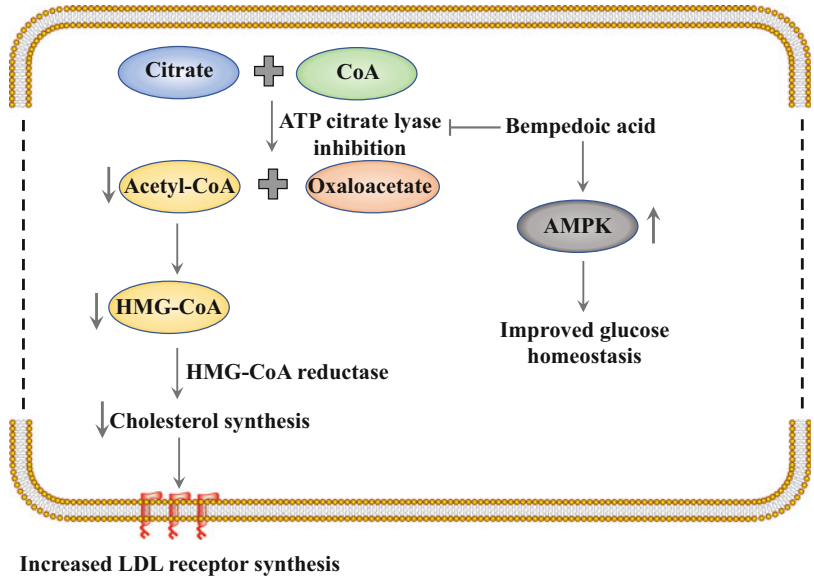
Inhibition of Adenosine Triphosphate Citrate Lyase with Bempedoic Acid

Adenosine triphosphate citrate lyase is a cytosolic enzyme expressed in liver and adipose tissue. It catalyzes the reaction in which citrate plus coenzyme A (CoA) are converted into acetyl-CoA and oxaloacetate (Fig. 2). Acetyl-CoA is an essential substrate in the pathway of cholesterol synthesis that acts upstream of HMG-CoA, the molecular target of statins. Pharmacological inhibition of adenosine triphosphate citrate lyase with bempedoic acid (also known as ETC-1002) reduces cytosolic acetyl CoA levels, decreases hepatic sterol synthesis, up-regulates the LDL receptor and reduces plasma LDL-C levels by up to 27%. Bempedoic acid also activates adenosine monophosphate (AMP), which can impact favorably on glucose homeostasis (Fig. 2). Effects of bempedoic acid on ASCVD risk are currently under investigation and it has been submitted for approval by the FDA.

Inhibitors of Lipoprotein (a) (Lp(a)) Synthesis

Lp(a) is an LDL particle to which an apolipoprotein(a) (apo(a)) molecule is attached via a disulfide bond. Lp(a) is pro-thrombotic, impairs fibrinolysis, and it activates endothelial cells (Kronenberg and Utermann 2013). It also transports pro-inflammatory, oxidized phospholipids that can increase cardiovascular risk. Elevated plasma levels of Lp(a) are genetically determined and associated with an increased risk of having an ASCVD event. Interventions that reduce the level of Lp(a) in plasma are therefore predicted to reduce ASCVD risk.

Inhibitors of PCSK9 reduce plasma Lp (a) levels by about 30% by an unknown mechanism. An almost 80% reduction in plasma levels of Lp(a) has been achieved with ASOs that inhibit apo(a) synthesis (Tsimikas et al. 2015). Apo(a) ASOs also reduce the levels of oxidized phospholipids that are associated with apoB-100-containing lipoproteins and Lp (a) (Tsimikas et al. 2015). Whether a therapeutic reduction in plasma levels of Lp (a) translates into a reduction in ASCVD events is currently not known.



Lipid-Lowering Drugs, Fig. 2 Bemedoic acid inhibits cholesterol biosynthesis and improves glycemic control. Bempedoic acid reduces cholesterol biosynthesis by inhibiting ATP citrate lyase, which is upstream of HMG-CoA reductase. Bempedoic acid also improves glucose homeostasis by upregulating AMPK

Inhibitors of ApoC-III Synthesis

ApoC-III is a pro-inflammatory protein that circulates in plasma as a component of VLDLs and HDLs. ApoC-III inhibits LPL and impacts adversely on HDL function. An elevated level of apoC-III in apoB-containing lipoproteins is an independent predictor of the risk of having an ASCVD event (Jorgensen et al. 2014). Transgenic overexpression of human apoC-III in mice increases both plasma triglyceride levels and susceptibility to atherosclerosis. Genetic studies in humans have shown that people with loss-of-function mutations in the *APOC3* gene have a significantly reduced risk of having an ASCVD event (Jorgensen et al. 2014), which makes the case for developing apoC-III inhibitors strong.

An apoC-III ASO that reduces the synthesis of apoC-III, decreases plasma triglyceride levels, and increases HDL-C levels in hypertriglyceridemic patients has been developed (Graham et al. 2013; Gaudet et al. 2015). The effects of inhibiting apoC-III synthesis on ASCVD risk remain to be established.

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Lipoprotein Receptors

▶ Low-Density Lipoprotein Receptor Gene Family

Local Anesthetic Drugs

▶ Local Anesthetics

Local Anesthetics

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Synonyms

Local anesthetic drugs

Definition

Local anesthetics are drugs that reversibly prevent impulse generation and conduction in peripheral nerves. This action can lead to loss of sensation in

a limited area of the body without the loss of consciousness. Although local anesthetics are often used as analgesics, they may cause other physiological changes in autonomic, somatic, or muscle function.

Mechanism of Action

Chemistry

Most local anesthetics contain hydrophobic and hydrophilic moieties that are separated by an intermediate ester or amide linkage (Table 1). The hydrophobic group is usually an unsaturated aromatic ring, and the hydrophilic group is mostly a tertiary amine. Both groups are important for the drug binding to the target channel (voltage-gated Na^+ channel), and an ester or amide linkage affects the metabolism of the drugs. Since the ester bond is more prone to hydrolysis than the amide bond, esters usually show a shorter duration of action. The blocking potencies of the drugs range from a few micromolar to millimolar half-maximal blocking concentration and highly dependent on lipid solubility (Table 1).

Most local anesthetics are weak bases with pK_a values ranging from 7.5 to 9 and exist either as the charged form or the uncharged form at physiologic pH. The relative proportions of these two forms are shown in the Henderson-Hasselbalch equation:

$$\log [\text{charged form}]/[\text{uncharged form}] = pK_a - pH$$

According to this equation, the larger fraction of local anesthetics is the charged form at physiologic pH. An exception is benzocaine with a pK_a of 3.5 and benzocaine exists solely as the uncharged form under normal physiologic conditions.

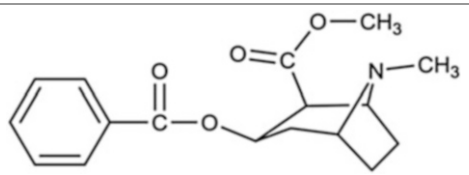
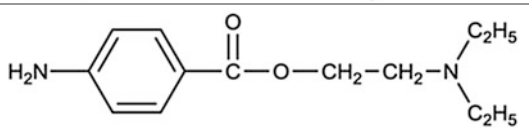
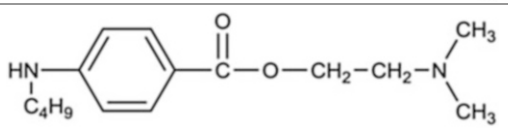
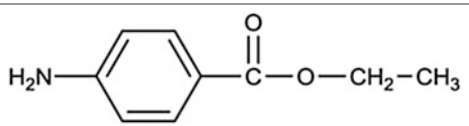
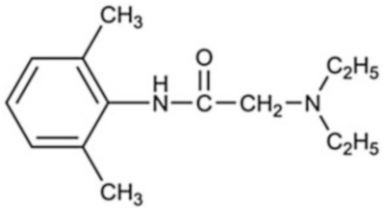
The charged form is thought to be the active form at the receptor site, but the uncharged form is important for penetration of the cell membrane (Butterworth and Strichartz 1990; Courtney and Strichartz 1987; Hille 1977). Since the local anesthetic binding site is in the inner pore of the Na^+ channel, the uncharged form first penetrates into the cytoplasm, and after equilibration of the drug, the charged form binds to the Na^+ channel. The uncharged form may also reach the binding site laterally through the hydrophobic pathway of the cell membrane. In an acidic environment, like inflamed tissues, local anesthetics except benzocaine are highly protonated, therefore hardly penetrate the axonal membrane, and have little effect.

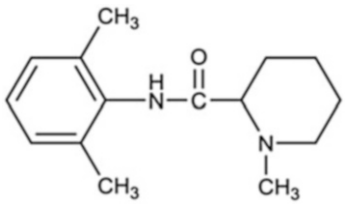
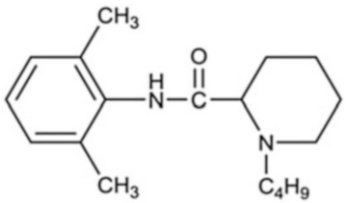
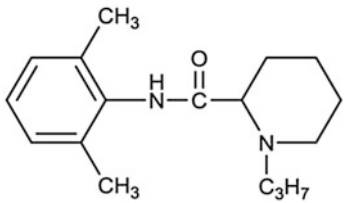
Sodium Channel Blockade

The major mechanism of action of local anesthetics is the blockade of voltage-gated Na^+ channels. Voltage-gated Na^+ channels are complexes of a large, about 2000-amino acid α-subunit, which contains the main channel functions, and one or two smaller β-subunits, which modulate membrane expression and channel function properties (Catterall 2000; Fozzard and Hanck 1996). The α-subunit is organized into four homologous, but not identical domains (I–IV), and each domain contains six transmembrane segments (S1–S6). The six transmembrane segments in each domain are further divided into a voltage-sensing unit (S1–S4) and a pore-forming unit (S5–S6), connected through the S4–S5 cytoplasmic linker. There are nine isoforms of α-subunits in mammals, which are 50–90% identical in amino acid sequence. Mutational studies allowed for the identification of the local anesthetic binding site on the α-subunit, which is formed by amino acid residues in the S6 segments in domains I, III, and IV (Ragsdale et al. 1994; Yarov-Yarovoy et al. 2001, 2002). Phenylalanine in domain IV, S6 is the most important residue for local anesthetic binding (Ragsdale et al. 1994).

Nerve fibers differ significantly in their susceptibility to local anesthetic block on the basis of differences in size and myelination. The relatively small and myelinated B fibers (autonomic preganglionic fibers) and the small and unmyelinated C fibers (mediating pain sensations or postganglionic sympathetic fibers) are blocked first, followed by the relatively small and myelinated Aδ fibers (mediating pain and temperature sensations) (Raymond and Gissen 1987). Then, the large and myelinated Aγ (mediating muscle tone) and Aβ fibers (mediating touch and pressure sensations) are blocked. The largest and myelinated Aα fibers (mediating proprioception and motor function) are blocked last.

Local Anesthetics, Table 1 Structure and properties of selected local anesthetics

Name	Structure	Potency (procaine = 1)	Duration of action	LogP	pK_a
Esters					
Cocaine		2	Medium	2.30	8.6
Procaine		1	Short	1.92	9.1
Tetracaine		16	Long	3.73	8.4
Benzocaine		–	–	1.86	3.5
Amides					
Lidocaine		4	Medium	2.26	7.9

Mepivacaine		2	Medium	1.95	7.8
Bupivacaine		16	Long	3.41	8.1
Ropivacaine		14	Long	2.90	8.2

Lipid solubility is given as the logarithm of the water/octanol partition coefficient, LogP

Another important factor determining the susceptibility of nerve fibers to local anesthetic block is the voltage-dependent effects of local anesthetics. Channels in the activated (open) and inactivated states, which predominate at more positive membrane potentials, have much higher affinities for local anesthetics than the rested channels, which predominate at more negative membrane potentials (Hille 1977). Therefore, local anesthetic blockade increases at higher frequencies of depolarizations (i.e., with an increased fraction of activated and/or inactivated channels) and with longer depolarizations (i.e., with an increased fraction of inactivated channels). Sensory fibers mediating pain sensations have a high frequency of firing and a relatively long action potential duration. Motor fibers fire with low frequency and have a shorter action potential duration. Since Aδ and C fibers exhibit high-frequency pain transmission, these fibers are blocked earlier with lower concentrations of local anesthetics than are Aα fibers (Drasner 2018).

Clinical Use

Local anesthetics provide regional analgesia and anesthesia. The routes of administration include a topical application (e.g., nasal mucosa), injection in the vicinity of peripheral nerve endings and major nerve trunks, and injection into the epidural or subarachnoid spaces surrounding the spinal cord. The patients usually stay awake under regional analgesia and anesthesia, but these can be combined with general anesthesia to reduce the requirement of narcotics and analgesics during major surgical procedures.

The choice of local anesthetic for a specific procedure is usually based on the duration of action required. Procaine is short-acting; lidocaine and mepivacaine have an intermediate duration of action; and tetracaine, bupivacaine, and ropivacaine are long-acting drugs (Table 1). The anesthetic effects of the drugs with short and intermediate durations of action can be prolonged by adding a vasoconstrictor agent (e.g., adrenaline) to reduce the absorption of local anesthetic molecules.

The clinical use of cocaine is restricted to topical anesthesia for ear, nose, and throat procedures because of its systemic toxicity and potential for abuse. Procaine was synthesized to reduce cocaine's toxicity and addictive nature and was the prototype for local anesthetics for nearly half a century. At present, procaine is not much used as a single entity. Tetracaine is widely used in spinal anesthesia when a drug of long duration is needed. Although tetracaine also is incorporated into several topical anesthetic preparations, tetracaine is rarely used in peripheral nerve blocks. Benzocaine is effective even under acidic conditions (e.g., gastric mucosa, infected tissues) because of its low pK_a and applied to topical anesthesia. Benzocaine, however, has a concern about its potential to induce methemoglobinemia. Lidocaine has a wide range of clinical uses as a local anesthetic. Lidocaine has utility in most applications except spinal administration where a local anesthetic of intermediate duration is needed. Lidocaine also is used as an antiarrhythmic drug. Mepivacaine exhibits clinical properties resembling those of lidocaine. However, mepivacaine is more toxic to the neonate and more slowly metabolized in the fetus, and thus is not used in obstetrical anesthesia. Mepivacaine is not effective as a topical anesthetic, which is different from lidocaine. Bupivacaine is widely used in almost any application when prolonged anesthesia is needed. Ropivacaine is an *S*-enantiomar of one of the bupivacaine derivatives and expected to be less cardiotoxic than bupivacaine. Ropivacaine is suitable for both epidural and regional anesthesia.

All local anesthetics are not isoform-specific and are capable of blocking different isoforms of voltage-gated Na^+ channels in the different tissues and organs. The major unwanted side effects of local anesthetics are disturbances of brain and heart function occurring during high blood levels of the drugs after absorption from the site of administration or after accidental intravascular injection.

An early symptom of side effects in the brain is circumoral and tongue numbness. At higher concentrations, muscular twitching occurs and is followed by tonic-clonic convulsions caused by a depression of cortical inhibitory pathways. Such excitation is then followed by generalized brain depression, producing respiratory depression and coma at the highest concentrations.

Following systemic absorption, local anesthetics potentially block cardiac Na^+ channels and thus depress excitability and conduction. Local anesthetics except cocaine also depress the strength of cardiac contraction and cause arteriolar dilation, which leads to hypotention. Cardiovascular side effects are usually seen only after high systemic concentrations are attained and central nervous system symptoms are evident. However, bupivacaine is more cardiotoxic than other local anesthetics and induces serious arrhythmias. Both levobupivacaine and ropivacaine being *S*-enantiomars are expected to be less cardiotoxic than bupivacaine in clinical use.

In rare cases, hypersensitivity to local anesthetics occurs. The ester-type local anesthetics are metabolized to *p*-aminobenzoic acid derivatives, and these metabolites cause allergic reactions. Although allergic responses to the amide-type drugs are not common, the solutions of the amide-type drugs may contain preservatives such as methylparaben, and they may elicit allergic reactions.

Cross-References

► Antiarrhythmic Drugs

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Low Molecular Mass GTPases

► Small GTPases

Low Molecular Weight Heparin (LMWH)

► Heparin and Related Drugs

Low Voltage-Activated (LVA) Calcium Channels

► Voltage-Dependent Ca^{2+} Channels

Low-Density Lipoprotein Receptor Gene Family

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Synonyms

ApoE receptors; Apolipoprotein E receptor family; Lipoprotein receptors; Low-density lipoprotein receptor-related proteins; LRP

Definition

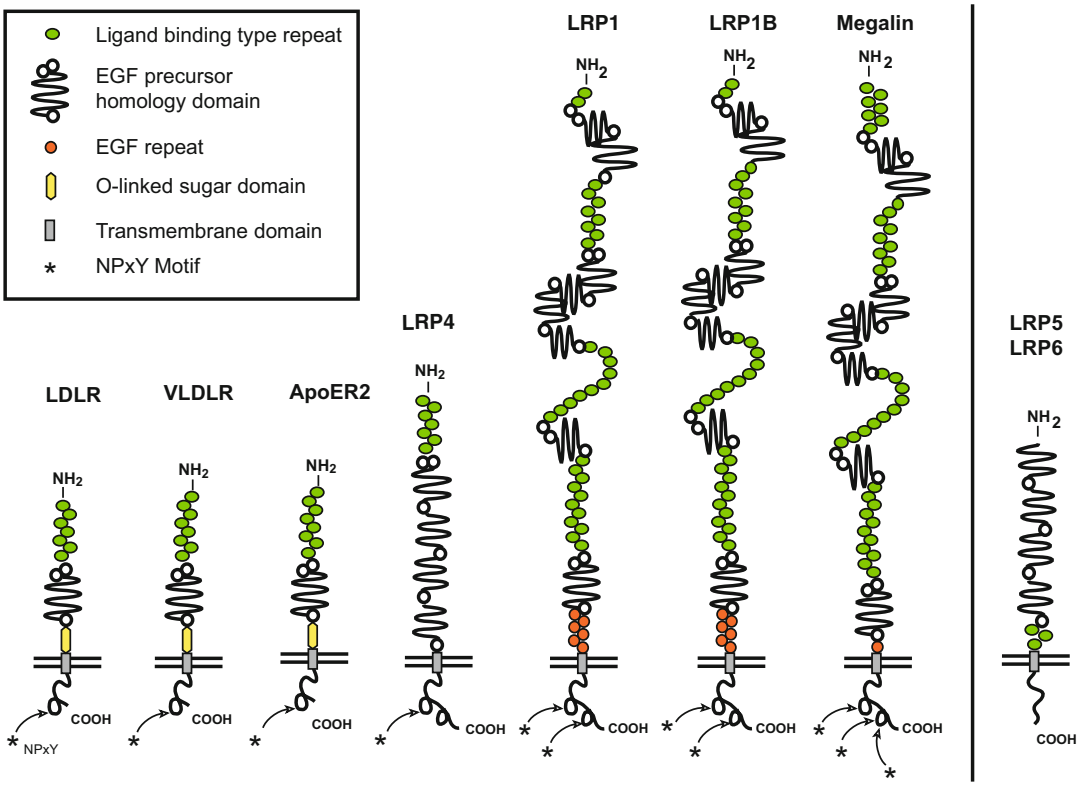
The low-density lipoprotein receptor gene family consists of structurally related cell surface proteins with diverse biological functions in numerous cell types and tissues. They are endocytic receptors that mediate binding and cellular uptake of extracellular cargo, including lipoproteins, complexes of lipids with lipid-binding proteins, proteases and protease-inhibitor-complexes, viruses, toxins, and extracellular signaling molecules. Several family members also participate directly in signal transduction processes by which cells communicate with each other and the extracellular environment. Members of the LDL receptor gene family fulfill essential roles in a number of biological processes, including lipid transport, regulation of protease activity, calcium homeostasis, cell migration, organ development, neurotransmission, and tumorigenesis.

Basic Characteristics

Structural Organization of the LDL Receptor Gene Family

The LDL receptor gene family (Fig. 1) consists of seven core members that share common structural elements in the extracellular and intracellular receptor domains, namely the family namesake LDL receptor, LRP1, LRP1b, LRP2 (megalin), LRP4 (MEGF7), LRP8 (APOER2), and very-low density lipoprotein receptor (VLDLR). They are type I transmembrane proteins with an extracellular domain that contains clusters of complement type repeats (= ligand binding type repeats), epidermal growth factor (EGF) precursor homology domains containing EGF repeats and 6 YWTD repeats that form a β-propeller structure, followed by a single transmembrane-spanning sequence and a comparatively short cytoplasmic domain that interacts with adapter and scaffolding proteins through various sequence motifs (Herz and Bock 2002; Nykjaer and Willnow 2002). The complement-type repeat clusters form calcium-dependent ligand-binding sites of different specificity, whereas the β-propeller domains allow the *pH*-dependent release of internalized ligands in the endosomes. The intracellular domains of all core members contain at least one Asn-Pro-X-Tyr (NPXY, where X designates any amino acid) tetra-amino acid motif, which is crucial for their involvement in receptor-mediated endocytosis and cellular signal transduction (May et al. 2003; Stolt and Bock 2006). The smaller members of the gene family (LDL receptor, ApoER2, and VLDLR) also contain an *O*-linked sugar domain, which may be absent due to alternative splicing and regulates proteolytic processing of the receptors. All core members of the LDL receptor gene family bind to the receptor-associated protein (RAP), an endoplasmic reticulum-associated chaperone that antagonizes binding of other lipoprotein receptor ligands. Moreover, all members of the receptor family can also interact with the cholesterol transport protein apolipoprotein E (ApoE), which exists in three major isoforms (ApoE2, 3, and 4) in humans (Lane-Donovan and Herz 2017). ApoE is the principal cholesterol-carrying apolipoprotein in the brain.

LRP5 and 6 are more distant members of the LDL receptor gene family. Their extracellular domains are arranged in a different order, with four β-propeller domains alternating with four epidermal growth factor (EGF) repeats and three complement-type repeats directly adjacent to the transmembrane-spanning segment. Moreover, their intracellular domains do not contain NPXY motifs.



Low-Density Lipoprotein Receptor Gene Family, Fig. 1 The LDL receptor gene family. The figure depicts the structural organization of mammalian members of the LDL receptor gene family

Receptor-Mediated Endocytosis and Signal Transduction

Members of this evolutionarily ancient gene family can be found in worms, insects, and vertebrates. This suggests that they evolved at an early stage of metazoan development. They can mediate the cellular uptake of extracellular cargo through a process known as receptor-mediated endocytosis, where a cell surface receptor binds a specific ligand from the extracellular space. The receptor-ligand complex is internalized via specialized regions of the plasma membrane called clathrin-coated pits and moves to an intracellular vesicle named endosome to release its cargo. The ligands are further directed to lysosomes for catabolism, while the unliganded receptor returns to the cell surface to initiate the next round of endocytosis. These concepts were first established for the LDL receptor and its role in cholesterol metabolism (Goldstein and Brown 2015).

Binding of some ligands to endocytic receptors may also trigger intracellular signaling cascades involved in the regulation of important (patho-)physiological processes such as embryonic patterning, neuronal cell migration, synaptic plasticity, or atherosclerosis. This is achieved by the interaction of conserved recognition motifs in the intracellular receptor tails with specific adaptor and scaffolding proteins, which functionally link the receptor tails to cellular trafficking, cellular signaling, and transcriptional regulation (Stolt and Bock 2006). Proteolytic processing of LRP family members offers an additional level of regulation. Soluble, released extracellular receptor domains may inhibit signaling by scavenging extracellular ligands. Sequential proteolytic cleavage of LRP family members by α- and γ-secretases can result in the release of a soluble intracellular receptor domain, which can translocate to the

nucleus and regulate transcription, as has been shown for LRP1 and LRP8.

Functions of LDL Receptor Gene Family Members

A number of human diseases are caused by sporadic or inherited forms of lipoprotein receptor deficiency (Table 1). In addition, animal models with spontaneous or induced receptor gene defects have been instrumental in elucidating the physiological roles of members of the LDL receptor gene family. The founding member of the gene family, the LDL receptor, was discovered as the gene that is defective in the autosomal-dominantly inherited disease called familial hypercholesterolemia (FH), characterized by high levels of low-density lipoprotein and premature cardiovascular disease.

LDL Receptor

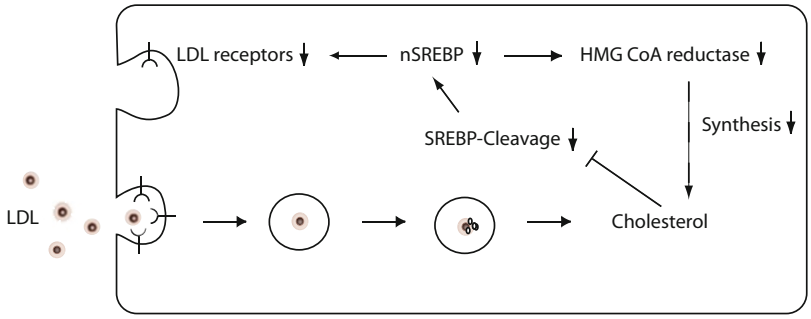
The LDL receptor (LDLR) is the founding member of the family (Goldstein and Brown 2015). It is the major endocytic receptor for cholesterol-rich lipoprotein particles. It binds lipoprotein particles that contain apolipoprotein B-100 (ApoB-100), which is the major structural apolipoprotein component of very low-density (VLDL), intermediate-density (IDL), and low-density lipoprotein (LDL) particles. In addition, LDLR binds to apolipoprotein E (ApoE), which is part of chylomicron remnants, VLDL and IDL, but not LDL.

After selective cellular uptake by receptor-mediated endocytosis in clathrin-coated vesicles, the internalized lipoprotein particle-receptor complexes are delivered to early endosomes where a decrease in *pH* allows the dissociation of the receptors from their cargo. The LDL receptors recycle back to the cell surface, whereas the lipoprotein particles are directed to lysosomes. Here, the apolipoproteins are broken down into amino acids while the cholesteryl esters are hydrolyzed and released into the cytosol for further metabolism (Fig. 2).

The uptake of lipoproteins by the LDL receptor serves two main functions. First, it delivers cholesterol to the cell which can be used for the formation of membranes, synthesis of steroid hormones and bile acids. Second, it regulates endogenous cholesterol synthesis. The importance of its regulating the concentration of cholesterol in the circulation is underscored by the phenotype observed in humans with LDL receptor gene defects, a syndrome called familial

Low-Density Lipoprotein Receptor Gene Family, Table 1 Human diseases of the LDL receptor gene family

Receptor	Type of mutation	Disease
LDL receptor	Loss-of-function (familial, autosomal dominant)	Familial hypercholesterolemia (impaired clearance of LDL)
LRP1B	Loss-of-function (somatic)	Esophageal squamous cell carcinoma, non-small-cell lung cancer
LRP4	Loss-of-function (familial, autosomal-recessive)	Cenani–Lenz syndrome (oligosyndactyly, metacarpal synostosis, phalangeal disorganization; variable facial dysmorphism)
	Loss-of-function (familial, autosomal-recessive)	Congenital myasthenic syndrome
VLDL receptor	Loss-of-function (familial, autosomal recessive)	VLDLR-associated cerebellar hypoplasia (ataxia, mental retardation, dysarthria, seizures)
LRP5	Loss-of-function (familial, autosomal recessive)	Osteoporosis-pseudoglioma-syndrome
	Gain-of-function (familial, autosomal dominant)	Increase in bone mass
	Loss-of-function	Polycystic liver disease
LRP6	Missense mutation (familial, autosomal dominant)	Hyperlipidemia, hypertension, diabetes mellitus, early coronary artery disease, osteoporosis
LRP2	Loss-of-function (familial, autosomal recessive)	Donnai–Barrow syndrome (brain and facial malformation, sensorineural hearing loss, renal tubular deficiency, diaphragmatic hernia)



Low-Density Lipoprotein Receptor Gene Family, Fig. 2 Regulation of cholesterol homeostasis by LDLR. Receptor-mediated uptake and lysosomal hydrolysis of LDL leads to accumulation of cholesterol in the ER membrane, which blocks SREBP cleavage and results in decreased nuclear SREBP (nSREBP) and decreased transcription of target genes including LDLR and HMG CoA reductase

hypercholesterolemia (FH). In patients with FH, lack of LDL receptor activity results in a massive increase in plasma LDL (approximately twofold in heterozygotes and six- to tenfold in homozygotes). As a consequence, FH patients suffer from premature atherosclerosis and coronary artery disease. To date, far more than 1000 different mutations have been identified in the human LDL receptor gene, which can be assigned to different classes: class I-mutations result in no detectable LDL receptor protein, class II-mutations prevent transport of the LDLR from the endoplasmic reticulum to the Golgi, class III-mutations result in defective ligand binding, class IV-mutations interfere with LDLR clustering in clathrin-coated pits, and class V-mutations result in defective receptor recycling to the cell surface.

Diet high in cholesterol leads to decreased transcription of the LDLR gene, which is mediated by the SREBP (sterol regulatory element-binding protein) pathway. SREBPs are membrane-anchored proteins that are transported to the Golgi and proteolytically cleaved when the cholesterol content of endoplasmic reticulum membranes drops, thus releasing a transcriptionally active fragment that translocates to the nucleus and activates genes involved in cholesterol synthesis and uptake (e.g., HMG CoA reductase, the rate-limiting enzyme in cholesterol synthesis, and LDLR itself; Fig. 2). Statins (HMG CoA-reductase inhibitors) block endogenous cholesterol synthesis in the cell. This leads to an increase in the production of LDL receptors and uptake of lipoproteins. The concomitant increase in HMG CoA reductase is inhibited by the statin. Therefore, statin treatment results in decreased plasma LDL concentrations, which reduces the risk for cardiovascular events and mortality.

The cytosolic adaptor protein ARH binds to the NPXY motif in the cytoplasmic tail of the LDLR and couples it to the endocytic machinery in hepatocytes. Mutations in the *ARH* gene cause autosomal-recessive hypercholesterolemia. An autosomal-dominant trait also causing familial hypercholesterolemia was linked to the *PCSK9* (proprotein convertase subtilisin/kexin type 9) gene, which encodes a secreted protein that binds to the extracellular domain of LDLR. PCSK9 binding to LDLR makes the receptor susceptible to enzymatic degradation by preventing a conformational change and directing it to the lysosome. Inherited gain-of-function mutations in the *PCSK9* gene cause familial hypercholesterolemia, whereas loss-of-function mutations result in low concentrations of plasma LDL cholesterol and protect against cardiovascular disease. Hence, pharmacological inhibition of PCSK9 has arisen as a novel therapeutic approach to lower LDL cholesterol to an unparalleled degree (Warden et al. 2020). Another pathway regulating lysosomal proteolysis of the LDLR and other members of the lipoprotein receptor family in various tissues involves the ubiquitin ligase IDOL (*inducible degrader of the LDLR*; a.k.a. MYLIP: myosin regulatory light chain interacting protein),

which is transcriptionally induced by activation of the sterol-responsive nuclear receptor LXR (liver X receptor).

LRP1

LRP1 (a.k.a. α2-macroglobulin receptor or CD91), one of the most versatile members of the LDLR family, is ubiquitously expressed (Herz and Strickland 2001). Conventional, constitutive inactivation of *LRP1* results in early embryonic lethality in mice. LRP1 binds more than 40 extracellular ligands including lipoproteins, protease/protease inhibitor complexes, coagulation factors, toxins, cytokines and growth factors, and has important functions both as an endocytic uptake and as a signaling receptor. The mature form of the receptor is derived from a large precursor protein of 600 kDa by proteolytic processing and consists of an 85-kDa membrane-spanning fragment including the intracellular domain and a noncovalently attached 515-kDa extracellular domain, which contains four ligand-binding domains. The intracellular domain can interact with a large number of cytoplasmic adaptor proteins, which contributes to its diverse roles in physiology and disease (May et al. 2007).

Together with the LDLR, LRP1 is the only other family member that is expressed at functionally significant levels in hepatocytes, where it mediates the uptake of ApoE-containing chylomicron remnants, lipoproteins that transport dietary lipids from the intestine to the liver. It also functions as a main uptake receptor for aggregated LDL in the vascular wall, a form of modified LDLs that promote foam cell formation and atherosclerotic lesion progression. By clearing proteases and protease inhibitor complexes from the extracellular space, LRP1 is an important regulator of extracellular proteolytic activity. This contributes to the regulation of cell motility and fibrosis, wound healing responses, tumor invasiveness, and vascular permeability, including that of the blood–brain barrier. In the lung and other tissues, LRP1 in conjunction with calreticulin can mediate the phagocytic uptake of apoptotic cells. Furthermore, bacterial toxins and viruses have been shown to use LRP1 (as well as other members of the gene family) as entry receptor on the cell surface. In the brain, neuronal LRP1 is a major receptor for astrocyte-derived ApoE-containing lipoprotein particles, and like APOER2 and VLDLR, it interacts with components of the postsynaptic density and regulates glutamatergic signaling.

In vascular smooth muscle cells, LRP1 acts as signaling receptor that suppresses the activity of the PDGF (platelet-derived growth factor) receptor-β (PDGFRB). Loss of LRP1 expression in vascular smooth muscle cells in mice enhanced activation of the PDGF receptor pathway, resulting in smooth muscle cell proliferation, hyperplasia of the aortic wall, and marked susceptibility to atherosclerotic lesion formation. Furthermore, LRP1 was shown to be identical to transforming growth factor-β (TGF-β) receptor V, which is coexpressed together with TGF-β receptors I and II. Thus, interactions between LRP1 and growth factor receptors in the vascular wall regulate signaling processes that are involved in aortic aneurysm formation and Marfan syndrome.

Another important function of LRP1 relates to the regulation of hepatic glucose and fatty acid metabolism in response to insulin, which regulates the cell surface translocation and endocytic function of LRP1 in various cell types. At the same time, LRP1 regulates the cell surface expression of the insulin receptor (IR). In consequence, loss of hepatic LRP1 attenuates insulin-dependent trafficking of glucose transporter 2 (GLUT2) to the plasma membrane, which ultimately leads to a state of insulin resistance. Thus, LRP1 is involved in the integration of hepatic lipid and glucose homeostasis and might be protective against development of the metabolic syndrome (van de Sluis et al. 2017).

Proteolytic shedding of LRP1 leads to release of a soluble form (sLRP1) that can be detected at nanomolar concentrations in human plasma. It contains the large extracellular α-chain and a 55 kDa fragment of the membrane-spanning β-chain (85 kDa subunit). The sLRP1 can act as a competitive inhibitor of ligand uptake by the uncleaved receptor. After shedding of sLRP1, the intracellular domain of LRP1 (ICD) can be released from the remaining receptor fragment by

a process called regulated intramembrane proteolysis (RIP). The ICD can translocate to the nucleus where it regulates transcription of target genes. RIP of LDLR family members has also been demonstrated for LRP8, VLDLR, and LRP2.

LRP1 also binds to amyloid precursor protein (APP), a transmembrane protein and precursor of amyloid-β (Aβ) whose fibrillar form is the major component of the neuritic plaques found in Alzheimer disease (AD). Their intracellular domains share common adaptor proteins, and processing and trafficking of APP is regulated by LRP1. Moreover, LRP1 is involved in the clearance of Aβ across the blood–brain barrier, which is modulated by ApoE in an isoform-dependent manner. Other ApoE receptor family members are also involved in APP- and Aβ-dependent processes to various degrees (Pohlkamp et al. 2017), linking them intrinsically to the pathophysiology of neurodegenerative disease.

VLDLR and APOER2 (LRP8)

Apolipoprotein E receptor 2 (APOER2, a.k.a. LRP8) and very low-density lipoprotein receptor (VLDLR) are the closest relatives of the LDL receptor (Dlugosz and Nimpf 2018). Both are expressed in the developing and adult nervous system and act as cell surface receptors of the secreted glycoprotein Reelin (Fig. 3). Binding of Reelin to LRP8 or VLDLR leads to receptor clustering and activation of an intracellular kinase signaling cascade, which is dependent on tyrosine phosphorylation of the intracellular adaptor protein Disabled-1 (DAB1) (Bock and May 2016). Defective Reelin signaling in mice or humans results in neurodevelopmental disorders with abnormal neuronal layering defects. In the adult brain, alterations in ApoE receptor-mediated Reelin signaling are involved in numerous neuropsychiatric disorders, including schizophrenia, bipolar disorder, and autism (Ishii et al. 2016).

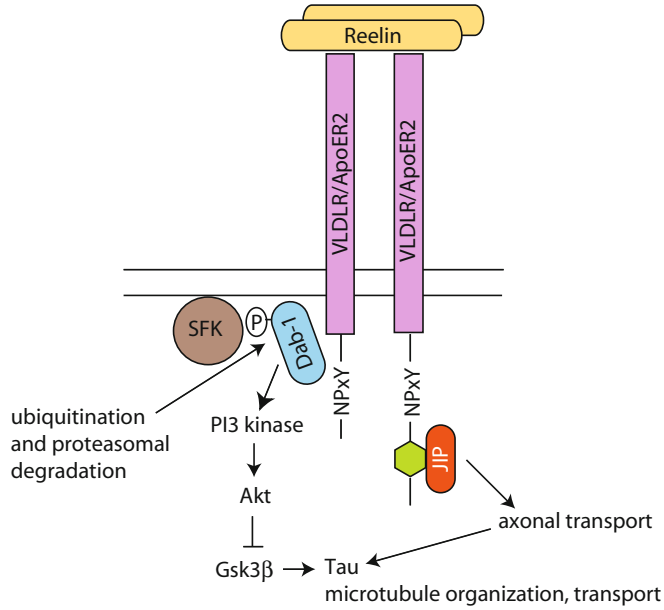
In humans, autosomal-recessive VLDLR-cerebellar hypoplasia is characterized by a predominantly truncal, nonprogressive congenital ataxia. Mice lacking VLDLR develop aberrant retinal neovascularization resembling age-related macular degeneration. This is caused by overactivation of Wnt signaling resulting in enhanced production of vascular endothelial growth factor. It was shown that the ectodomain of VLDLR inhibits Wnt signaling through heterodimerization with the LRP6 ectodomain.

In the brain, LRP8 and VLDLR associate with components of the postsynaptic density, including glutamate receptors. Reelin signaling modulates NMDA receptor function, thereby regulating synaptic plasticity. By antagonizing the synaptic suppression induced by oligomeric amyloid-β, Reelin and LRP8 may have a protective role in synaptic dysfunction associated with Alzheimer disease (Lane-Donovan and Herz 2017). The ε4 isoform of apolipoprotein E, ApoE4, constitutes a major genetic risk for the sporadic, late-onset form of Alzheimer disease. By preventing LRP8-glutamate receptor complexes from recycling to the cell surface, ApoE4 blocks Reelin's effect on synaptic plasticity.

In brain and testis, LRP8 functions as an endocytic uptake receptor for selenoprotein P (SEPP1) and contributes to the supply with selenium to these organs. Binding of SEPP1 to LRP8 occurs through the β-propeller domain and does not require the ligand-binding domain. *Lrp8*-deficient male mice display structural sperm defects and are infertile. LRP8 is also expressed in endothelial cells and platelets, where it can interact with dimerized β2-glycoprotein I bound to antiphospholipid antibodies, contributing to the pathophysiology of this autoimmune disorder.

LRP4

Various mutations of the human gene encoding LRP4 (a.k.a. *multiple epidermal growth factor-like domains 7*, MEGF7) are associated with Cenani–Lenz (C–L) syndrome, which is inherited in an autosomal-recessive manner. It is characterized by oligosyndactyly, abnormal limb development, and variable facial and systemic features (Al-Qattan and Alkuraya 2019), features that are shared by *Lrp4* mutant mice. In C–L syndrome, loss of function-mutations of *LRP4* release the antagonizing effect of LRP4 on LRP6, which is an essential coreceptor of frizzled in the canonical WNT signaling cascade (Niehrs 2006). This results in overactivation of the canonical WNT signaling pathway, ultimately leading to



Low-Density Lipoprotein Receptor Gene Family, Fig. 3 Signal transduction by members of the LDL receptor gene family: simplified scheme of Reelin signaling in neurons via lipoprotein receptors VLDLR and APOER2. Binding of Reelin induces clustering of DAB1 on the cytoplasmic receptor tails, which activates SRC family tyrosine kinases (SFKs) that phosphorylate the adaptor. Phosphorylated DAB1 activates phosphatidylinositol-3-kinase (PI3K) and protein kinase B (PKB). PKB inhibits the activity of glycogen synthase kinase 3β (GSK3β), resulting in reduced phosphorylation of Tau (τ) and stabilization of microtubules. A proline-rich region in the intracellular domain of APOER2, which is encoded by an alternatively spliced exon that is absent from other lipoprotein receptors, interacts with intracellular proteins including JNK-interacting protein (JIP) and postsynaptic density protein-95 (PSD95)

increased β-catenin stability and target gene transcription.

LRP4 is required for the formation and maintenance of the neuromuscular junction (NMJ) – a chemical synapse between skeletal muscle fibers and motor neurons – as well as for the signal transduction occurring there (Ohno et al. 2017). Autoantibodies against LRP4 have been reported in patients with seronegative myasthenia gravis, an autoimmune disease where inhibitory antibodies block acetylcholine receptor (AChR)-mediated neurotransmission. At the NMJ, LRP4 forms a heteromeric complex with muscle-specific receptor tyrosine kinase (MuSK). This complex functions as a transmembrane receptor for the motor neuron-derived proteoglycan agrin, which binds to the extracellular domain of LRP4 and indirectly activates the intracellular kinase domain of MuSK. MuSK phosphorylation leads to the recruitment and activation of downstream proteins, ultimately driving aggregation of the AChR-clustering scaffold protein rapsyn. Studies in gene-targeted mice suggest that LRP4 is also involved in synaptogenesis and neurotransmission in the central nervous system.

LRP5 and 6

LRP5 and 6 are distant relatives of the core members of the LDL receptor gene family (Fig. 1). Both receptors are highly homologous proteins that are integral components of the wingless (Wnt) signaling cascade by acting as coreceptors to a member of the Frizzled (Fz) family of seven-transmembrane proteins (Joiner et al. 2013). In the extracellular receptor domain, four β-propeller motifs, each connected by an EGF-like domain, are followed by three ligand binding type repeats (LBRs), which can mediate interaction with apolipoprotein E. In contrast to core members of the LRP family, the ligand binding domains and

β-propeller motifs are arranged in an inverse order, with the LBRs adjacent to the transmembrane-spanning segment rather than at the aminoterminal end, and the intracellular domain does not contain an *NPXY* tetraamino-acid motif.

Simultaneous binding of Wnt ligands to LRP5 or 6 and Fz induces canonical Wnt signaling, ultimately leading to increased β-catenin levels in the cytoplasm and nucleus, where it forms complexes with LEF/TCF family members to control transcription of target genes. Secreted proteins such as Dickkopf-1 (Dkk1), Wise, or sclerostin antagonize Wnt signaling by binding to LRP5/6, thereby disrupting the Wnt-induced Fz/LRP complex. In addition, Dkk1 binding to its coreceptor Kremen may inhibit Wnt signaling by promoting endocytosis of a ternary Kremen-Dkk1-LRP6 complex, removing LRP6 from the plasma membrane. Moreover, β-catenin independent LRP5/6 signaling has been reported.

Loss of LRP6 function in fruit fly, *Xenopus*, and mouse causes aberrant pattern formation and early embryonic lethality. A causal *LRP6* mutation in a family with early coronary disease, hypercholesterolemia, and glucose intolerance has been described. LRP5 activity is mainly required for the regulation of bone formation as concluded from loss- and gain-of-function mutations in humans. Sclerostin is produced by osteocytes and inhibits Wnt signaling by binding to LRP5/6. Its inactivation causes sclerosteosis, a rare disorder that is characterized by overgrowth of bone tissue. *LRP5* missense mutations were associated with polycystic liver disease. In mice, simultaneous inactivation of *Apoe* and *Lrp5* led to dyslipidemia and atherosclerosis.

LRP1B

LRP1B is a giant member of the LDL receptor gene family that shares extensive homology with LRP1. It was originally named LRP-DIT (*deleted-in-tumors*), because it was found to be frequently inactivated in non-small cell lung cancer (NSCLC) cell lines. Inactivation of the *LRP1B* gene has been observed in several types of human cancers. Therefore, it has been suggested to function as a tumor suppressor gene. Moreover, genome-wide association (GWA) studies have shown its genetic association with obesity. Mice carrying inactivating mutations of the *Lrp1b* gene display early embryonic lethality, whereas expression of a truncated allele predicted to allow secretion of the complete extracellular domain of LRP1b is sufficient to allow normal development without any obvious phenotype. Both LRP1 and LRP1B display an overlap in expression patterns and share common extracellular ligands, but in contrast to LRP1, LRP1B is not cleaved by furin and has a very slow endocytosis rate. This suggests that both receptors might partially antagonize each other's functions (May et al. 2007).

LRP2

LRP2 (a.k.a. megalin or gp330) is the largest member of the LDL receptor gene family. It was initially identified as the antigen giving rise to Heymann nephritis, a rat experimental model of human membranous nephropathy. It functions as an endocytic clearance receptor for a variety of extracellular ligands and is specifically expressed by absorptive epithelia in the kidney, brain, lung, eye, and reproductive organs (Willnow and Christ 2017). The interaction of the cytoplasmic receptor tail with different adaptor proteins governs the fate of internalized LRP2, which is either directed for lysosomal degradation or recycled back to the apical cell surface. In addition, trafficking from the apical to the basolateral plasma membrane enables transcytosis through polarized epithelia, e.g., in thyroid cells.

A major function of LRP2 in the kidney is its role as uptake receptor for plasma proteins from the tubular lumen, including low-molecular weight transport proteins for the vitamins D, A, and B_{12}. Lack of megalin in *Lrp2* deficient mice results in excessive urinary loss of filtered 25-OH vitamin D_3 bound to the vitamin D-binding protein (DBP), leading to osteomalacia due to vitamin D deficiency. In a similar manner, megalin is involved in vitamin A homeostasis by renal reuptake of retinol bound to retinol-binding protein (RBP).

Megalin interacts with cubilin (CUBN), a cargo protein that is mainly expressed in the kidney and small intestine. Because cubilin lacks a

transmembrane domain, the interaction with megalin is required for its endocytosis in proximal tubule cells. Mutations in *LRP2* and *CUBN* have been recognized as being involved in congenital forms of renal Fanconi syndrome, characterized by proximal tubular dysfunction with low-molecular weight proteinuria, phosphaturia, and renal tubular acidosis (Willnow and Christ 2017). Autosomal-recessive mutations in the *LRP2* gene give rise to the Donnai–Barrow syndrome, a rare human inherited disorder characterized by facial dysmorphology, agenesis of the corpus callosum (a form of holoprosencephaly), sensorineural hearing loss, developmental delay, progressive vision loss, and renal dysfunction. Despite the importance of megalin for renal reabsorption processes, including nephrotoxic drugs such as aminoglycoside antibiotics, and vitamin homeostasis, no therapeutic applications related to LRP2 have been developed yet.

Another important function of megalin is the control of morphogen signaling during organogenesis. By forming a receptor complex with the transmembrane protein Patched 1 (PTCH1), a receptor of the morphogen sonic hedgehog (SHH), on the apical surface of neuroepithelial cells, megalin facilitates binding and internalization of SHH bound to PTCH1. This in turn reduces the suppressive action of PTCH1 on Smoothened (SMO), which mediates the cellular effects of SHH signaling in the primary cilium. Consequently, inactivation of *LRP2* in mice results in holoprosencephaly resembling mice carrying mutations in components of the sonic hedgehog pathway.

Drugs

Statins

Statins lower plasma LDL cholesterol by inhibiting HMG CoA-reductase, the rate-limiting step of endogenous cholesterol synthesis in the cell (Goldstein and Brown 2015). The reduced cellular membrane cholesterol content activates SREBP-2, which results in increased LDLR cell surface expression and LDL uptake. HMG CoA reductase expression is also increased but cholesterol synthesis is inhibited by the statin. Lowering LDL-C by statins reduces the risk for cardiovascular events and mortality, and is a cornerstone of primary and secondary prevention of atherosclerosis and coronary artery disease (Boekholdt et al. 2014). There are six statins available, namely simvastatin, atorvastatin, fluvastatin, pravastatin, rosuvastation, and lovastatin, the latter being the first statin approved for medical use in 1987. Cerivastatin was withdrawn due to an increased risk of rhabdomyolysis. Other common side effects include myalgia and an increase in liver enzymes.

PCSK9 Inhibitors

Two monoclonal antibodies that bind to and inhibit PCSK9, thereby increasing cell-surface LDLR and reducing plasma LDL-C, have been approved for human therapeutic use both by the FDA and the European Union (EU) in 2015, namely alirocumab (Praluent™) and evolocumab (Repatha™). A third inhibitor, bococizumab, was withdrawn due to a relatively high incidence of neutralizing antidrug antibodies (Warden et al. 2020).

Alirocumab was approved as a second-line therapy for adults with familial hypercholesterolemia and people with atherosclerosis who require additional lowering of LDL cholesterol when diet and statin treatment alone have not worked, or when statins are not tolerated or are contraindicated. Evolocumab was the first PCSK9 inhibitor approved in the EU. Based on the results of two large-scale phase 3 trials, FOURIER (Further cardiovascular OUTcomes Research with PCSK9 Inhibition in subjects with Elevated Risk) for evolocumab, and ODYSSEY OUTCOMES (Evaluation of Cardiovascular Outcomes After an Acute Coronary Syndrome During Treatment With Alirocumab), both monoclonal antibodies also carry an indication for the reduction of cardiovascular events in patients with established atherosclerotic cardiovascular disease. Whether overall mortality reduction can be achieved by PCSK9 inhibition remains to be established. Side effects mainly included local injection site reactions. Adverse effects related to

cognitive impairment were not confirmed in the abovementioned trials.

Other approaches to lower PCSK9 that have not reached clinical practice (yet) include siRNA oligonucleotides (Inclirisan), vaccination or oral small-molecule inhibitors of PCSK9.

Romosozumab

The LRP5/6 antagonist sclerostin (SOST) is targeted by the inhibitory humanized monoclonal antibody romosozumab (Evenity™), which has been approved in the United States and EU for the treatment of osteoporosis in postmenopausal women with high risk of fracture in 2019 (Markham 2019). The drug is contraindicated in patients with hypocalcemia. In clinical trials, an increase in serious cardiovascular events in romosozumab-treated patients was observed. Therefore, the antibody is contraindicated in patients with a history of myocardial infarction or stroke.

Lipoprotein Receptors for Drug Delivery

In principle, lipoproteins or lipoprotein-like synthetic particles can be conceived as carriers to transport therapeutic or diagnostic agents (especially lipophilic compounds) to their cellular destination, making use of the highly efficient and specific ligand-receptor uptake systems provided by LDLR family members. To date, however, no such application has found its way into clinical practice yet.

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Low-Density Lipoprotein Receptor-Related Proteins

► Low-Density Lipoprotein Receptor Gene Family

LRP

▶ Low-Density Lipoprotein Receptor Gene Family

Lymphokines

▶ Cytokines

Lysoglycerophospholipids

▶ Lysophospholipids

Lysolipid Mediators

▶ Lysophospholipids

Lysophosphatidic Acid

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Definition

The term lysophosphatidic acid describes a class of structurally related monoacyl-glycerol-3-phosphates which belong to the lysophospholipids.

Basic Characteristics

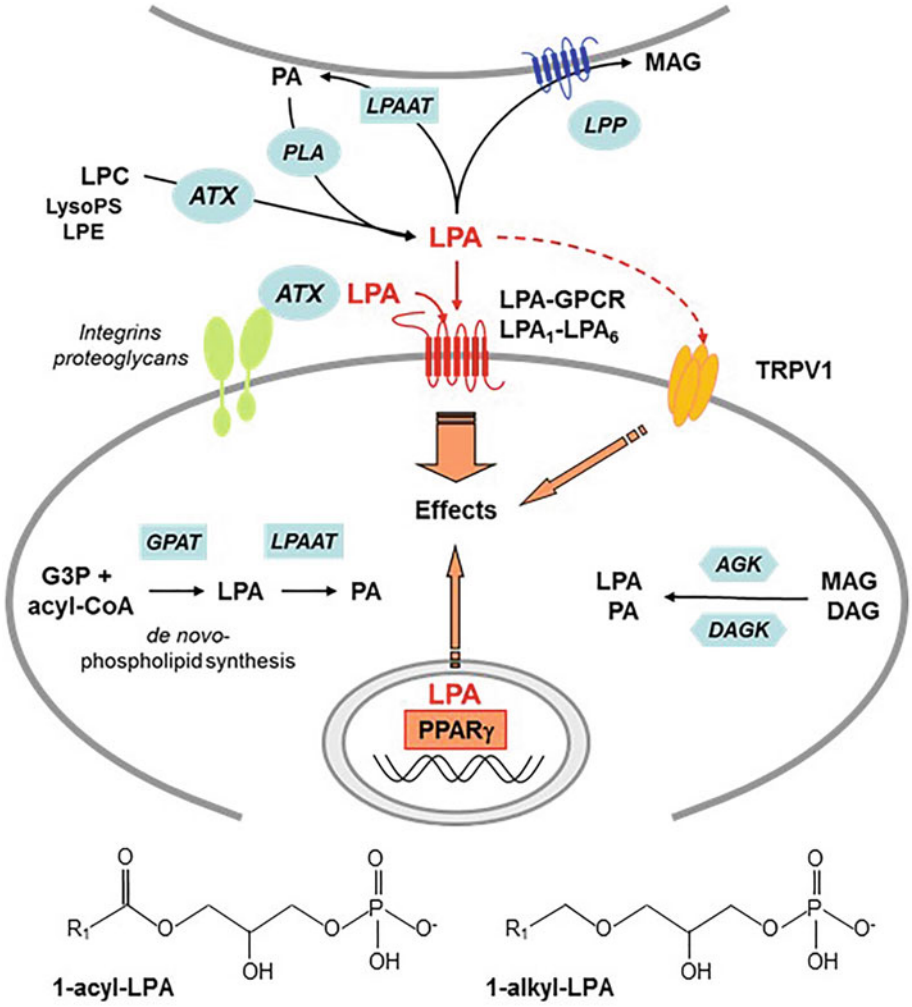
Metabolism

Lysophosphatidic acid (LPA) can be formed and degraded by multiple metabolic pathways (Fig. 1). Depending on the precursor molecule and respective pathway, the fatty acid chain in LPA differs in length, degree of saturation, and position (sn-1 or sn-2). LPA analogues with ether-bound alkyl or alkenyl chains are quantitatively less abundant than those with ester-bound acyl chains. LPA occurs in plasma (~200 nM), is formed during coagulation, and is present in serum in micromolar concentrations. Differences in the fatty acid composition of LPA in plasma and serum suggest that the sources of these LPA pools are different. LPA's chemical structure determines its interaction with LPA receptors and its biological activity (Yung et al. 2014).

Extracellular LPA is generated primarily from LPC by a lysophospholipase D named autotaxin (ATX; human gene name: *ENPP2*). LPA production by ATX is essential for embryonic development in mice and contributes most of LPA in plasma. In fact, ATX inhibitors can reduce plasma LPA by >95% (Yung et al. 2014; Benesch et al. 2018). Binding of ATX to cell surface integrins and proteoglycans facilitates the direct delivery of LPA to its GPCR (Peyruchaud et al. 2019). Extracellular LPA can furthermore be generated from surface-exposed phosphatidic acid by phosphatidic acid-selective PLA_1 or secretory type-II PLA_2. Intracellularly, LPA can be formed by glycerol-3-phosphate acyltransferases ($GPAT_{1-4}$) (Yu et al. 2018) or by phosphorylation of monoacylglycerol. Phosphorylation may be catalyzed by mitochondrial acylglycerol kinase (AGK) or by diacylglycerol kinase (DAGK) isoforms with partial monoacylglycerol kinase activity (Tigyi et al. 2019). Degradation of LPA occurs via dephosphorylation, catalyzed by lipid phosphate phosphatases (LPP_{1-3}; human gene names: *PLPP1-3*), or by acylation, catalyzed by LPA acyltransferases (LPAATα-ε; human gene names: *AGPAT1-5*) (Tang et al. 2015; Bradley and Duncan 2018). LPPs are integral membrane proteins with their catalytic activity directed to the extracellular space or to the luminal side of organelles. They can dephosphorylate diverse phospholipids and lysophospholipids and thereby regulate extra- and intracellular levels of both LPA and sphingosine-1-phosphate (Tang et al. 2015).

LPA Receptors

An overview of LPA receptors as listed in the IUPHAR/BPS Guide to Pharmacology is



Lysophosphatidic Acid, Fig. 1 The LPA signaling system. AGK, acylglycerol kinase; ATX, autotaxin; DAG, diacylglycerol; DAGK, diacylglycerol kinase; G3P, glycerol-3-phosphate; GPAT, glycerol-3-phosphate acyltransferase; GPCR, G-protein-coupled receptor; LPAAT, LPA acyltransferase; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPP, lipid phosphate phosphatase; LysoPS, lysophosphatidylserine; MAG, monoacylglycerol; PA, phosphatidic acid; PPARγ, peroxisome proliferator-activated receptor-γ; TRPV1, transient receptor potential cation channel subfamily V member 1

presented in Table 1. These receptors, $LPA_{1\text{-}6}$ (human gene names: *LPAR1-6*), are class A GPCR and widely expressed. $LPA_{1\text{-}3}$ belong to the EDG family of GPCR, while $LPA_{4\text{-}6}$ form a subcluster within the purinergic P2Y receptor family. LPA-GPCR differentially couple to $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$ proteins, and LPA_4 and LPA_6 also couple to G_s (Table 1). Thus, they regulate classical signaling pathways such as adenylyl cyclase, phospholipase C, $[Ca^{2+}]_i$, protein kinase C, mitogen-activated protein kinases, Akt, Ras, Rac, and Rho. The LPA_2 receptor has a unique C-terminus which binds LIM domain-containing proteins and has a PDZ binding motif for G-protein-independent signaling (Tigyi et al. 2019). Non-GPCR targets of LPA comprise the nuclear transcription factor, peroxisome proliferator-activated receptor-γ (PPARγ), and diverse ion channels. While several ion channels are downstream targets of LPA-GPCR, direct binding of LPA to

Lysophosphatidic Acid, Table 1 G-protein-coupled LPA receptors

Receptor (previous names)	Signaling	Main biological effects
LPA_1 (EDG2, VZG1)	$G_{i/o}$, $G_{q/11}$, $G_{12/13}$	Cell proliferation, survival, migration, neurite retraction, brain development, olfaction, neuropathic pain −/− mice: 50% perinatal lethality, defective olfaction/impaired suckling, decreased body size, craniofacial dysmorphism
LPA_2 (EDG4)	$G_{i/o}$, $G_{q/11}$, $G_{12/13}$ PDZ binding	Cell proliferation, survival, migration, facilitation of DNA damage repair −/− mice: mostly normal
LPA_3 (EDG7)	$G_{i/o}$, $G_{q/11}$	−/− mice: defective embryo implantation and spacing
LPA_4 (P2Y9, GPR23)	G_s, $G_{i/o}$, $G_{q/11}$, $G_{12/13}$	Rho-mediated neurite retraction, cell motility ↓ −/− mice: enhanced LPA-induced cell migration, reduced numbers of hematopoietic stem/progenitor cells
LPA_5 (GPR92)	$G_{q/11}$, $G_{12/13}$	Neurite retraction, cell motility ↓, release of proinflammatory cytokines, neuropathic pain −/− mice: reduced pain sensitivity, faster recovery from inflammation, nocturnal hyperactivity and anxiety
LPA_6 (P2Y5)	G_s, $G_{i/o}$, $G_{12/13}$	Hair growth, neurite retraction, telencephalon development, vascular stability Human mutations: woolly hair, hypotrichosis

the transient receptor potential (TRP) ion channels, TRPV1 and TRPA1, may contribute to LPA-mediated pain and itch (Juárez-Contreras et al. 2018).

Biological Actions

The ATX/LPA receptor axis plays an important role in organismal **development**. In mice, ATX is required for vessel formation in yolk sac and embryos, neural crest development, and axial turning. *Lpar1* is highly expressed in the neurogenic ventricular zone of the embryonic cerebral cortex and important for neural development, olfaction, and craniofacial development in mice. *Lpar3* is expressed in the endometrial epithelium during embryo implantation and involved in cyclooxygenase-2-dependent implantation and embryo spacing in mice. Blood and lymphatic vessel development, also in extraembryonic vessels, depends furthermore on *Lpar4*. The LPA_6 receptor regulates hair growth and texture, and its mutation causes woolly hair with or without hypotrichosis in humans. In contrast to ATX knockout, deletion of single LPA receptors in mice is not fully lethal during embryonic development, indicating some redundancy. LPA/LPA_1 is furthermore involved in post-hemorrhagic fetal hydrocephalus (Yung et al. 2014; Sheng et al. 2015; Benesch et al. 2018).

In the adult organism, the ATX/LPA axis is involved in **tissue remodelling and wound healing** (Benesch et al. 2018). Platelets release high amounts of LPA during blood clotting. LPA in turn induces platelet aggregation, vasoconstriction, and proliferation and migration of fibroblasts. In endothelial cells, LPA promotes surface expression of leukocyte adhesion molecules. LPA is present in oxidized low-density lipoproteins (LDL) and accumulates in atherosclerotic plaques where it acts proinflammatory and promotes monocyte recruitment, macrophage-foam cell transformation, proliferation and migration of vascular smooth muscle cells, and neointima and thrombus formation. Therefore, LPA plays a role in atherosclerosis (Zhao et al. 2019; Zhou et al. 2019). A major source of ATX in plasma is adipose tissue, but the role of ATX/LPA in obesity remains contradictory. ATX/$LPA_{1,3}$ signaling impairs insulin sensitivity and deteriorates glucose tolerance (D'Souza et al. 2018). LPA furthermore regulates functions of T cells, macrophages, mast cells, neutrophils, and dendritic cells and is involved in **immunity, inflammation, and fibrosis**. ATX, LPA_4, and LPA_6 promote T cell entry into lymph nodes.

The role for ATX/LPA in fibrosis of the lung, kidney, liver, and skin is underlined by the clinical development of ATX inhibitors and LPA_1 receptor antagonists in these areas of indication. LPA is also involved in chronic inflammatory diseases such as bronchial asthma and rheumatoid arthritis (Yung et al. 2014; Ninou et al. 2018; Magkrioti et al. 2019). The ATX/LPA receptor axis plays an important role in the adult nervous system and has been implicated in numerous diseases of the central and peripheral nervous systems (Herr et al. 2019). ATX, LPA_1, LPA_3, and LPA_5 contribute to **neuropathic pain**. Different LPA receptor antagonists/ATX inhibitors were protective in mouse models of chemotherapy-induced or diabetic neuropathy (Velasco et al. 2017). Finally, ATX and LPA are secreted by many **cancers**, both by tumor cells and by cells of the tumor microenvironment. They promote invasion and metastasis and inhibit antitumor immunity. Furthermore, both ATX and LPA_2 play a role in DNA damage repair and counteract apoptosis-inducing therapies such as ionizing radiation or chemotherapy (Tigyi et al. 2019; Leblanc et al. 2018; Benesch et al. 2018).

Drugs

Several LPA_1 receptor antagonists and ATX inhibitors are in clinical development for treatment of fibrotic diseases (Table 2). SAR100842 is a negative allosteric modulator of the LPA_1 receptor and in phase II clinical trials for systemic sclerosis (Allanore et al. 2018). A phase II study with the LPA_1 antagonist, BMS-986020, for treatment of idiopathic pulmonary fibrosis, was terminated because of three cases of cholecystitis related to BMS-986020 (Palmer et al. 2018) (ClinicalTrials.gov Identifier: NCT01766817). An orthosteric LPA_1 antagonist, BMS-986202, is in phase I with the future indication psoriasis (ClinicalTrials.gov Identifier: NCT02763969). Several ATX inhibitors are listed in the clinical trial database. GLPG1690 (Maher et al. 2019) has been granted orphan drug designation for idiopathic pulmonary fibrosis and systemic sclerosis. Other ATX inhibitors, such as BBT-877 (future indication, idiopathic pulmonary fibrosis) and BLD-0409 (future indication, nonalcoholic steatohepatitis), are currently entering clinical development (Table 2).

Lysophosphatidic Acid, Table 2 Drugs targeting the LPA signaling system

Drug	Target	Indication	Stage
SAR100842	LPA_1 negative allosteric modulator	Systemic sclerosis	Phase II
BMS-986020	LPA_1 antagonist	Idiopathic pulmonary fibrosis (study terminated because of serious adverse events)	Phase II
BMS-986202 (AM152)	LPA_1 antagonist	Psoriasis	Phase I
GLPG1690	ATX inhibitor	Idiopathic pulmonary fibrosis Systemic sclerosis	Orphan drug
BBT-877	ATX inhibitor	Idiopathic pulmonary fibrosis	Phase I
BLD-0409	ATX inhibitor	Chronic liver disease, nonalcoholic steatohepatitis	Phase I

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Lysophospholipids

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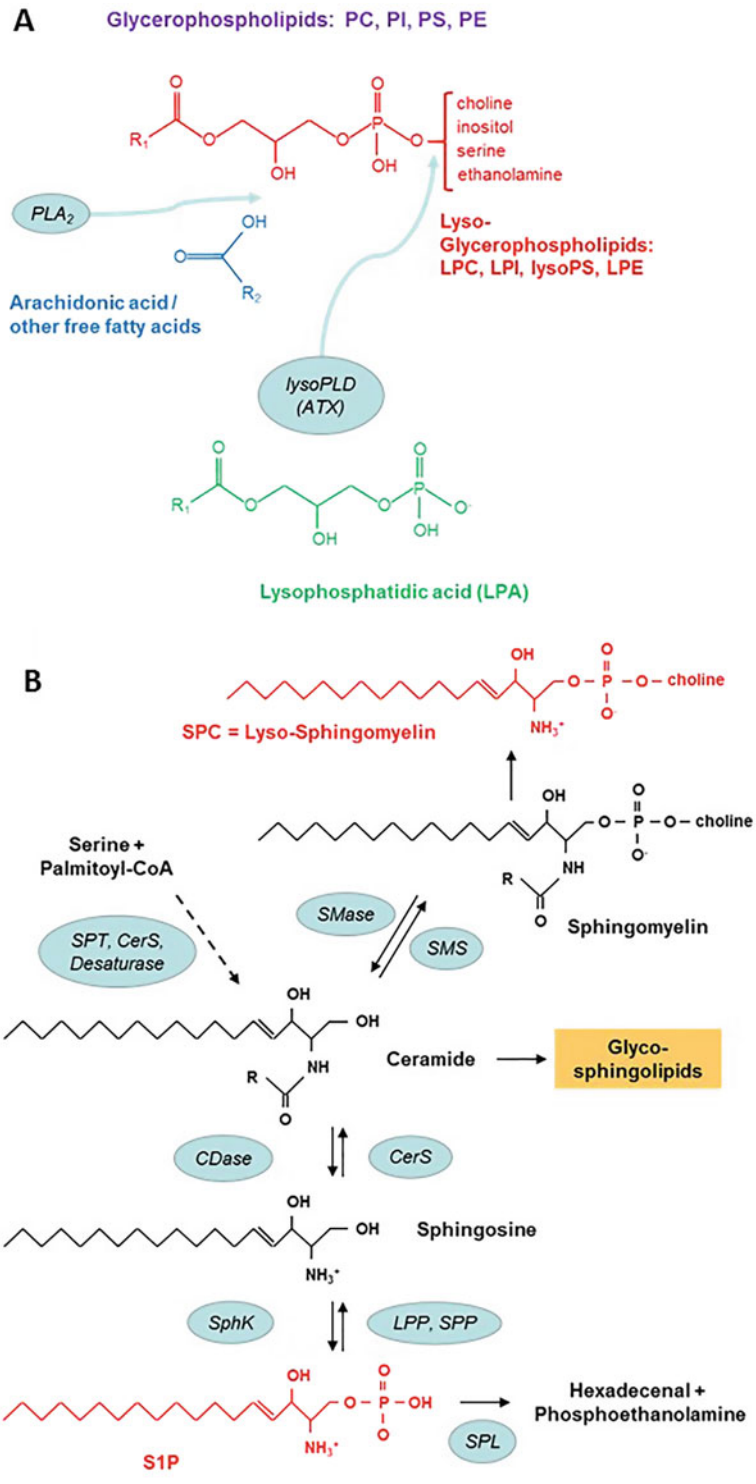
Synonyms

Lysoglycerophospholipids; Lysolipid mediators; Lysosphingolipids

Definition

Lysophospholipids are small bioactive lipid molecules characterized by a single carbon chain and a phosphate-containing polar head group. Two subgroups can be distinguished: molecules based on the glycerol backbone (lysoglycerophospholipids) and molecules based on the sphingoid base backbone (lysosphingolipids). The lysolipid structure renders these lipids more hydrophilic and versatile than their corresponding phospholipids. Cleavage



Lysophospholipids, Fig. 1 (**a**) Generation of lysoglycerophospholipids and arachidonic acid/other free fatty acids by phospholipase A_2 (PLA_2), and generation of lysophosphatidic acid (LPA) by autotaxin (ATX). LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; lysoPS, lysophosphatidylserine; PC,

of glycerophospholipids by phospholipases A_2 (PLA_2) simultaneously generates free fatty acids such as arachidonic acid, the precursor of eicosanoids, and lysoglycerophospholipids such as lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylserine (lysoPS), or lysophosphatidylinositol (LPI) (Fig. 1a). Further cleavage of their head groups results in lysophosphatidic acid (LPA), the simplest lysophospholipid (Fig. 1a). The lysosphingolipids, sphingosine-1-phosphate (S1P) and sphingosylphosphorylcholine (SPC), are products of sphingolipid metabolism as shown in Fig. 1b. Other lysosphingolipids, namely, psychosine (galactosylsphingosine) and glucopsychosine (glucosylsphingosine), lack a phosphate group.

Lysophospholipids may act as extracellular agonists at specific G-protein-coupled receptors (GPCR) and/or as versatile intracellular mediators. Bona fide lysophospholipid receptors as listed in the IUPHAR/BPS Guide to Pharmacology comprise the class A GPCR, $LPA_{1\text{-}6}$ (human gene names: *LPAR1-6*), and $S1P_{1\text{-}5}$ (human gene names: *S1PR1-5*) (Blaho et al. 2019a, b). Other lysophospholipid GPCR are still listed as orphan receptors. They comprise the potential LysoPS-GPCR, GPR34, GPR174, and P2Y10 (Kihara et al. 2015; Yatomi et al. 2018) and the potential LPI-GPCR, GPR55 (Alhouayek et al. 2018). Previous reports on G2A, TDAG8, OGR1, or GPR4 as SPC- and/or LPC-GPCR have in part been retracted; the role of these GPCR remains largely unclear.

Of all lysophospholipids, LPA and S1P have been characterized in greatest detail so far. They are auto- or paracrine regulators of cell proliferation and survival, cell migration and chemotaxis, cytoskeletal architecture, cell-cell contacts and adhesion, Ca^{2+} homoeostasis, and Ca^{2+}-dependent functions. By regulating these cellular responses, they play a role in organism development and homeostasis of many tissues within the cardiovascular, nervous, immune, and metabolic systems.

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Lysosphingolipids

▶ Lysophospholipids

Lysophospholipids, Fig. 1 (continued) phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLD, phospholipase D; PS, phosphatidylserine. (**b**) Generation of sphingosine-1-phosphate (S1P) and sphingosylphosphorylcholine (SPC) by sphingolipid metabolism. CDase, ceramidase; CerS, ceramide synthase; LPP, lipid phosphate phosphatase; SMase, sphingomyelinase; SMS, sphingomyelin synthase; SphK, sphingosine kinase; SPL, S1P lyase; SPP, S1P phosphatase; SPT, serine palmitoyltransferase. R, R1, R2, residues containing carbon chains with different lengths and degrees of saturation

M

Macroautophagy

► Autophagy

MAP Kinase Cascades

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Synonyms

Serine/threonine protein kinase phosphorelay modules

Definitions

Mitogen activated protein kinase (MAPK) cascades are three kinase modules activated by phosphorylation. These modules are composed of a MAPK, a MAPKK, and a MAPKKK. Each component in the MAPK cascade is composed of multiple family members that are conserved from yeast to human. MAPK modules are selectively activated by different, specific stimuli including growth factors, cytokines, and cellular stresses. Activation of MAPK modules results in changes in gene expression and the control of diverse cellular functions such as growth, migration, and apoptosis.

Basic Characteristics

Characteristics of MAPK Cascades

MAPK cascades are signaling modules that mediate signal transduction of diverse, cellular stimuli to the nucleus to control gene expression (Widmann et al. 1999; Cuevas et al. 2007). These stimuli include growth factors, cytokines, and bacterial products. MAPK cascades are also activated by numerous cellular stresses such as irradiation, cold and heat shock, hyperosmolarity, and oxidative stress. Selective stimulation of MAPK modules leads to changes in cellular function, including proliferation, differentiation, survival, apoptosis, migration, and adhesion.

MAPK cascades are composed of three cytoplasmic kinases, the MAPKKK (MAP3K), MAPKK (MAP2K), and the MAPK that are regulated by phosphorylation (Fig. 1). The MAP3Ks are a large family of structurally diverse, serine/threonine kinases that are differentially activated by upstream stimuli. Activated MAP3Ks phosphorylate specific MAP2Ks, also called MEKs for MAP/ERK kinase. The MAP2K is phosphorylated by the MAP3K on two specific serine/threonine residues in its activation loop. The MAP2Ks are a family of seven, dual specificity kinases that phosphorylate threonine and tyrosine residues in the activation loop of specific MAPKs.

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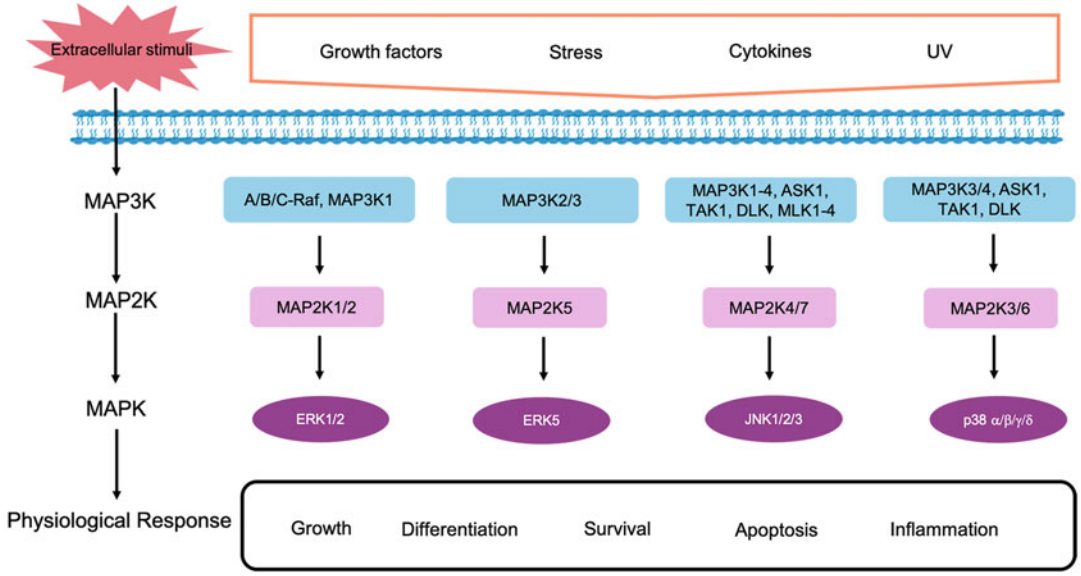

MAP Kinase Cascades, Fig. 1 Organization of MAPK cascades. See text for details

The MAPKs are a family of 13 serine/threonine kinases. Phosphorylation and activation of MAPKs by MAP2Ks result in MAPK phosphorylation of specific cytoplasmic substrates. In addition, phosphorylated MAPKs translocate to the nucleus where they phosphorylate transcription factors and regulate gene expression. MAPKs are divided into the following six subfamilies: extracellular signal-regulated kinases (ERKs) 1 and 2, ERK5, Jun amino-terminal kinases (JNKs) 1, 2, and 3, p38 kinase members p38α, β, γ, and δ, atypical ERK3/4, and atypical ERK7/8. This review will focus on the four most characterized subfamilies, ERK1/2, ERK5, JNK, and p38 kinase (Fig. 1).

Characteristics of the ERK1/2 Pathway

Extracellular signal-regulated kinase (ERK) 1 and ERK2 are also called p44 MAPK and p42 MAPK, respectively. These MAPKs are expressed ubiquitously in most cell types. ERK1 and ERK2 play critical roles in the promotion of cell proliferation and survival, making them critical targets of anticancer therapies. ERK1/2s are the end components of several three kinase signaling modules that are activated in response to growth factors and cellular adhesion. These stimuli activate specific MAP3Ks, including RAF kinases and MEK kinase 1 (MEKK1/MAP3K1). MAP3K1 and RAF kinases phosphorylate and activate specific MAP2Ks called MAP2K1 and MAP2K2, more commonly called MAP/ERK kinase (MEK) 1 and MEK2. MEK1/2 dual phosphorylate threonine and tyrosine in a conserved threonine-glutamate-tyrosine (TEY) motif in the catalytic domain of ERK1/2. Active ERK1/2 phosphorylate many substrates in the cytoplasm, including ribosomal S6 kinase, phospholipase A2, and microtubule associated proteins. Further, active ERK1/2 translocate to the nucleus to promote the phosphorylation and transcriptional activity of several transcription factors, including ELK1, ETS1, and MYC. Dual specificity protein phosphatases (DUSP1 and DUSP2) dephosphorylate both threonine and tyrosine in the ERK1/2 catalytic domain, decreasing ERK1/2 activity (Widmann et al. 1999).

The selective deletion of ERK1 or ERK2 in mice has been used to define their biological functions. Although animals with the deletion of ERK1 are overtly normal, deletion of ERK2 results in early embryonic lethality due to defective placental development. Interestingly, deletion of one upstream activator of this MAPK cascade, the MAP3K CRAF, also results in early embryonic lethality due to placental defects. Knockout of the MAP3K BRAF or the MAP2K MEK1 results in

embryonic lethality due to vascular and angiogenesis defects (Pearson et al. 2001). These studies show the critical roles of the ERK1/2 MAPK cascades in cell proliferation, migration, differentiation, and angiogenesis during development. Further, aberrant activation of the ERK signaling module is responsible for the majority of cancers.

Characteristics of the ERK5 Pathway

ERK5 was originally named Big MAP Kinase (BMK1) owing to it being over twice the size of ERK1/2 (Nithianandarajah-Jones et al. 2012). This larger size is due to the presence of a long C-terminal extension not found in other MAPKs. Similar to ERK1/2, ERK5 is expressed in most cell types, tissues, and organs, and is activated in response to growth factors similar to ERK1/2. ERK5 is also activated by cellular stresses such as oxidative stress and hyperosmolarity similar to the MAPK members JNK and p38. Although ERK5 is activated by growth factors and cellular stresses, it is phosphorylated by distinct MAP3K and MAP2K family members. MAP3K2 and MAP3K3 are the sole MAP3Ks that activate MAP2K5 (MEK5). MAP3K2, MAP3K3, and MEK5 share a Bem1P (PB1) domain that is used for binding between the upstream components of this MAP3K/MAP2K module. MEK5 selectively phosphorylates the TEY motif in the catalytic domain of ERK5, and MEK5 is not used to activate other MAPKs. Active ERK5 phosphorylates many transcription factors, including MEF2A, C, and D, and increases their transactivation.

Individual components of the MAP3K2/3/MEK5/ERK5 pathway have been deleted in mice to determine the biological roles of these components. Although mice lacking MAP3K2 are overtly normal, MAP3K3 deletion results in early embryonic lethality due to defects in angiogenesis. Deletion of MEK5 is also early embryonic lethal due to defects in cardiac development, and increased apoptosis in several regions including the heart. ERK5 knockout results in early embryonic lethality due to defects in both the embryo and the placenta. Defects include defective heart development and angiogenesis. Deletion of MEF2C also results in death due to cardiac and vascular defects. Together, these studies demonstrate the critical function of this MAP3K3/MEK5/ERK5 signaling module in regulation of development. In addition to roles in blood vessel and cardiac development, ERK5 expression has been implicated in promoting several diseases including cancer.

Characteristics of the JNK Pathway

Jun amino-terminal kinases (JNKs) are a subfamily of MAPKs composed of three members, JNK1, 2, and 3 (Zeke et al. 2016). This family is named based on their shared ability to phosphorylate and activate the transcription factor Jun. They were also called stress-activated protein kinases (SAPKs) due to their activation in response to cellular stresses, including osmotic shock and irradiation. JNKs are also activated in response to stimulation by many growth factors and cytokines. Activation by these stimuli leads to JNK-mediated regulation of a diverse set of genes including growth factors, cytokines, extracellular matrix proteins, and matrix metalloproteinases. JNK activation is induced by over a dozen different MAP3Ks, including MAP3K1-4 and MLK1-4. These MAP3Ks selectively phosphorylate and activate MAP2K4 and 7, which in turn phosphorylate the threonine-proline-tyrosine (TPY) motif in the catalytic domain of JNKs. The presence of this proline is unique to JNKs. There are approximately 100 reported JNK substrates that include receptors, enzymes, cytoskeletal proteins, and transcription factors (Zeke et al. 2016).

JNKs play key roles in several cellular processes, most notably apoptosis. However, the biological roles of individual JNK family members have been difficult to determine. JNK1 and 2 are ubiquitously expressed in almost all cell types. In contrast, JNK3 expression is restricted mainly to the central nervous system with some expression in the heart and testis. The complexity of JNK signaling is increased by alternative splicing of JNK family members. For example, there are four isoforms each of JNK1 and 2, and eight different isoforms of JNK3. Individual deletion of JNK family members results in modest defects, suggesting their overlapping roles. For example, JNK1 knockouts show defects in metabolism, whereas JNK2 knockouts display epidermal hyperplasia. Individual deletion of JNK1 or JNK 2 also disrupts immune cell function. However,

co-deletion of JNK1 and JNK2 results in embryonic lethality due to failed neural tube closure. These studies have identified key roles for JNK in neuronal development and immune function. JNK dysfunction is associated with tumorigenesis, neurological disorders, insulin resistance and diabetes, and altered immune function.

Characteristics of the p38 Kinase Pathway

The p38 kinases are a subfamily of MAPKs identified by several groups as 38 kDa proteins that are phosphorylated in response to bacterial lipopolysaccharide (LPS) (Cuadrado and Nebreda 2010). The p38 kinase family is composed of four members, p38α, β, γ, and δ, based on a shared threonine-glycine-tyrosine (TGY) motif in their activation loop. The four p38 kinase family members are approximately 60% identical. They are found in both the cytoplasm and the nucleus where they phosphorylate and regulate a diverse set of proteins, including kinases, chromatin remodelers, and transcription factors. Similar to JNK family kinases, p38 MAPKs are activated in response to cellular stresses like hyperosmolarity, heat shock, and irradiation, and by growth factor stimulation. p38 MAPKs play critical roles in inflammation and cellular stress. p38 MAPKs are activated by several MAP3Ks, including MAP3K3, MAP3K4, ASK1, and TAK1. Active MAP3Ks phosphorylate MAP2K3 and MAP2K6, which phosphorylate the TGY motif in p38 kinases. MAP2K4 (MEK4) also phosphorylates p38. Interestingly, p38 kinases can also be activated by MAP2K independent mechanisms. Transient activation of p38 can lead to survival, whereas sustained activation of p38 often leads to cell death. Activation of p38 is terminated by both serine/threonine phosphatases like PP2A and by DUSPs.

The individual biological roles of p38 isoforms have been difficult to determine due to their overlapping expression and redundant substrate phosphorylation. The p38α isoform is expressed ubiquitously and at high levels in most cell types. In contrast, the other isoforms show reduced and more selective expression. p38β, p38γ, and p38δ show highest expression in the brain, muscle, and pancreas, respectively. Deletion of p38α in mice results in embryonic lethality due to placental defects. Although mice with individual deletion of MAP2K3 or MAP2K6 are viable, co-deletion of MAP2K3/6 results in embryonic lethality due to placental defects, demonstrating their key role in activating p38α during placental development. Mice with single deletion of MAP2K3 or MAP2K6 show immune function defects. In contrast to deletion of p38α, mice with deletion of p38β, γ, and δ are viable and overtly normal. Importantly, studies of adult knockout animals have revealed unique roles for these isoforms in muscle stem cells (p38γ) and in pancreatic beta cells (p38δ). Studies in mice have revealed key roles for p38 MAPKs during development and disease progression, especially inflammation.

Drugs

Due to the critical roles played by MAPKs in many pathological conditions including inflammation and cancer, MAPK modules have been very actively targeted for pharmacological intervention. Selective inhibitors for many of the MAPK family members have been created (Table 1). The most extensively targeted are the ERK1/2 and p38 MAPK modules. However, inhibitors have also been developed that target ERK5 and JNK MAPKs.

The RAF/MEK1/2/ERK1/2 MAPK signaling module is the most heavily mutated pathway in cancer. Aberrant activity of this MAPK cascade is responsible for the majority of cancers, making members of this MAPK cascade critical targets for drug therapy. Selective inhibitors have been developed for each component of the RAF/MEK1/2/ERK1/2 MAPK signaling module (Liu et al. 2018). First to be developed, PD98059 and U0126 inhibit MEK1/2 decreasing ERK1/2 activity at low concentrations that did not inhibit other MAPK cascades. Both act as allosteric inhibitors, binding at sites distinct from the ATP binding pocket. However, the pharmacological properties of these inhibitors made them unsuitable for clinical use. More recent work has developed new inhibitors for this cascade. RAF inhibitors that target the MAP3K

MAP Kinase Cascades, Table 1 Pharmacological inhibitors of MAPK cascades

Kinase	Inhibitors	Mechanisms of action	Biological consequences	Clinical trials
RAF	1. Sorafenib 2. Vemurafenib 3. Dabrafenib	ATP competitive	Reduces MAP2K1/2 activity and cancer growth	1. Phase IV liver cancer, Phase III kidney cancer 2/3. FDA approved for metastatic melanoma
MAP2K1/2 (MEK1/2)	1. PD98059 2. U0126 3. PD184352 4. PD0325901 5. Selumetinib 6. Binimetinib	Allosteric inhibitors bind to MAP2K1/2 at a site distinct from the ATP binding pocket	Reduces ERK1/2 activity and cancer cell growth	3. Phase II Cancer 4. Phase II neurofibromatosis type 1 5. Phase III cancer, FDA-approved low-grade glioma 6. Phase II metastatic colorectal cancer, FDA-approved melanoma
ERK1/2	1. SCH772984 2. VX-11e 3. BVD-523 4. GDC-0994	ATP competitive	Reduces ERK1/2 activity and cancer cell growth	3. Most advanced ERK inhibitor Phase I/II advanced solid tumors 4. Phase I melanoma and solid tumors
MAP2K5 (MEK5)	1. PD98059 2. U0126 3. PD184352 4. BIX02188 5. BIX02189	1-3. Allosteric MEK1/2 inhibitors that can also inhibit MEK5. 4/5. MEK5 selective ATP competitive	Reduces ERK5 activity	
ERK5	1. XMD8-92 2. ERK5-IN-1	Inhibit ERK5 kinase activity and EGF induced ERK5 autophosphorylation	Reduce ERK5 activity	
JNK1/2/3	1. SP600125 2. JNK-IN-8 3. JNK Inhibitor IX 4. XG-102	1. ATP competitive 2. Irreversible covalent modification of cysteine residue in ATP binding site 3. ATP binding site of JNK2/3 4. Cell-permeable peptide competes for docking site	1-3. Blocks JNK activity, including induction of TNFα and Jun phosphorylation 3. Selective for JNK2/3 4. Inhibit JNK interaction with substrates	4. Phase III in pain and inflammation
p38 kinase	1. SB203580 2. SB202190 3. Vertex 745 4. Vertex 702 5. BIRB-796 6. Ralimetinib 7. PH-797804	1-4, 6, 7. ATP competitive 5. Allosteric	Reduces activation of p38 and decreases production of inflammatory cytokines	3. Phase II Alzheimer's disease 4/5. Phase II rheumatoid arthritis 6. Phase I advanced cancers, phase I/II in adult glioblastoma 7. Phase II COPD, osteoarthritis, and rheumatoid arthritis

component of the cascade selectively inhibit RAF monomers. These inhibitors are in clinical trials to treat liver and kidney cancers, and have been approved for treating metastatic melanoma. Since the early development of PD98059 and U0126, multiple new inhibitors have been developed for

MEK1/2. These allosteric inhibitors are in clinical trials for several cancer types, and are approved for melanoma. Recently, ATP competitive ERK1/2 inhibitors have been developed. These inhibitors are in clinical trials to treat melanoma and advanced solid tumors. Several studies have shown an association of ERK5 expression in pathologies inducing cancer. Recent work has developed inhibitors specific for the MAP2K5 (MEK5) and ERK5 MAPK module (Table 1). These MEK5/ERK5 inhibitors have been tested in preclinical models of cancer, and may also be applicable to treat ischemia and chronic pain.

Similar to the ERK1/2 MAPK module, p38 kinases have been subjected to extensive development of pharmacological inhibitors, mostly due to the well-defined role of p38 kinase in inflammation. There are more than 20 candidate p38 kinase inhibitors in clinical trials, but no selective, p38 inhibitors have been approved (Haller et al. 2020). Inhibition of p38 kinase activity decreases the production of inflammatory cytokines and decreases inflammation. Several diseases strongly associated with inflammation including rheumatoid arthritis, osteoarthritis, and chronic obstructive pulmonary disease (COPD) have been targeted in clinical trials with p38 kinase inhibitors. Early p38 kinase inhibitors with pyridinyl imidazole compounds like SB203580 competitively inhibit p38 kinase by binding the ATP-binding pocket. Most of the new p38 inhibitors also act as ATP competitive inhibitors that bind to the ATP-binding pocket of p38 kinases. However, BIRB-796 is thought to function as an allosteric inhibitor that causes a conformational change in p38 kinase, preventing ATP binding and activation. Although p38 kinase inhibitors have been successful in preclinical trials, these inhibitors have failed in clinical trials due to poor efficacy and/or liver toxicity. Newer strategies include local application of p38 inhibitors and creation of inhibitors that block the interaction of p38 with specific substrates. In addition to targeting p38, there have been efforts to target JNK. For example, the JNK inhibitor SP600125 is an ATP competitive inhibitor, but it also inhibits several other kinases. Newer JNK inhibitors using a different strategy have recently been developed. For example, XG-102 acts by competing with the docking site, inhibiting the interaction of JNK with its substrates. XG-102 is currently in clinical trials to treat pain and inflammation.

In summary, MAPK cascades mediate the transduction of diverse cellular stimuli to the cytoplasm and nucleus. These cascades play critical roles during development, and their activity is altered during pathologies such as inflammation and cancer. Members of MAPK cascades have been actively targeted for pharmaceutical intervention with some success. However, key issues in the pharmacological targeting of the MAPK components include poor efficacy and organ toxicity. In addition, cross-talk between different MAPK modules has also resulted in issues. Further, compensatory activation of alternative kinase components or modules has frequently been observed. For example, initial targeting of MEK1/2 with inhibitors effectively decreases activation of ERK1/2 and blocks tumor cell proliferation. However, compensation by increased expression of ERK1/2 or activation of other kinases allows increased tumor cell proliferation and survival, and cancer recurrence. Approaches to mitigate these issues are actively being pursued.

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Matrix Metalloproteinases

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Synonyms

MMPs

Definition

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases and belong to a larger family of proteases known as the metzincin superfamily. MMPs are a major group of enzymes that regulate cell–matrix composition. They require a zinc ion in their active site for catalytic activity and are critical for maintaining tissue allostasis. MMPs are active at neutral pH and can therefore catalyze the normal turnover of extracellular matrix (ECM) macromolecules. Members of the MMP family include the "classical" MMPs (extracellular proteolytic enzymes), the membrane-bound MMPs (MT-MMPs), the ADAMs (a disintegrin and metalloproteinase; adamlysins), and the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motif).

Basic Characteristics

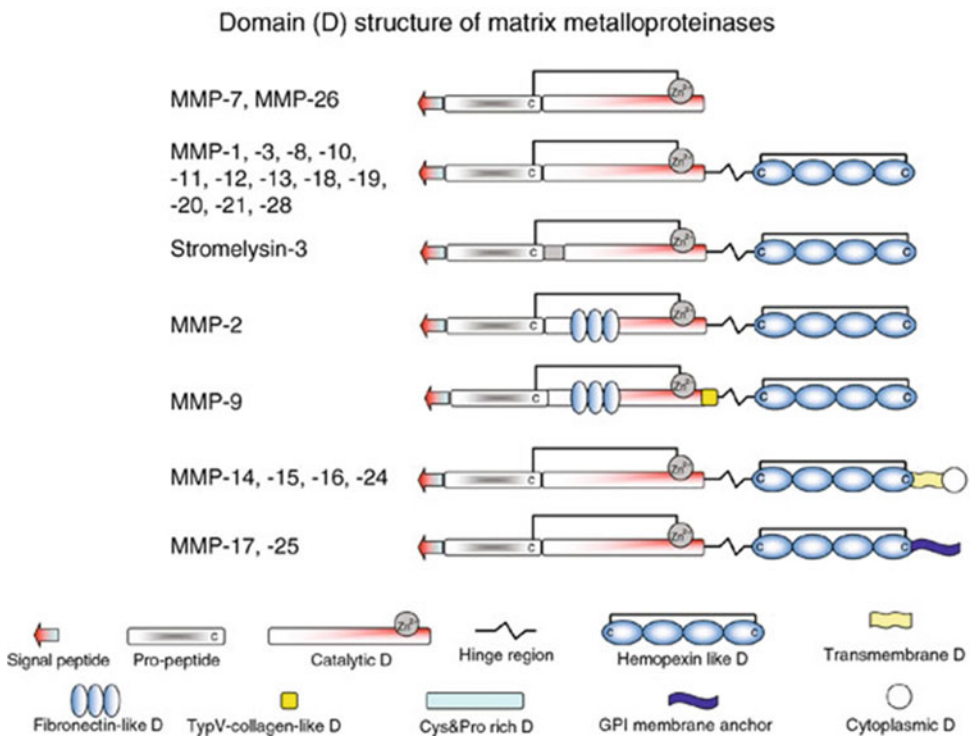
MMP genes show a highly conserved modular structure. They were first detected in vertebrates (1962), and human beings, but have since been found in invertebrates and plants as well. The MMP family consists of zinc-dependent proteolytic enzymes, which comprise at least 24 different members so far (Somerville et al. 2003) (Table 1). Although all of them exhibit a broad substrate spectrum, they are divided based on their main substrate into collagenases, gelatinases, stromelysins, matrilysins, metalloelastase, membrane-type MMPs (MT-MMPs), and others. MMPs are secreted as zymogens and become activated by cleavage of their propeptide. Figure 1 (domain structure) depicts an overview of the domain organization of different MMPs. They have several structural features in common that include a propeptide domain containing the "cysteine switch," the catalytic zinc-binding domain with the sequence HEXGHXXGXXHS, and a hemopexin-like domain. Although each MMP exhibits substrate specificity toward individual matrix proteins, there is considerable overlapping within the whole family. Collectively, they are capable of degrading all kinds of ECM proteins, such as the interstitial and basement membrane collagens, proteoglycans (e.g., aggrecan, decorin, biglycan, fibromodulin, and versican), as well as accessory ECM proteins such as fibronectin. MMPs can also process a number of bioactive molecules. They are involved in the cleavage of cell surface receptors, the release of apoptotic ligands (such as the FAS ligand), and chemokine (in-)activation. They also play a major role on cell proliferation, migration (adhesion/dispersion), differentiation, angiogenesis, apoptosis, and host defense.

In addition, MMPs influence endothelial cell function as well as vascular smooth muscle cell migration, proliferation, Ca^{2+} signaling, and contraction. MMPs play a role in vascular tissue remodeling during various biological processes such as angiogenesis, embryogenesis, morphogenesis, and wound repair. Alterations in specific MMPs might influence arterial remodeling and lead to various pathological disorders such as hypertension, preeclampsia, atherosclerosis, aneurysm formation, as well as venous dilation and lower extremity venous disease.

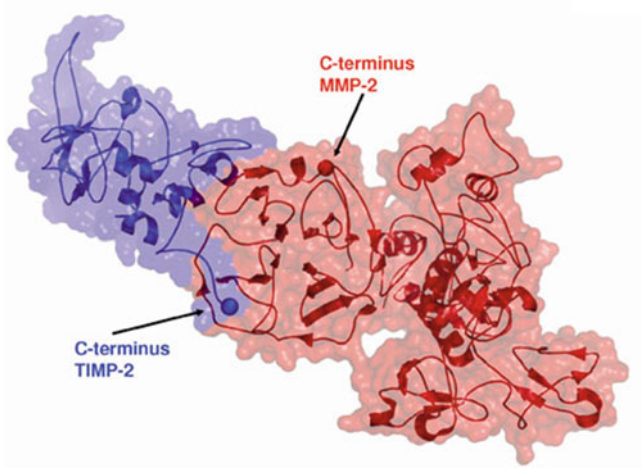
The enzymatic activity of these potentially harmful enzymes is tightly controlled. Once transcribed into protein, MMPs are expressed as inactive zymogens and require distinct activation processes to convert them into active enzymes. After secretion, MMP-activity is regulated by the noncovalent binding of tissue inhibitors of metalloproteinases (TIMPs) as shown in Fig. 2 for MMP-2 and TIMP-2. Four TIMPs have been identified so far: TIMP-1, TIMP-2, TIMP-3, and

Matrix Metalloproteinases, Table 1 MMPs and their genes known so far

Gene	Name	Location	Comments
MMP-1	Interstitial collagenase	Secreted	One of three collagenases that can degrade the interstitial collagens, types I, II, and III
MMP-2	Gelatinase-A, 72 kDa gelatinase	Secreted	
MMP-3	Stromelysin 1	Secreted	
MMP-7	Matrilysin, PUMP 1	Secreted	Smallest member of MMPs
MMP-8	Neutrophil collagenase	Secreted	
MMP-9	Gelatinase-B, 92 kDa gelatinase	Secreted	MMP-9 plays a regulatory role in angiogenesis not only through proteolytic activity but also through other downstream angiogenic factors
MMP-10	Stromelysin 2	Secreted	
MMP-11	Stromelysin 3	Secreted	MMP-11 shows more similarity to the MT-MMPs, is convertase-activatable and is secreted therefore usually associated to convertase-activatable MMPs.
MMP-12	Macrophage metalloelastase	Secreted	
MMP-13	Collagenase 3	Secreted	
MMP-14	MT1-MMP	Membrane-associated	Type-I transmembrane MMP
MMP-15	MT2-MMP	Membrane-associated	type-I transmembrane MMP
MMP-16	MT3-MMP	Membrane-associated	Type-I transmembrane MMP
MMP-17	MT4-MMP	Membrane-associated	Glycosyl phosphatidylinositol-attached
MMP-18	Collagenase 4, xcol4, xenopus collagenase	–	No known human orthologue
MMP-19	RASI-1, occasionally referred to as stromelysin-4	–	
MMP-20	Enamelysin	Secreted	
MMP-21	X-MMP	Secreted	
MMP-23A	CA-MMP	Membrane-associated	Type-II transmembrane cysteine array
MMP-23B	–	Membrane-associated	Type-II transmembrane cysteine array
MMP-24	MT5-MMP	Membrane-associated	Type-I transmembrane MMP
MMP-25	MT6-MMP	Membrane-associated	Glycosyl phosphatidylinositol-attached
MMP-26	Matrilysin-2, endometase	–	
MMP-27	MMP-22, C-MMP	–	
MMP-28	Epilysin	Secreted	Discovered in 2001, given its name due to have been discovered in human keratinocytes. Highly expressed in lung, placenta, salivary glands, heart, uterus, skin



Matrix Metalloproteinases, Fig. 1 Domain organization of different mammalian MMPs



Matrix Metalloproteinases, Fig. 2 ProMMP-2-TIMP-2 structure. (Adopted from Morgunova et al. 2002). TIMP-2 cartoon and transparent surface structure is shown in blue, MMP-2 in red. The C-terminal ends of both molecules are marked as spheres

TIMP-4. All known MMPs can be inhibited by at least one of the four known TIMPs. Nevertheless, individual differences with regard to bond strength and thus the magnitude of inhibition of a particular MMP do exist.

Drugs

Ample evidence exists on the role of MMPs in normal and pathological processes, including tissue remodeling associated with various

physiological and pathological processes such as morphogenesis, embryogenesis, angiogenesis, tissue repair, inflammation, cirrhosis (Hemmann et al. 2007), arthritis, cancer, and metastasis. The gelatinases MMP-2 and MMP-9 are thought to be important, e.g., in metastasis and MMP-1 in rheumatoid and osteoarthritis.

MMP inhibitor development constitutes an important branch of research in both academic and industrial settings and advances our knowledge on the structure–function relationship of these enzymes. Targeting MMPs for therapeutic interventions is complicated by the fact that MMPs are indispensable for normal development and physiology and by their multi-functionality, possible functional redundancy or contradiction, and context-dependent expression and activity. This complexity was revealed by previous efforts to inhibit MMP activity in the treatment of cancer patients that yielded unsatisfactory results. Doxycycline, at subantimicrobial doses, inhibits MMP activity and has been used in various experimental systems for this purpose. It is used clinically for the treatment of periodontal disease and is the only MMP inhibitor that is widely available clinically.

A number of rationally designed MMP inhibitors have shown some promise in the treatment of pathologies, which MMPs are suspected to be involved in. However, most of these, such as Marimastat (BB-2516), a broad spectrum MMP inhibitor, or trocade (Ro 32–3555), an MMP-1 selective inhibitor, have performed poorly in clinical trials. The failure of Marimastat was partially responsible for the folding of British Biotech, which developed it. The failure of these drugs has been largely due to toxicity (particularly musculo-skeletal toxicity in the case of broad-spectrum inhibitors) and failure to show expected results (in the case of trocade, promising results in rabbit arthritis models were not replicated in human trials). The reasons behind the largely disappointing clinical results of MMP inhibitors are still unclear, especially in light of their activity in animal models. New evidence suggests that MMPs may also generate angiogenesis inhibitors (Stetler-Stevenson 1999). Because of this dual role in tumor tissue, it is important to identify correctly the MMPs involved in function, activity, and origin as a prerequisite to creating highly selective MMP inhibitors as potential therapeutics.

Development of MMP null mice carrying specific MMP deletions has provided an opportunity to explore the role of MMPs in normal development as well as in such diverse conditions and diseases such as skeletal dysplasia, coronary artery and heart disease, arthritis, cancer, and brain disorders. MMP-28, e.g., the latest discovered MMP, has a role in promoting chronic lung inflammation and tissue remodeling induced by cigarette smoke and highlights a potential target to modulate COPD as has been seen in MMP-28$^{-/-}$ mice.

Adeno-associated virus (AAV) has emerged as a promising gene delivery vector. A protease-activatable AAV vector was developed, that responds to elevated extracellular protease activity commonly found in diseased tissue microenvironments. The provector is able to deliver transgenes site specifically to high-MMP-activity regions of the damaged tissue, with concomitant decreased delivery to many off-target organs, including the liver (Guenther et al. 2019). The AAV provector may be useful in the future for enhanced delivery of transgenes to sites of, e.g., cardiac damage.

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Mechanistic/Mammalian Target of Rapamycin Complex 1 Signaling Network

▶ mTOR Signaling Pathways

Melatonin

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Synonyms

5-Methoxy-N-Acetyltryptamine; N-Acetyl-5-methoxytryptamine

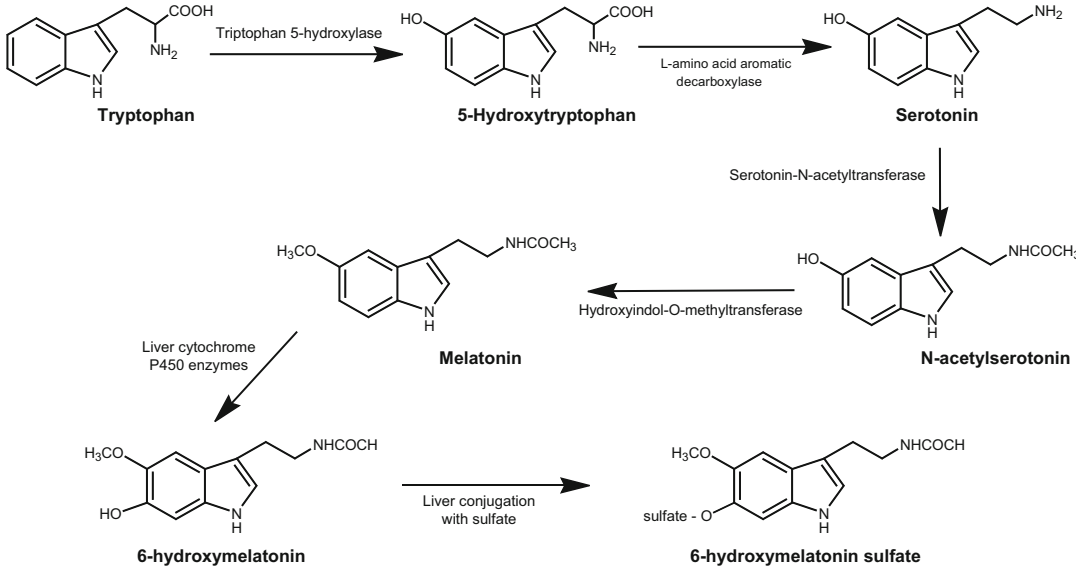
Definition

Melatonin (MLT, N-acetyl-5-methoxytryptamine) is a pleiotropic neurohormone primarily synthesized by the pineal gland during darkness that plays a key role in several physiological and biochemical functions including sleep, pain, thermoregulation, circadian rhythms, immunity, metabolism, and cardiovascular functioning (Reiter 1991). Notably, the synthesis of MLT is not restricted to mammalian species, but it is a unique universal substance with the molecular structure unchanged among non-mammalian vertebrates, some invertebrates, and plants. It was first isolated from bovine pineal tissue and structurally identified in 1958 by Aaron Lerner. Since this new molecule was blanching the melanophores in the skin of frogs and was derived from serotonin, it was named melatonin (i.e., melanophore-contracting hormone; Greek: μέλας, black; τόνος tension, in the sense of contraction).

MLT is also synthesized by several extrapineal organs among which the most important is represented by the gastrointestinal tract. Other organs include the retina, bone marrow cells, platelets, skin, lymphocytes, Harderian gland, and cerebellum (Acuna-Castroviejo et al. 2014).

MLT is synthesized from its precursor, the essential amino acid L-tryptophan, which is hydroxylated by the enzyme tryptophan-5-hydroxylase into 5-hydroxytryptophan that is then decarboxylated by the 5-hydroxytryptophan decarboxylase enzyme into serotonin. Two enzymes then transform serotonin into MLT: serotonin is first acetylated to form N-acetylserotonin by the arylalkylamine-N-acetyltransferase (AA-NAT) that is the rate-limiting enzyme for MLT synthesis, and then N-acetylserotonin is methylated by the N-acetylserotonin-O-methyltransferase (ASMT, also called hydroxyindole-O-methyltransferase or HIOMT) to form MLT. MLT is then mostly metabolized by cytochrome P450 enzymes in the liver to 6-hydroxymelatonin that is further conjugated and excreted with the urine as 6-hydroxymelatonin sulfate (Fig. 1). Degradation of MLT by deacetylation into 5-methoxytryptamine as well as by reaction with reactive oxygen and nitrogen species due to its important radical scavenging properties has been also reported.

MLT synthesis and secretion into body fluids including blood and cerebrospinal fluid is controlled by the external light/dark cycle. Its synthesis occurs during darkness and is inhibited by light in both nocturnal and diurnal species. Photosensory information is transmitted via a polyneuronal pathway which begins from the retina



Melatonin, Fig. 1 Biosynthetic and main degradation pathways of melatonin

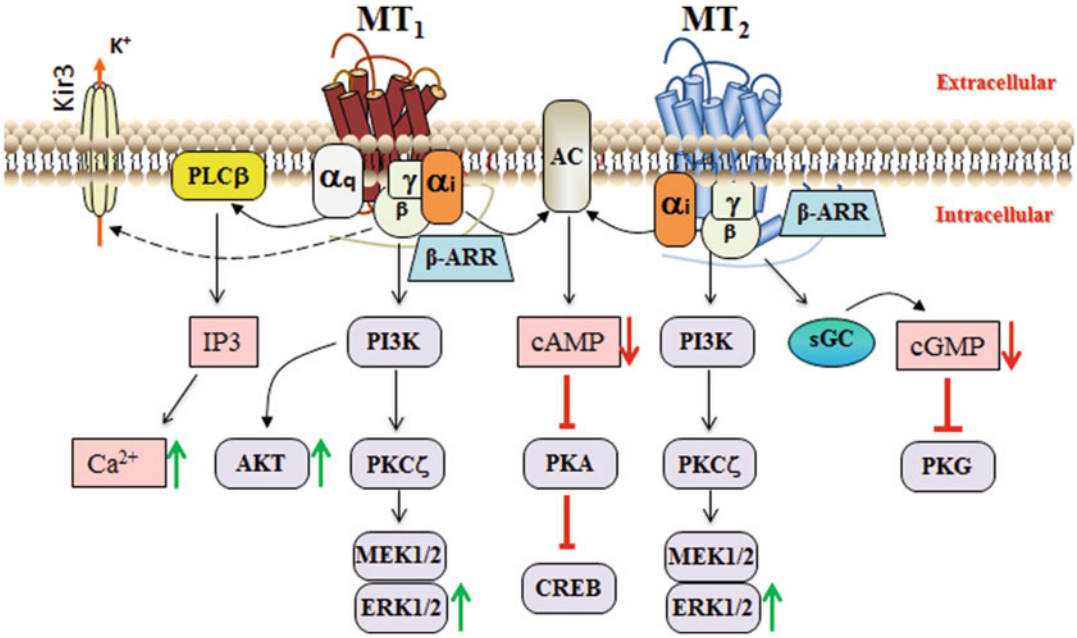
and terminates in the pineal gland and passes through the suprachiasmatic nucleus (SCN), the "master clock" that governs physiological circadian rhythms within the body. Postganglionic sympathetic nerve fibers that end in the pineal gland, when activated, stimulate the release of noradrenaline which produces a cascade of biochemical events within the pinealocyte. Noradrenaline binds to pinealocyte β-adrenergic receptors (and partially α-adrenergic receptors) and activates the adenylate cyclase through a GTP-binding protein in the cell membrane which leads to an increase in intracellular cyclic adenosine monophosphate (cAMP) levels. The increased cAMP levels stimulate the AA-NAT enzyme and finally the synthesis of MLT.

In humans, MLT secretion follows a well-defined circadian pattern with a peak (80–150 ng/mL) in the middle of the night – between 12 PM and 03 AM – that decreases gradually during the second half of the night, offset at 07–09 AM, reaching the level of 10–20 ng/mL during the day. Nearly 80% of MLT is synthesized during the night, with the peak of its serum concentrations decreasing with aging (Reiter 1991).

Basic Characteristics

Melatonin Receptor Types

MLT has been shown to bind to several proteins with various affinities. Most of its physiological effects are mediated through high-affinity MLT receptors belonging to the superfamily of the GTP-binding protein-coupled receptors (GPCR). In humans two MLT receptors exist, called MT_1 and MT_2, both binding MLT with high affinity (0.1–1 nM). A third protein called melatonin-related receptor or GPR50 belongs to the same family because of its high degree of sequence homology with MT_1 and MT_2, but this receptor is unable to bind to MLT or any other known ligand. The atomic structure of the human MT_1 and MT_2 has been solved in 2019. Among the low-affinity MLT targets, the best characterized target is the enzyme quinone reductase 2 (QR2), initially named MT3 or ML2. QR2 binds MLT with an affinity of 1 μM and has been suggested to participate in toxification/detoxification processes. The binding of MLT to QR2 has been confirmed in co-crystals (see Dubocovich et al. (2010) and Jockers et al. (2016) for reviews).



Melatonin, Fig. 2 Overview of main melatonin receptor signaling pathways

MT_1 and MT_2 are expressed in several brain regions (hypothalamic suprachiasmatic nuclei, ventrolateral preoptic nucleus, striatum, hippocampus, pituitary and pineal gland) in the retina and in several peripheral tissues (liver, adipose tissue, pancreas, kidney, etc.). Signaling of GPCRs is initiated by the activation of heterotrimeric GTP binding proteins and ß-arrestins followed by the activation of further downstream signaling cascades. MT_1 and MT_2 are coupling to GTP binding proteins of the Gi and Gq type. Coupling to Gi has been observed in many tissues, while coupling to Gq seems to be restricted to some tissues. Both receptors recruit also ß-arrestin1 and 2. Downstream effector pathways of MLT receptors include the cAMP/PKA, ERK1/2, PI3K/AKT, PLCß and soluble guanylate cyclase pathways, as well as calcium-activated Kir3 inward-rectifier potassium and the N-type calcium Cav2.2 channels. These signaling properties of MT_1 and MT_2 can be profoundly modified when they are engaged into heteromeric complexes with other receptors such as the serotonin $5\text{-}HT_{2c}$ receptor (Cecon et al. 2017). Figure 2 shows the main molecular signaling pathways activated by MLT receptors.

M

Physiology

Aging

The night peak of circulating levels of MLT varies according to aging. It is low during the first 6 months of life, it reaches the maximum between 1 and 3 years of life, and it remains almost constant until puberty from when it starts to decline progressively. During daylight hours, the serum concentrations of MLT are low (10–20 pg/ml) (Reiter 1991). Several potential mechanisms yet to be clearly elucidated may account for the decline in the peak of circulating MLT during senescence: a degeneration of the pineal gland which has a tendency to calcify with aging, altered clearance of MLT, and/or reduced activity of noradrenergic neurons stimulating the synthesis of MLT. In addition, during aging there is also a degeneration of the SCN that in parallel with the reduction of MLT synthesis may explain

the higher incidence and severity of circadian rhythm disturbances such as sleep disorders in elderly patients (Reiter 1991).

Sleep and Circadian Rhythms

The synthesis of MLT is under the control of the external light/dark cycle through the SCN. In turn, MLT by acting on MT_1 and MT_2 receptors present in the SCN synchronizes the circadian rhythm of the nucleus by affecting both the phase and amplitude of the rhythm. The MT_1 is highly expressed in the SCN and seems to control the circadian rhythm. Because of its ability to modulate the activity of the SCN and thus physiological circadian rhythms, MLT has the so-called chronobiotic properties. Indeed, it has shown efficacy for the treatment of disorders associated with abnormal timing of the circadian system including jet lag, shift work, and delayed sleep phase syndrome.

Among the many circadian rhythms, the sleep-wake cycle is governed by the Circadian process "C" and the Sleep homeostatic process "S." The "C" is the regulation of sleep by a light/night pattern, while the "S" is the regulation of sleep following the need of sleeping (i.e., if your sleep is of 7 h and yesterday you slept only 2 h, tonight you will need 9 h sleep to recover). For a long time it has been thought that MLT modulates sleep by acting on the SCN and thus on the circadian process. Research performed mostly during the last decade has instead been demonstrating that MT_1 and MT_2 receptors are located in several brain regions/nuclei involved in the homeostatic processes of sleep including the reticular thalamus, the lateral hypothalamus, and the ventrolateral preoptic nucleus. Therefore, MLT can directly affect sleep stages. In particular, preclinical research in rodents has shown that MLT by acting on MT_2 receptors modulates non-rapid eye movement (NREM) sleep whereas by acting on MT_1 receptors mostly controls REM sleep. Consequently, selective pharmacological activation of MT_2 receptors enhances NREM sleep duration, while that of MT_1 receptors increases REM sleep duration.

Overall, many studies have investigated the effects of MLT on sleep, and although hypnotic effects of MLT in humans have been reported, results are still controversial. Indeed, a meta-analysis has shown that MLT has soporific effects, thus helping people to fall asleep, rather than affecting sleep quality and maintenance. Selective MLT receptor agonists or antagonist may instead have more therapeutic effects on sleep disorders. See Gobbi and Comai (2019) for a review detailing the current knowledge on the MLT system and sleep.

Metabolic Regulation

The contribution of MLT in metabolic regulation occurs at different tissues and timescales (Karamitri and Jockers 2019). To better understand this diversity, it is helpful to recognize that MLT can mediate different types of effects, immediate effects that occur during the night, prospective or delayed effects that are typically primed during the night with functional consequences during the day, chronobiotic effects that rely on the direct effect of MLT on the circadian clock, and seasonal effects that depend on the duration of the night. Immediate effects of MLT are observed on glucose uptake and insulin secretion in insulin-sensitive tissues and insulin-producing pancreatic beta-cells, respectively. Important differences have been reported between nocturnal rodents and diurnal humans. Delayed effects have been observed on the expression of the glucose transporter type 4 (GLUT4) upon long-term MLT treatment and the modulation of diurnal insulin sensitivity via the regulation of PI3K expression by nocturnal MT_1 receptor activation. Most metabolic processes are under rhythmic regulation and desynchronization leads to metabolic dysfunction. MLT is involved in the synchronization of these processes. This chronobiotic effect of MLT can either occur by synchronizing peripheral tissue clocks by acting directly on the clock machinery in metabolic tissues or by acting on the biological master clock in the hypothalamic SCN. MLT plays a critical role in the photoperiodic control of reproduction in seasonal breeders by decoding the length of the night by means of the duration of MLT secretion. In this way, MLT can induce massive changes not only in gonad size but also in adipose tissue mass and associated metabolic adaptations in preparation of

hibernation in seasonal animals such as Siberian hamsters. Details can be found in Karamitri and Jockers (2019).

Non-receptor-Mediated Properties: Antioxidant and Anti-Inflammatory Effects

MLT is a high lipophilic compound that on top of binding to its receptors also has powerful free-radical scavenger and anti-inflammatory properties. As example, MLT has a greater efficiency in neutralizing the hydroxyl and the peroxyl radicals than reduced glutathione (GSH) and vitamin E, respectively (Acuna-Castroviejo et al. 2014). These effects likely derive by a non-enzymatic process of electron donation due to its chemical structure. MLT also seems to have the ability of regulating cytokine production, in particular to reduce the synthesis of pro-inflammatory cytokines, and of inhibiting cyclooxygenase 2 and the inducible nitric oxide synthase enzymes. Increased oxidative stress and inflammation, which may lead to cell and tissue damages, have been implicated in physiological aging and in a large number of pathologies including neurodegenerative, metabolic, and cardiovascular disorders and cancer (Acuna-Castroviejo et al. 2014). Many clinical trials have consequently studied the effects of MLT in these diseases, and despite some encouraging findings, the results are still contrasting and preliminary, and thus further work is necessary before validating a possible therapeutic use of the hormone.

Pathophysiology

Neuropsychiatric Disorders

Mood disorders including major depression (MDD) and bipolar (BD) disorders involve disruption of intrinsic biological rhythms, and therefore it is not surprising that several studies have demonstrated changes in MLT levels associated with MDD and BD. Low serum levels of MLT were shown to be a trait marker in BD, even though MLT levels did not show state-dependent changes across mania or depression states. A shift in the nocturnal MLT peak was also observed in BD patients. MDD was associated with an altered diurnal rhythm of MLT output, including a blunted nighttime surge. Indeed, MLT production may be lower in depression, and an enhancement in circulating levels of MLT has been correlated with effective antidepressant treatment. Interestingly, concerning MLT receptors, it was demonstrated a dysregulation of MT_1 but not of MT_2 receptors, namely, a high number of MT_1-immunoreactive cells, in the SCN of MDD and BD patients postmortem. Similarly to depression, anxiety disorders have been linked to MLT activity, but evidence is still limited. In preclinical paradigms of anxiety-like behavior, MLT has displayed anxiolytic-like effects. In human studies, MLT showed anxiolytic effects similar to benzodiazepines but without side effects including sleep disturbances (Comai and Gobbi 2014).

In patients with psychosis, most of the studies reported decreased nocturnal secretion of MLT. Interestingly, antipsychotic treatments ameliorating psychotic symptoms do not normalize the reduced levels of MLT. While MLT treatment alone is not effective for the therapeutics of psychosis, some studies have shown that in addition to antipsychotics, it is able to significantly improve sleep impairments often present in psychotic patients.

In age-related neurodegenerative disorders including Alzheimer's disease (AD) and Parkinson's disease (PD), the serum and cerebrospinal fluid levels of MLT are lower than in controls, and the loss of the diurnal rhythm of MLT synthesis is closely associated with the symptoms of these diseases. However, these impaired MLT levels are likely a consequence rather than a cause of the diseases. Reduced hippocampal MT_2 receptor expression was observed in AD patients. In PD, a significant decrease of both MT_1 and MT_2 receptor expression in both substantia nigra and amygdala, two brain regions affected by the disease, has been found.

Overall, several neuropsychiatric and neurodegenerative disorders are associated with reduced secretion and/or phase shift in MLT levels, but current knowledge does not allow to establish if circulating MLT could be a biomarker of disease, disease severity, or disease progression (Comai and Gobbi 2014).

Type 2 Diabetes Mellitus and Obesity

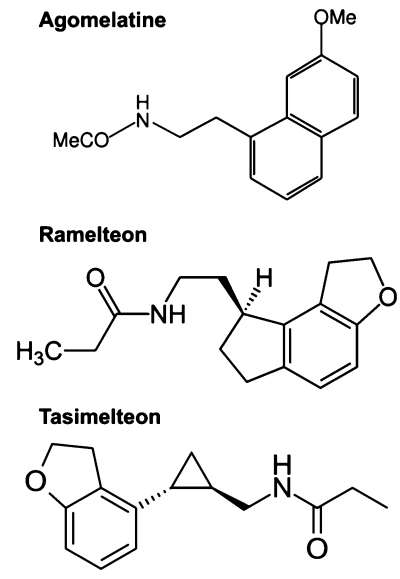
The role of MLT in the regulation of energy homeostasis implies a possible contribution to metabolic diseases like obesity and type 2 diabetes (T2D) (Karamitri and Jockers 2019). In rodents, MLT has a beneficial effect on body weight regulation by limiting body weight gain and the amount of adipose tissue. In humans, there is no strong support for a major role of MLT in body weight regulation and lipid metabolism in adults. However, notable effects of maternal MLT have been reported on fetal birth weight in humans.

The absence of MLT in rodents leads to glucose intolerance and peripheral and central insulin resistance. MLT treatment reverses these adverse effects. The absence of MT_1 receptors in mice induces systemic insulin resistance. The absence of MT_2 receptors reduces hepatic insulin sensitivity, but in contrast, MT_1 knockout mice show increased insulin release. In humans, the most convincing evidence for the role of MLT receptors in T2D development comes from genetic association studies of frequent and rare variants of the *MTNR1B* locus, encoding the MT_2 receptor, with T2D risk. Cumulative evidence indicates that the effect of the frequent intronic risk allele rs10830963 probably starts early during the development of prediabetic fasting hyperglycemia by affecting insulin secretion. Further contribution to T2D risk comes from multiple rare variants in the coding region of the *MTNR1B* gene that result in the expression of defective MT_2 receptors.

Drugs

The activation of the MLT system has shown to induce antidepressant, hypnotic, analgesic, and anxiolytic effects, although the results are not consistent among different preclinical and clinical studies. MLT's pharmacokinetic profile still limits its efficacy and applicability in therapy as a multipurpose drug. Indeed, although it is characterized by high lipophilicity which enables it to cross cell membranes as well as to distribute quickly also in the brain, when administered orally, MLT displays a poor bioavailability (due to first-pass metabolism) and short half-life (less than 30 min). One important aspect to consider when using MLT and selective or non-selective MLT receptors drugs is the time of administration due to the fact that (1) by acting on MLT receptors we may affect physiological circadian rhythms and (2) the pharmacological effect is dependent on the expression of MLT receptors which may vary according to the time of the day and endogenous levels of MLT (Atkin et al. 2018).

In many countries, MLT is classified as a dietary supplement to be used as a sleep inducer or for the treatment of irregular phase shifting, for example, in the case of jet lag. To overcome the pharmacokinetic problems of MLT when given orally, a long-release formulation of MLT (2 mg) has been approved in many countries as monotherapy for the short-term treatment of primary insomnia in patients aged more than 55 years (Atkin et al. 2018). During the past decades, other compounds targeting MLT receptors have been developed. One of them is agomelatine (Fig. 3), a structural



Melatonin, Fig. 3 Chemical structure of melatonergic drugs approved for use in humans: agomelatine, ramelteon, and tasimelteon

analog of MLT that acts as a non-selective MT_1/MT_2 agonist and as an antagonist for 5-HT_{2C} receptors and that is registered in Europe as an antidepressant drug. Ramelteon (Fig. 3) is another non-selective MT_1/ MT_2 agonist that has been approved only in the USA, in July 2005, for the treatment of insomnia, characterized by long sleep onset latency. Finally, tasimelteon (Fig. 3) is another non-selective MT_1/MT_2 agonist that has entered the clinical practice specifically for the treatment of non-24 h sleep-wake disorder in blind subjects (Atkin et al. 2018).

While very elevated doses of MLT that bring circulating levels of MLT up to 1000 times the physiological levels do not seem to induce a significant toxicity in either humans or rodents, except for headache, nausea, dizziness, and drowsiness (at doses higher than 10 mg/day), the use of agomelatine is associated with hepatotoxicity as well as with other common minor symptoms including drowsiness, headache, nausea, and dizziness.

The main adverse effects of ramelteon and tasimelteon include headache, elevated liver enzymes (likely due to an idiosyncratic issue similar to agomelatine), nightmares, and upper respiratory tract infections. Hepatic toxicity was instead not reported even after the administration of MLT for long periods of time and at high doses, likely indicating that the hepatic issues reported with MLT receptor agonists like agomelatine and ramelteon are probably not the consequences of the activation of MLT receptors (Atkin et al. 2018).

Despite the limited clinical efficacy of these non-selective MLT receptor agonists, MLT receptors seem to be a promising target in neuropsychopharmacology (Comai and Gobbi 2014; Gobbi and COmai 2019). As abovementioned, research is showing that the two MLT receptor subtypes may mediate complementary or opposite functions, and thus from a pharmacological point of view, selectivity toward either MT_1 or MT_2 receptors may enhance the therapeutic potential of targeting the MLT system. During the past decades, several selective ligands for the MT_2 receptors have been developed, some of which have been also tested for their pharmacological properties at preclinical level. Compounds including the selective MT_2 receptor agonist IIK7 or the partial agonists UCM765 and UCM924 have displayed in rodents hypnotic properties. In particular, they enhanced selectively NREM sleep. UCM924 also showed anxiolytic and analgesic effects at a dose lower than that inducing sleep. Research aimed at identifying MT_1-selective ligands has been not yet very successful, but with the very recent characterization of the crystallographic structure of both MT_1 and MT_2 receptors, it is plausible to think that MT_1 agonists/antagonists to be tested at preclinical level will be soon developed. In this direction, compounds such as the MT_1-selective partial agonist UCM871 and the MT_1-selective inverse agonist UCSF7447 seem to be promising. Some of the distinct physiological properties mediated by MT_1 and MT_2 receptors are reported in Table 1. Drugs that will be selectively developed to target MT_1 or MT_2 receptors may thus be endowed with peculiar and specific pharmacological activity (Gobbi and Comai 2019).

Melatonin, Table 1 Distinct pharmacological effects obtained following the selective activation of MT_1 or MT_2 receptors

	MT_1 receptor activation	MT_2 receptor activation
REM sleep	Increase duration	No effects
NREM sleep	Not investigated	Increase duration
Circadian rhythm	Deceleration of re-entrainment; phase advance when administered at subjective dusk[a]	Not investigated
Pain	Not investigated	Analgesia
Vascular level	Vasoconstriction	Vasodilatation
Body temperature	Increase	Decrease

[a]pharmacological effect shown in vivo by MT_1-selective inverse agonists

Not investigated: findings are yet too preliminary

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Mepolizumab

▶ Interleukin-5

5-Methoxy-N-Acetyltryptamine

▶ Melatonin

Microsomal Triglyceride Transfer Protein Inhibition

▶ Lipid-Lowering Drugs

Misfolded Proteins

▶ Unfolded Protein Response

Mitochondrial Selective Autophagy

▶ Mitophagy

Mitogens

▶ Growth Factors

Mitophagy

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Synonyms

Mitochondrial selective autophagy

Definition

Mitophagy is an evolutionary conserved cellular process that sustains mitochondrial homeostasis by mediating the elimination of superfluous and/or defective mitochondria. Mitophagy can be classified as basal, stress-induced, and programmed based on physiological context. Failure to properly carry out mitophagy deregulates mitochondrial metabolism and causes progressive accumulation of defective organelles leading to the deterioration of biological systems, often culminating in tissue collapse.

Basic Characteristics

Molecular Mechanisms of Mitophagy

Cells have evolved several molecular signaling pathways to eliminate defective organelles and adjust their mitochondrial pool in response to environmental and/or intracellular stimuli. Hence, different signals can promote mitophagy via multiple signaling cascades in distinct cellular contexts. Although mitophagy pathways are classified as ubiquitin-dependent or -independent, several studies highlight the intricate interplay between different signaling and execution mechanisms, and emphasize the conservation of mitophagy regulators in eukaryotes (Table 1) (Harper et al. 2018; Palikaras et al. 2018).

The PINK1/Parkin Pathway

The PINK1 (phosphatase and tensin homolog (PTEN)-induced putative kinase 1)/Parkin is the most well-studied molecular pathway known to mediate ubiquitin-dependent mitochondrial removal. Several aspects of mitochondrial metabolism, such as mitochondrial dynamics, biogenesis, transport, and recruitment of autophagic machinery, are associated with the induction of the PINK1/Parkin pathway to assure the degradation of damaged organelles.

In healthy mitochondria, the PINK1 kinase is transported into the inner mitochondrial membrane (IMM), where it is processed and cleaved by mitochondrial proteases. In turn, the truncated form of PINK1 is degraded by the proteasome system. Upon stress conditions, mitochondrial membrane is depolarized mediating the stabilization of PINK1 on the outer mitochondrial membrane (OMM). Subsequently, the enzymatic activity of PINK1 is enhanced by its auto-phosphorylation and promotes the recruitment of the E3 ubiquitin ligase Parkin to mitochondrial surface (Sekine and Youle 2018). PINK1 phosphorylates both Parkin triggering its E3 ligase activity and ubiquitin molecules or poly-ubiquitin chains on depolarized mitochondria. Inactive Parkin is translocated and bound to phopho-ubiquitin molecules or chains leading to its subsequent activation by PINK1. Then, Parkin mediates a feed-forward mechanism generating poly-ubiquitin chains, which are substrates for PINK1, amplifying mitophagy signal (Harper et al. 2018). In addition to its beneficial effects on Parkin activation and recruitment, PINK1-dependent phosphorylation of ubiquitin and poly-ubiquitin chains ameliorates hydrolysis by deubiquitinating enzymes (Harper et al. 2018). Several deubiquitinases, such as USP15, USP30, and USP35, block mitophagy by removing Parkin-generated ubiquitin chains from its mitochondrial substrates (Harper et al. 2018). Hence, a fine-tuned balance between ubiquitination and deubiquitination events regulates energy metabolism and underlines poly-ubiquitination as an "eat-me" signal for defective mitochondria.

Mitophagy, Table 1 Mitophagy components are evolutionary conserved

Model organisms					
S. serevisiae	*C. elegans*	*D. melanogaster*	*M. musculus*	*H. sapiens*	Molecular function
–	SQST-1	Ref(2)a	p62/SQST1	p62/SQST1	Adaptor protein
–	PINK-1	Pink1	PINK1	PINK1	Mitochondrial kinase
–	PDR-1	Park	Parkin/PARK2	Parkin/PARK2	E3 ubiquitin ligase
Atg8	LGG-1/-2	Atg8a	MAP1LC3	MAP1LC3	Autophagosomal protein
–	DCT-1	–	BNIP3	BNIP3	Mitophagy receptor
–	–	–	NIX	NIX	Mitophagy receptor
Atg32		–	BCL2L13	BCL2L13	Mitophagy receptor
–	FNDC-1	–	FUNDC1	FUNDC1	Mitophagy receptor
Phb2	PHB-2	Phb2	PHB2	PHB2	Mitophagy receptor
–	FZO-1	Fzo, Dmfn	MFN2	MFN2	Mitochondrial GTPase
Dnm1	DRP-1	Drp1	DRP1	DRP1	Mitochondrial GTPase
Mgm1	EAT-3	DmeI/Opa1	OPA1	OPA1	Mitochondrial GTPase

Following its translocation, Parkin mediates poly-ubiquitination of several OMM proteins leading eventually to their recognition by autophagy adaptors or proteasomal degradation. Parkin-dependent proteasomal turnover of mitofusins (MFN1/2) diminishes mitochondrial fusion mediating the isolation of damaged organelles from the healthy mitochondrial population (Harper et al. 2018). MFN2 is also phosphorylated by PINK1 and thereby functions as a scaffold protein for Parkin recruitment upon mitochondrial stress. Additionally, PINK1 influences indirectly DRP1 activity promoting fission of defective mitochondria for their autophagic elimination. Furthermore, mitochondrial motility is also reduced during energetic stress. Mitochondria Rho-GTPase (Miro), an OMM protein that anchors mitochondria to cytoskeleton, is degraded upon Parkin-driven ubiquitination. Miro turnover mediates inhibition of mitochondrial transport in response to mitochondrial membrane dissipation (Harper et al. 2018; Palikaras et al. 2018; Sekine and Youle 2018). Collectively, inhibition of mitochondrial motility and enhanced fission might promote mitophagy, presumably by generating smaller organelles that can easily be engulfed by autophagosomes.

Although Parkin is a major mitophagy regulator, mitochondrial elimination does not exclusively rely on its activity. Several ubiquitin E3 ligases, such as Gp78, SMURF1, SIAH, MUL1, and ARIH1, have been reported to ubiquitinate mitochondrial proteins facilitating mitophagy (Harper et al. 2018; Montava-Garriga and Ganley 2019; Palikaras et al. 2018). Once poly-ubiquitin chains have been generated on mitochondrial surface, they stimulate the recruitment of autophagy adaptor molecules, including p62/SQST1, optineurin 1 (OPTN1), nuclear dot protein 52 (NDP52) among others. In turn, autophagy adaptors interact directly with the autophagosomal light chain 3 (LC3) protein via their LIR (LC3-interacting region) motifs promoting autophagosome biogenesis and sequestration of dysfunctional mitochondria (Harper et al. 2018; Montava-Garriga and Ganley 2019; Palikaras et al. 2018).

Receptor-Mediated Mitophagy

Multiple OMM proteins serve as mitophagy receptors interacting directly with autophagosomal machinery and fine-tuning mitochondrial number in response to various stimuli in mammalian cells. Mitophagy receptors bind directly to autophagosomal membrane proteins via their LIR motifs promoting mitochondrial degradation.

Genetic studies in *Saccharomyces cerevisiae* have identified Atg32 as an essential protein for mitochondrial removal. Atg32 is an OMM protein associating with cargo-specific protein Atg8 and Atg11 adaptor to facilitate autophagosome formation. The kinases Hog1 and CK2 (casein kinase 2) regulate Atg32 phosphorylation status promoting its association with Atg11 adaptor (Montava-Garriga and Ganley 2019). Interestingly, Atg11 recruits also Dnm1 (the homologue of DRP1 in yeast) to enhance mitochondrial fission leading to the isolation of damaged organelles. Hence, Atg32 mediate the formation of a multiprotein complex together with Atg8 and Atg11 regulating mitochondrial network morphology and mitophagy stimulation. Recently, BCL-2-like protein 13 (BCL2L13) has been characterized as a functional homologue of Atg32 in mammals. BCL2L13 is localized on the OMM and interacts directly with LC3 via its LIR motif upon mitochondrial stress (Sekine and Youle 2018; Montava-Garriga and Ganley 2019).

NIX has a pivotal role in the elimination of entire mitochondrial population during erythrocytes differentiation. Indeed, NIX-deficient cells display mitochondrial accumulation leading to elevated apoptotic events and subsequently to developmental deficits (Montava-Garriga and Ganley 2019; Palikaras et al. 2018). In addition to NIX, BNIP3 (BCL2 interacting protein 3) acts as a mitophagy receptor mediating mitochondrial removal. Interestingly, BNIP3 also regulates mitochondrial fission and fusion through its direct association with OPA1 and DRP1 in response to mitochondrial damage. Mutations in the LIR motifs of both NIX and BNIP3 disturb their physical interaction with autophagosomal proteins and deregulate mitophagy. Multiple lines of

experimental evidence indicate a complex interplay between mitophagy receptors and the PINK1/Parkin signaling pathway. Indeed, both NIX and BNIP3 require Parkin recruitment to preserve mitochondrial metabolism. Notably, Parkin targets and ubiquitinates NIX promoting its recognition by autophagy adaptors to enhance mitochondrial clearance (Montava-Garriga and Ganley 2019; Palikaras et al. 2018). Recently, DCT-1, the mammalian homologue of BNIP3 and NIX in nematodes, is underlined as a key mediator of mitophagy sustaining cellular homeostasis and viability during challenged conditions. In congruent with its mammalian counterparts, DCT-1 is also ubiquitinated by PDR-1 (the nematode Parkin homologue) in a PINK1-dependent manner during stress-induced mitophagy. Interestingly, BNIP3 prevents PINK1 proteolytic cleavage and mediates its stabilization on the mitochondrial surface (Montava-Garriga and Ganley 2019; Palikaras et al. 2018).

FUN14 domain-containing protein 1 (FUNDC1) is a highly conserved OMM protein that facilitates the selective elimination of damaged organelles during hypoxic stress (Georgakopoulos et al. 2017; Montava-Garriga and Ganley 2019; Palikaras et al. 2018). The Sc and CK2 kinases regulate the activation of FUNDC1 by modulating the phosphorylation status of its LIR motif and thereby inhibiting the recruitment of autophagic machinery under non-stress conditions. FUNDC1 influences mitochondrial network morphology through its associations with both fission and fusion components in response to hypoxia. Indeed, the mitochondrial phosphatase PGAM5 dephosphorylates FUNDC1 promoting its dissociation with OPA1 and eventually preventing mitochondrial fusion. Then, FUNDC1 is recruited to ER-mitochondrial contact sites stimulating DRP1 translocation and subsequently mitochondrial network fragmentation. In addition to FUNDC1, both NIX and BNIP3 have been involved in the execution of mitochondrial removal upon low oxygen levels (Georgakopoulos et al. 2017; Montava-Garriga and Ganley 2019; Palikaras et al. 2018). Interestingly, HIF1 (hypoxia inducible factor 1) regulates transcriptionally both BNIP3 and NIX enhancing mitophagy during hypoxic-like conditions (Palikaras et al. 2018).

Recent studies revealed an unexpected role of IMM proteins and phospholipids in mitochondrial turnover. OMM is disrupted in response to excessive stress conditions resulting in elevated ROS levels, mtDNA release, and cytoplasmic externalization of several IMM proteins. Notably, prohibitin 2 (PHB2) and cardiolipin, which are primarily distributed in IMM, are exposed to the cytoplasm and trigger mitophagy through the direct association with their LIR motifs with LC3 autophagosomal protein (Montava-Garriga and Ganley 2019; Palikaras et al. 2018).

Taken together, this experimental evidence underscores that the coordinated and compensatory action of OMM and IMM mitophagy receptors might assure the efficiency of mitochondrial surveillance and energy metabolism in response to multiple stimuli.

Basal, Stress-Induced, and Programmed Mitophagy

Until recently, information on basal mitophagy levels was limited. However, the generation of transgenic animals expressing mitophagy reporters endorsed the in vivo investigation of mitochondrial degradation under physiological conditions (Palikaras et al. 2018; Montava-Garriga and Ganley 2019). Although most cell types undergo continuously basal mitophagy during routine mitochondrial quality control, the extent differs across tissues or even between cells within the same tissue. Notably, mitophagy is highly induced in heart, skeletal muscles, brain, liver, and kidney, whereas thymus and spleen display low levels of mitochondrial removal (Palikaras et al. 2018; Montava-Garriga and Ganley 2019). Surprisingly, the PINK1/Parkin pathway does not regulate basal mitophagy both in mammals and flies, highlighting the existence of different mitophagy regulators under physiological and stress conditions (Montava-Garriga and Ganley 2019).

Mitochondrial homeostasis is impaired by extracellular and/or environmental stimuli that

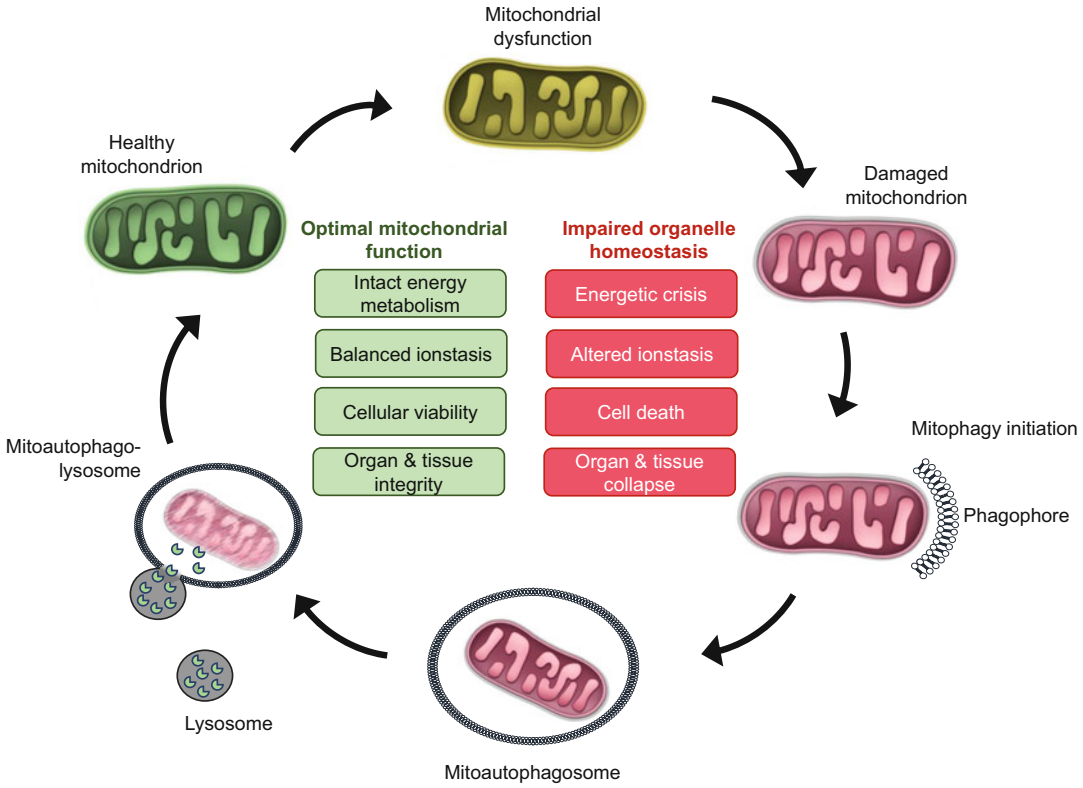
induce acute clearance of damaged mitochondria, by stress-induced mitophagy. Nutrient starvation, hypoxia, insecticides, and mitochondrial toxins deregulate electron transport chain (ETC) function and subsequently trigger energetic stress. In turn, stress-induced mitophagy facilitates mitochondrial quality control mediating the adjustment and adaptation of cellular metabolism to the external challenge. Nutrient deprivation promotes re-shaping of mitochondrial network and autophagy induction to degrade cytoplasmic materials and supply cells with building blocks for re-use and metabolism (Murphy and Hartley 2018; Palikaras et al. 2018; Montava-Garriga and Ganley 2019). Damaged and small sized mitochondria are initially removed through mitophagy to sustain a healthy mitochondrial pool during short period of starvation. Then, mitochondrial fission is prevented leading to unopposed fusion events. Elongated mitochondrial network is protected against autophagic degradation and display enhanced capacity of energy production (Montava-Garriga and Ganley 2019). On the other hand, prolonged starvation stimulates excessive mitochondrial elimination to renew and optimize mitochondrial population to the new environmental conditions. These observations underline the ability of eukaryotic cells to differentially regulate mitophagy under certain conditions of starvation. Indeed, yeast cells induce or inhibit mitophagy depending on carbon source in response to nitrogen starvation (Palikaras et al. 2018). General toxicants and mitochondrial uncouplers, including paraquat, valinomycin, oligomycin, and CCCP among others, have been widely used to induce mitochondrial depolarization and subsequently mediate PINK1/Parkin-dependent mitophagy (Georgakopoulos et al. 2017). Alternatively, hypoxia mainly engages receptor-mediated mitophagy to mediate mitochondrial quality and quantity (Georgakopoulos et al. 2017; Montava-Garriga and Ganley 2019; Palikaras et al. 2018). Lack of oxygen results in defective mitochondrial respiration and energetic stress. Thus, a metabolic switch from oxidative phosphorylation (OXPHOS) to glycolysis is taking place attenuating the features of mitochondrial population. HIF1, the master regulator of hypoxic responses, orchestrates and coordinates both metabolic switch and mitophagy through the transcriptional regulation of glycolytic and mitophagic genes (Georgakopoulos et al. 2017; Montava-Garriga and Ganley 2019; Palikaras et al. 2018). Cytoplasmic iron chelation is an alternative procedure to trigger hypoxic-like response (Georgakopoulos et al. 2017). Although the impact of iron deprivation on mitochondrial physiology is not well studied, iron chelating agents, such as deferiprone (DFP) and 2′,2-bipyridyl (BP), are potent mitophagy inducers both in vitro and in vivo. Interestingly, DFP and BP activate different molecular signaling pathways to mediate mitochondrial removal underlining the complexity and diversity of mitophagy induction even under similar cellular responses (Georgakopoulos et al. 2017).

The induction of programmed mitophagy is a critical event during organismal development. Erythrocytes maturation requires the removal of all the internal organelles from immature erythroblast. NIX-mediated mitochondrial elimination is essential for erythrocytes differentiation and organismal physiology, since NIX deficient mice retain mitochondria in their peripheral blood cells displaying defective erythroid maturation and anemia (Montava-Garriga and Ganley 2019; Palikaras et al. 2018). Furthermore, mitophagy regulates the degradation of sperm-derived mitochondria upon oocytes fertilization in nematodes, flies, and mice preventing paternal mtDNA inheritance (Palikaras et al. 2018). Mitophagy has a prominent role in cardiomyocyte maturation. Adult heart is an organ with enhanced metabolic demands that preferentially utilizes fatty acids for energy production in mitochondria. During cardiomyocyte maturation, programmed mitophagy is taking place to eliminate fetal mitochondria, which primarily use glucose for energy generation. Therefore, mitochondrial population is functionally and morphologically altered to meet the contractile demands of the adult heart (Palikaras et al. 2018). Re-shaping and re-adjusting mitochondrial network fine-tune the transition from OXPHOS to glycolysis and vice versa. Indeed, mitophagy-mediated glycolytic shift is pivotal both for retinal ganglion cells

differentiation and macrophage polarization (Montava-Garriga and Ganley 2019). Moreover, mitophagy-driven mitochondrial rejuvenation dictates several features of stem cell biology. Although stem cells rely on glycolytic metabolism and exhibit few spherical mitochondria, accumulating evidence underscores the impact of mitochondrial homeostasis and mitophagy on stem cells activation, fate, and senescence (Zhang et al. 2018).

Drugs

Mitochondria are cellular organelles specialized for energy production and critically influence several features of cell metabolism and physiology. The maintenance of healthy mitochondrial population is a prerequisite for cellular and tissue homeostasis. Compromised mitochondrial function results in the transformation of cellular powerhouses to "hotspots" of metabolic stress. Hence, it is not surprising that mitochondrial damage is associated with a broad spectrum of pathologies, such as ageing, myopathies, cardiovascular, metabolic and neurodegenerative diseases (Fig. 1). Given that the consequences of defective mitochondrial function can be detrimental for cellular viability, interventions modulating mitochondrial turnover hold a promise for considerable therapeutic potential and have been in the spotlight of scientific research. Pharmacological screenings are taking place to identify novel chemical compounds that may be used to manipulate the



Mitophagy, Fig. 1 Contribution of proper mitochondrial function to cellular and tissue homeostasis. In physiological conditions, intact mitochondrial population sustain energy metabolism, ionstasis, and cellular survival resulting in subsequent organ and tissue integrity. Excessive mitochondrial damage disrupts energy production and promotes ROS elevation and ionic imbalance, which trigger cell death pathways and often culminating in tissue collapse. Efficient elimination of dysfunctional organelles via mitophagy ensures mitochondrial homeostasis protecting against deterioration of biological systems

clearance of defective mitochondria and restore cellular energetic status. To this direction, several synthetic and natural chemical molecules have been utilized to modulate mitophagy (Georgakopoulos et al. 2017; Murphy and Hartley 2018; Palikaras et al. 2017).

Recently, mitophagy modulators, including general mitochondrial targeting agents, NAD^+ precursor molecules, and naturally occurring products among others, have been shown to enhance energy metabolism, healthspan, and organismal survival.

General Mitochondrial Targeting Agents to Manipulate Mitochondrial Turnover

Various pharmacological agents have been shown to interfere with mitochondrial ETC function and trigger mitophagy. Mitochondrial toxicants, such as paraquat, carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), and carbonyl cyanide-*p*-(trifluoromethoxy) phenyl hydrazone (FCCP), have been widely utilized to induce mitochondrial elimination through initiation of the PINK1/Parkin signaling cascade in mammalian cells and nematodes (Georgakopoulos et al. 2017). On the other hand, well-established ROS-generators, including rotenone, 6-OHDA (6-hydroxyldopamine) and MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), are not able to promote PINK1/Parkin-mediated mitophagy due to their moderate effects on mitochondrial membrane potential (Georgakopoulos et al. 2017). Notably, cardiolipin is released and binds to LC3 facilitating mitochondrial removal upon rotenone and 6-OHDA treatment. Despite the widespread application of general mitochondrial toxicants in basal research, their nonselective properties and off-target effects limit their use for therapeutic interventions (Fig. 2).

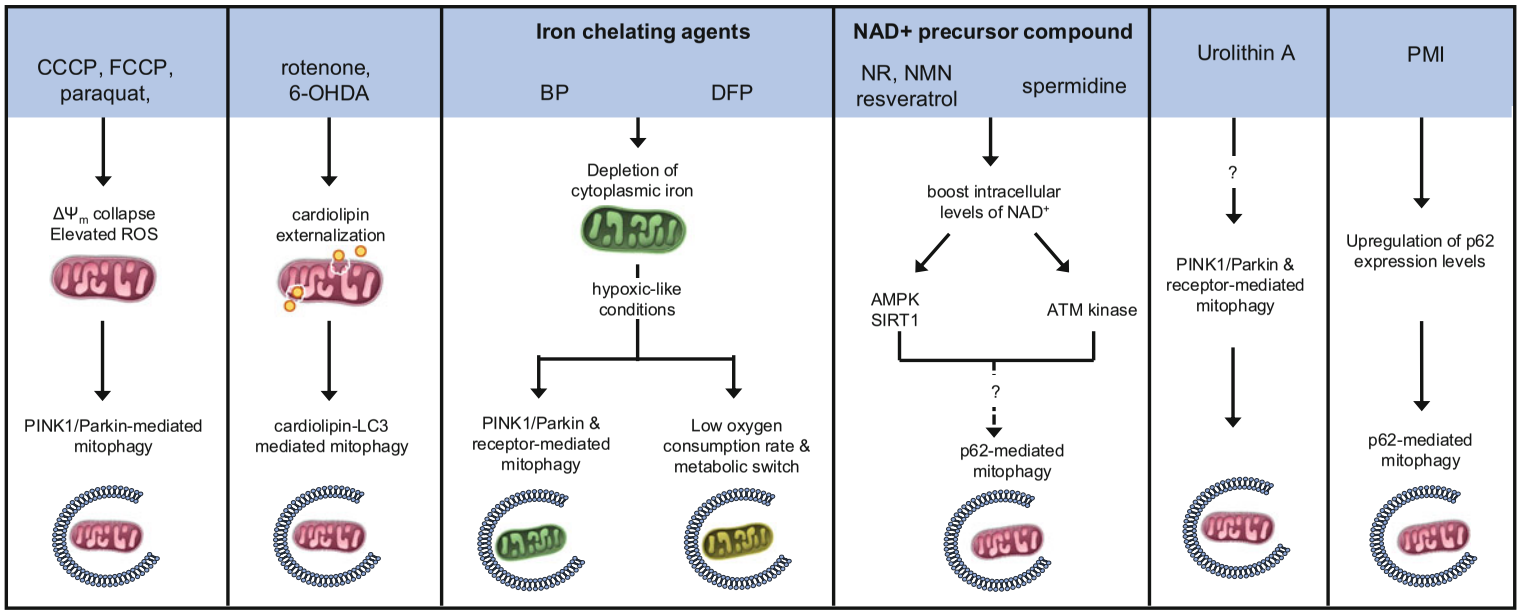
Kinetin triphosphate (KTP; 6-furfurylaminopurine) is a modified analog of ATP that binds and amplifies PINK1 catalytic activity both in vitro and in vivo. Supplementation of KTP diminishes mitochondrial motility, enhances Parkin translocation on depolarized organelles, and ameliorates apoptosis in a PINK1-dependent manner. Although KTP was initially identified and applied in clinical trials as mRNA splicing moderator, its safety and ability to cross the blood-brain barrier highlight its therapeutic potential in pathological conditions with defective PINK1 enzymatic activity, such as Parkinson's disease (Georgakopoulos et al. 2017).

To avoid nonspecific and toxic effects of uncontrolled mitochondrial membrane potential collapse by proton ionophores, a synthesized chemical compound called p62-mediated mitophagy inducer (PMI) was developed. PMI promotes the expression of p62/SQST1 adaptor protein forcing mitochondria to be sequestered and degraded by autophagosomes (Fig. 2). Interestingly, PMI does not affect mitochondrial bioenergetics and network morphology suggesting that its supplementation enhances the levels of basal mitophagy. This notion is further supported by the fact that PMI-induced mitophagy does not require the presence of PINK1 and/or Parkin (Georgakopoulos et al. 2017; Palikaras et al. 2017).

Maintenance of Mitochondrial Homeostasis Through the Regulation of NAD^+ Metabolism

Nicotinamide adenine dinucleotide (NAD^+) is an intracellular metabolite and a critical denominator of energy metabolism and organismal fitness. Age-dependent reduction of NAD^+ concentration is associated with premature ageing and several age-related pathologies, including cancer, metabolic syndrome, and neurodegeneration (Fang et al. 2017; Katsyuba and Auwerx 2017).

NAD^+ acts as a co-factor regulating the enzymatic activity of several proteins, including sirtuins and poly ADP-ribose polymerase 1 (PARP1) among others. Sirtuins are deacetylating enzymes and their activity relies mainly on NAD^+ intracellular levels. Sirtuins are involved in the regulation of multiple cellular processes, such as DNA damage responses, autophagy, and mitochondrial function (Katsyuba and Auwerx 2017). Supplementation of the NAD^+ precursor molecules, nicotinamide (NAM), nicotinamide



Mitophagy, Fig. 2 Overview of mitochondrial targeting agents that stimulates mitophagy. The molecular mechanisms and mitophagy-inducing properties of several synthetic and natural small molecules, such as CCCP, FCCP, paraquat, rotenone, 6-OHDA, iron chelating agents, NAD^+ precursors, spermidine, urolithin A, and PMI, are described

riboside (NR), and nicotinamide mononucleotide (NMN), boosts the intracellular NAD^+ levels enhancing energy metabolism, neuroprotection, and lifespan extension (Lou et al. 2019). Notably, NAM and NMN is recently documented to induce mitophagy in mammals and nematodes. The beneficial effects of NAD^+ replenishment on mitochondrial metabolism and organismal homeostasis are mediated, at least in part, by sirtuins (Fang et al. 2017). Indeed, NR and NMN treatment have been shown to induce NAD^+ – SIRT1 axis preventing protein aggregates accumulation and cognitive decline in Alzheimers' disease (AD) animal models via the restoration of mitochondrial activity and mitophagy (Lou et al. 2019). Moreover, administration of NR stimulates both the expression and activity of additional sirtuins, such as SIRT3 and SIRT6, ameliorating the hearing-loss defects in AD mouse models (Fang et al. 2017; Lou et al. 2019).

PARP1 is a NAD^+-consuming enzyme that is activated to maintain genome integrity through the recruitment of DNA repairing complexes during genotoxic stress. Accumulation of age-dependent DNA damage triggers the overstimulation of PARP1 resulting in diminished NAD^+ levels, reduced SIRT1 activity, and impairment of mitochondrial metabolism and mitophagy (Fang et al. 2017). Olaparib is a very well-studied inhibitor of PARP1 enzymatic activity. Interestingly, olaparib administration promotes mitophagy and restores mitochondrial homeostasis. Congruently, genetic ablation of PME-1 (the homolog of the mammalian PARP1 in *Caenorhabditis elegans*) enhances mitochondrial homeostasis and lifespan extension by augmenting intracellular NAD^+ content and sirtuins activity (Fang et al. 2017; Katsyuba and Auwerx 2017).

Altogether, these results underscore that genetic and chemical-induced modulation of intracellular NAD^+ pool might propel the development of novel therapeutic strategies to sustain energy homeostasis and cellular viability by stimulating the degradation of damaged mitochondria (Fig. 2). Although the beneficial outcome of NAD^+ replenishment methods has been investigated in several animal models, their off-target effects are questioned, highlighting the crucial and multifactorial role of NAD^+ in several cellular processes, including mitochondrial biogenesis, general autophagy, and tumorigenesis among others (Fang et al. 2017; Katsyuba and Auwerx 2017).

Natural Chemical Agents with Mitophagic Capacities

Mitophagy-inducing abilities of natural small-molecule drugs have been assessed in several model organisms, including yeast, flies, nematodes, and mice. Resveratrol, spermidine, urolithin A are first-in class mitophagy inducers that have been shown to adjust, preserve, and restore mitochondrial population and energy metabolism (Berman et al. 2017; Lou et al. 2019; Madeo et al. 2018; Palikaras et al. 2018).

Resveratrol is a natural phenol compound, which is highly concentrated in red grapes skin. Resveratrol has been characterized as a caloric restriction mimetic exerting anti-inflammatory and anti-ageing effects. This natural product has attracted the attention of scientific community due to its potential therapeutic properties against several human pathologies, such as neurological disorders, cardiovascular diseases, and diabetes (Berman et al. 2017). Experimental evidence indicates that resveratrol increases the intracellular NAD^+ concentration via AMP-protein kinase (APMK) activity resulting in the subsequent stimulation of SIRT1 (Fig. 2). Moreover, mitochondrial metabolism and biogenesis is upregulated in response to resveratrol treatment alleviating muscular and metabolic defects in obese mice (Palikaras et al. 2017).

Polyamines, putrescine, spermidine, and spermine, are involved in the regulation of multiple cellular processes, such as mitochondrial homeostasis, innate immunity, cell growth, and proliferation. Therefore, the biosynthetic and metabolic pathways of polyamines are tightly associated with organismal survival and viability. Indeed, the intracellular content of spermidine is gradually reduced in several cell types with age leading to the deterioration of biological systems

(Madeo et al. 2018). Chronic supplementation of spermidine enhances memory and lifespan in yeast, flies, nematodes, and mice, in an autophagy-dependent manner. Recent studies demonstrate that chronic administration of spermidine promotes also mitophagy sustaining heart function and renal homeostasis in aged rodents (Madeo et al. 2018). Ataxia telangiectasia mutated (ATM) kinase stimulates the PINK1/Parkin pathway upon spermidine treatment. ATM is involved in DNA damage responses preserving cellular physiology by altering mitochondrial activity and mitophagy upregulation. Notably, spermidine-induced mitochondrial membrane potential collapse leads to ATM activation and, in turn, PINK is stabilized on OMM and recruits Parkin on mitochondrial surface to initiate autophagosomal generation (Fig. 2) (Palikaras et al. 2018).

Urolithin A (UA) is the most abundant ellagitannin-derived metabolite in human body and is mainly generated by intestinal microflora upon consumption of fruits and nuts, such as pomegranate, raspberries, strawberries, walnuts, and almonds. Although the molecular mechanism of UA function is poorly understood, its antioxidant, anti-inflammatory, and anti-tumor properties are very well established (Palikaras et al. 2017). Recently, supplementation of UA is shown to improve muscle function and lifespan by stimulating mitophagy in both nematodes and mice (Palikaras et al. 2017, 2018). Furthermore, UA-induced mitophagy is shown to ameliorate cognitive defects, inflammatory responses, and protein aggregates in neuronal and microglia cells of AD nematode and mouse models (Lou et al. 2019). Interestingly, genetic studies in *C. elegans* uncovered that UA relies on BEC-1, PINK-1, SQST-1, and DCT-1 (the mammalian homologs of Beclin, PINK1, p62/SQST1, and BNIP3/NIX, respectively) to promote longevity, whereas PINK-1 and PDR-1 (the mammalian homolog of Parkin) are required for its neuroprotective effects indicating that there is an intricate communication between mitophagy regulators to sustain energy metabolism and cellular homeostasis in a cell-type and tissue-specific manner (Fig. 2). Moreover, the UA beneficial effects on cellular and organismal physiology are independent of dietary conditions and age. Thus, UA treatment could be used as a novel therapeutic strategy to rejuvenate mitochondrial metabolism and protect against age-dependent decline of muscular activity and mobility deficits (Palikaras et al. 2017, 2018). Indeed, the report of the first-in-human clinical study in which UA is orally administrated to healthy, sedentary elderly individuals highlights the successful translation of its benefits to humans, underlined by UA-mediated improvements on mitochondrial homeostasis and muscle activity together with its safety and bioavailability profile (Andreux et al. 2019).

Mitophagy Inhibitors

Despite the beneficial effects of mitophagy in mitochondrial metabolism and cellular homeostasis, runaway mitochondrial clearance promotes shrinkage of mitochondrial pool, overstressing the remaining organelles, triggering energetic crisis, and eventually leading to cell death (Kubli and Gustafsson 2012). Hence, several chemical substances have been identified and used to prevent mitophagy.

The most commonly used method to inhibit mitophagy relies on lysosomal inhibitors, such as bafilomycin, chloroquine, and berbamine, which could impair lysosomal acidification or the process of fusion between autophagosomal and lysosomal membranes (Georgakopoulos et al. 2017). An alternative indirect approach to block mitochondrial removal is via the regulation of fission/fusion machinery. Mitochondrial network fragmentation is a perquisite event for mitophagy under stress conditions. Administration of mitochondrial division inhibitor-1 (mdivi-1) disturbs mitochondrial morphology by preventing fission and subsequently mitophagy in yeast and mammalian cells (Georgakopoulos et al. 2017; Murphy and Hartley 2018). Mechanistically mdivi-1 disturbs enzymatic activity of DRP1 protecting against oxygen-glucose deprivation and glutamate driven neurotoxicity. Recently, a novel peptide inhibitor was generated to manipulate FUNDC1-meidated mitochondrial clearance. This specific peptide is

cell-permeable and associates directly with the LIR motif of FUNDC1. Indeed, this LIR mimetic peptide diminishes the physical interaction between FUNDC1 and LC3 preventing FCCP- and hypoxia-induced mitophagy (Georgakopoulos et al. 2017). Notably, general autophagy was not affected underlining peptide selectivity and endorsing the rationale for the designing of specific peptides mimetics to modulate mitophagy execution.

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Mixed-Function Oxidases

▶ P450 Monooxygenase System

MMPs

▶ Matrix Metalloproteinases

Molecular Chaperones

▶ Chaperones

Molecular Dynamics

▶ Molecular Modelling

Molecular Modelling

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Synonyms

Biocomputing; Molecular dynamics; Molecular simulation; Structural bioinformatics

Definition

Molecular modelling itself can be simply described as the computer-assisted calculation,

modulation, and visualization of realistic three-dimensional molecular structures and their physical-chemical properties using *molecular mechanics/force fields*.

Moreover, molecular modelling is one key method of a wide range of computer-assisted methods to analyze and predict relationships between protein sequence, three-dimensional molecular structure, and biological function (sequence-structure-function relationships). In molecular pharmacology, these methods focus predominantly on analysis of interactions between different proteins and between ligands (hormones, drugs) and proteins as well gaining information at the amino acid and even to atomic level.

Description

The constantly increasing amount of data coming from high throughput experimental methods, from genome sequences, from functional and structural genomics has given rise to a need for computer-assisted methods to elucidate sequence-structure and function relationships.

Sequence Similarity

Protein sequences encoded by genome sequences encode biological functions.

There are two different dimensions, breadth and depth, used to reveal sequence-structure-functional relationships by computational methods (Luscombe et al. 2001).

The aim of the first dimension "breadth" is to reveal sequence-function relationships by comparing protein sequences by sequence similarity. Simple bioinformatics algorithms can be used to compare a pair of related proteins or for sequence similarity searches, e.g., BLAST (Basic Local Alignment Search Tool). Improved algorithms allow multiple alignments of larger number of proteins and extraction of consensus sequence pattern and sequence profiles or structural templates which can be related to some functions, see, e.g., under http://www.expasy.ch/tools/#similarity.

The aim of the second dimension "depth" is to consider protein three-dimensional structures to uncover structure-function relationships. Starting from the protein sequences, the steps in the depth dimension are structure prediction, homology modelling of protein structures, and the simulation of protein-protein interactions and ligand complexes.

Protein Structure Predictions

Starting from the protein sequence (primary structure), several algorithms can be used to analyze the primary structure and to predict secondary structural elements like beta-strands, turns, and helices. The first algorithms from Chou & Fasman occurred already in 1978. The latest algorithms are to find, e.g., under https://www.expasy.org/proteomics/protein_structure where also predictions of transmembrane and coiled coil regions just from the primary sequence are possible.

Approaches of de novo predictions, which try to calculate how the structural elements are folded into the three-dimensional structure (tertiary structure) of complete proteins, are under strong development (Marks et al. 2012) indicated by recent successful results at the contest for structural prediction methods in the Critical Assesment of Structure Prediction, CASP14 in 2020. With the fast growing number of experimentally solved 3D structures of protein and new promising approaches like artificial inteligence, threading tools combined with evolutionary and experimental structural constraints, one can expect increasingly reliable de novo predictions for 3D protein structures in the future.

Homology Models of Protein Structures

The primary sequence of proteins with identical function varies within different species by natural mutations of amino acids. With increasing distance in the evolutionary process, the number of variations between the sequences of proteins increase.

In the protein structure database PDB (http://www.rcsb.org/pdb), all experimentally solved three-dimensional protein structures are publicly accessible. Structural data from X-ray crystallography, NMR spectroscopy and cryo-electron microscopy are available. Homology model building for a query sequence uses protein

portions of known three-dimensional structures as structural templates for proteins with high sequence similarity.

If the sequence of a protein has more than 90% identity to a protein with known experimental three-dimensional structure, then it is an optimal case to build a homologous structural model based on that structural template. The margins of error for the model and for the experimental method are in similar ranges. The different amino acids have to be mutated virtually. The conformations of the new side chains can be derived either from residues of structurally characterized amino acids in a similar spatial environment or from side chain rotamer libraries for each amino acid type which are stored for different structural environments like beta-strands or alpha-helices.

The discrepancies between homologous protein sequences occur predominantly at the surfaces and in the so-called loop regions. With decreasing sequence identities, insertions and deletions of loop chains or modelling of completely different loop chains are necessary. Segmentation of the loop in overlapping sequence fragments (3–10 residues) and searching for sequence similarity using BLAST or FASTA in the PDB database was proven to be most successful in loop modelling. Web platforms for loop modelling are, for example, https://modbase.compbio.ucsf.edu/modloop/; https://bioserv.rpbs.univ-paris-diderot.fr/services/DaReUS-Loop/.

Those fragments occurring several times in different protein structures with a common backbone conformation have a high probability of adopting the same backbone conformation also in the query sequence. The overlap almost allows knowledge-based assembly of the fragments to a new loop conformation. This segmentation strategy is also part of the successful algorithm Rosetta (http://boinc.bakerlab.org/rosetta/) for predicting complete folds for new proteins.

Similar residues in the cores of protein structures, especially hydrophobic residues at the same positions, are responsible for common folds of homologous proteins. Certain sequence profiles of conserved residue successions have been identified which give rise to a common fold of protein domains. They are organized in the smart database (simple modular architecture research tool) http://smart.embl-heidelberg.de.

Natural mutation of amino acids in the core of a protein can stabilize the same fold with different complementary amino acid types, but they can also cause a different fold of that particular portion. If the sequence identity is lower than 30%, it is much more difficult to identify a homologous structure. Other strategies like secondary structure predictions combined with knowledge-based rules about reciprocal exchange of residues are necessary.

If there is a reliable assumption for common fold, then it is possible to identify intra- and intermolecular interacting residues by search for correlated complementary mutations of residues by correlated mutation analysis, CMA (https://biokinet.belozersky.msu.ru/visualcmat/). Analysis of multiple sequence alignments for proteins of known structure has revealed that pairs of alignment columns corresponding to residues in spatial contact often tend to show patterns of correlated mutations (when the amino acid in one column changes, the amino acid in the other column is also likely to change correspondingly). This covariation between alignment columns has been attributed to the need to preserve favorable residue-residue interactions such as hydrogen bonds or tight packing.

Predicting a likely conformation or fold of a particular region of a protein with less or no sequence similarity to protein structures recorded in the PDB is the main challenge for homology modelling of proteins. Depending on the molecule type, different kinds of online web servers are available to generate homology models of 3D structures. For protein structure predictions, three web servers should be mentioned here as examples: ITASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER/); PHYRE (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) and RAPTORX (http://raptorx.uchicago.edu/). These servers take the primary sequence of the target protein as input. After the construction phase of a model follows the optimization of the geometrical structure utilizing force fields and provide as output the lowest energy 3D modeled structure of the protein. Thereby the contributions of force field terms to

an energy equation are minimized. A typical all atom form of a molecular force field is constituted by bonded (bond, angle, dihedral, and torsion) interactions and no bonded (electrostatic and van der Waal's) interaction energy terms. There are many all atom force fields available, such as CHARMM, AMBER, GROMOS, and OPLS among others. Each force field has a unique set of parameters that are optimized to reproduce experimental reference data of a particular molecule type (e.g., small molecules, peptides, proteins, glycans) and environment (solvent, membrane). For this reason, it is essential to ensure that the application of the corresponding force field is in accordance with the conditions to be investigated, especially in the case of online server use.

Sequence conservation is, in general, much weaker than structural conservation. There are proteins which are clearly not related in sequence but are closely related in 3D structure and fold, like hemoglobin and myoglobin, which have similar functions. In many proteins, fold elements like 4-helical bundles are repeated. Classifications of known structural folds of proteins are organized in the SCOP or CATH database, see, e.g., http://scop.mrc-lmb.cam.ac.uk/scop/.

Template-free modelling approaches can be applied to proteins without global structural similarity to a protein in the PDB. Lacking a structural template, these methods require a conformational sampling strategy for generating candidate models, as well as a ranking criterion by which native-like conformations can be selected. Advanced strategies are described in Kuhlman and Bradley (2019).

Taken together the procedure to build a starting protein structural model for a protein combines similarity searches by sequences and by folds in different 3D structure databases and filling in remaining unknown conformations by information resulting from bioinformatics, knowledge-based approaches, and overlapping segmentation of sequence fragments.

Molecular Simulation

Simulation in general describes calculations with models where different options and combinations of variables can be quickly played through. Molecular dynamics simulations allow the characterisation of molecular properties during the movements of the molecular models, over a period of time.

Starting with an energy for an unfavorable molecular geometry, the algorithm searches for the next local energy minimum at the energy hypersurface. Starting with different unfavorable geometries can lead to different conformations at other local energy minima. For larger molecular structures and especially for structural models built on templates with lower sequence identity, it is necessary to evaluate the geometrical stability.

Importantly, all biological procedures are operating at a temperature of 310 Kelvin, not at 0 Kelvin as the potential energy is calculated by the force fields. The kinetic energy must also be considered. Molecules and proteins at room temperature change the conformation at least at the surface and in the loop region. Molecular dynamics simulation (MD) is an approach to tackle these kinetic and stability problems. Simulations of biological systems are performed at multiple resolutions—atomistic, coarse-grained, and mesoscale. The all-atom simulations (MD engines GROMACS, NAMD, CHARMM, AMBER) are restricted to small time-steps to capture the intra- and intermolecular motion of atoms over time that result in detailed structural, thermodynamic, and kinetic data that can be compared to experimental data.

The coarse-grain (CG) resolution approach considers instead of all single atoms a group of atoms of an amino acid as one common interacting entity (e.g., CG force field MARTINI). Mesoscale models consider even clusters of amino acids as one common entity. These lower resolution approaches have the advantage of considering larger protein ensembles, but with the limitation of greater inaccuracy.

An approach to overcome the multi minima problem of proteins is the simulated annealing (SA) run. Besides global molecular properties such as structural and thermal motions, functional properties of fast biological reactions can also be studied by MD.

Docking

The goal of virtual docking is to calculate the binding of a large number of ligand-protein or of protein towards a binding site of a target protein and to predict their binding affinity. To solve the docking problem, both the flexibility of the ligand and the binding site at the target must be taken into account, as changes in the orientation of the amino acids involved can be induced during binding. This leads to a combinatorial explosion of possible binding modes of a single ligand, each of which must be evaluated for binding affinity to find the most likely one. For this reason, many of the currently available virtual docking programs neglect the conformational flexibility of the binding site.

Docking approaches are simple geometry-based methods in which large numbers of ligand-protein or protein-protein interfaces are generated via geometric rigidbody motions, followed by a scoring scheme that ranks the interfaces using scoring algorithms. Popular docking web server include among others SwissDock (http://www.swissdock.ch/) for small molecule docking and HADDOCK (http://www.bonvinlab.org/software/haddock2.2/) for protein-protein docking. The latter is an information-driven flexible protein-protein docking approach for the modelling of biomolecular complexes. It distinguishes itself from ab initio docking methods in the fact that it encodes information from identified or predicted protein interfaces in ambiguous interaction restraints (AIRs) to drive the docking process.

Accuracy and Limitations

Molecular models are only an approximation to reality, but experimentally guided good models can often closely approach reality.

The margin of error of a final structural model depends on the sequence or fold similarity to the starting structural template.

An important measure for quality is the verification by MD or SA of the stability of a molecular model. Other programs (e.g., PROCHECK) can also be used to check the globular geometrical quality of a structure to avoid serious defects in the geometry of proteins.

Even the most elaborate models are worthless if there is no experimental examination at all.

Functional insights based on structural relationships can only rise to the level of hypotheses, and these hypotheses must be tested by direct functional experiments.

The strongest verification for a 3D protein model comes from the experimental 3D structure. This is the objective of the Critical Assessment of Techniques for Protein Structure Prediction, CASP (http://predictioncenter.org), where the structural models are made in advance of the experimental structure of a particular protein. A similar contest is available for docking studies predicting protein-protein interaction, which is called Critical Assessment of PRediction of Interactions, CAPRI (http://www.ebi.ac.uk/msd-srv/capri).

In molecular pharmacology research, an indirect proof of a structural model is possible by functional examinations, e.g., by molecular biological experiments. Well-selected site directed mutagenesis and their functional characterization allows confirmation or rejection of a molecular protein model. The process is organized as an iterative procedure, where the biological answer of suggested mutations is used to refine the model. The iteration continues until the model is consistent with the biological experiments and the functional predictions of mutations are confirmed.

Pharmacological Relevance

In general the relevance of predictions of structure-function relationships based on molecular modelling and structural bioinformatics are threefold. First they can be used to answer the question of which partners (proteins) could interact. Second, predictions generate new hypotheses about binding site, about molecular mechanisms of activation and interaction between two partners, and can lead to new ideas for pharmacological intervention. The third aim is to use the predictions for structure-based drug design.

Common to all three aims is that in silico-derived predictions can rationalize experimental

efforts either by well-directed very specific molecular biological experiments like site-directed mutations or, e.g., by reducing the number of compounds to screen experimentally for drug design.

Structure-Function Prediction

From the human genome project, it is known that roughly 30,000 proteins exist in humans. Currently only the 3D structures of a few thousand human proteins or protein domains are known. Structures of membrane bound proteins are several magnitudes rarer. Beside efforts to solve further structures like structural genomics, there is a challenge for computational approaches to predict structures and function for homologous proteins.

This is eminently necessary for large protein classes with important functions, e.g., the G-protein coupled receptors (GPCR), where several hundred different human GPCR are known. Out of this large family of seven transmembrane helix proteins, there are currently crystal structures of approx. 60 different GPCRs stored in the PDB database with a constantly growing number. Of the more than 200 structural entries for GPCRs, there are fragments as well as dimers/oligomers (https://alf06.uab.es/dimerbow/) and different activation states of the receptors including bound antagonists, agonists, and G-proteins.

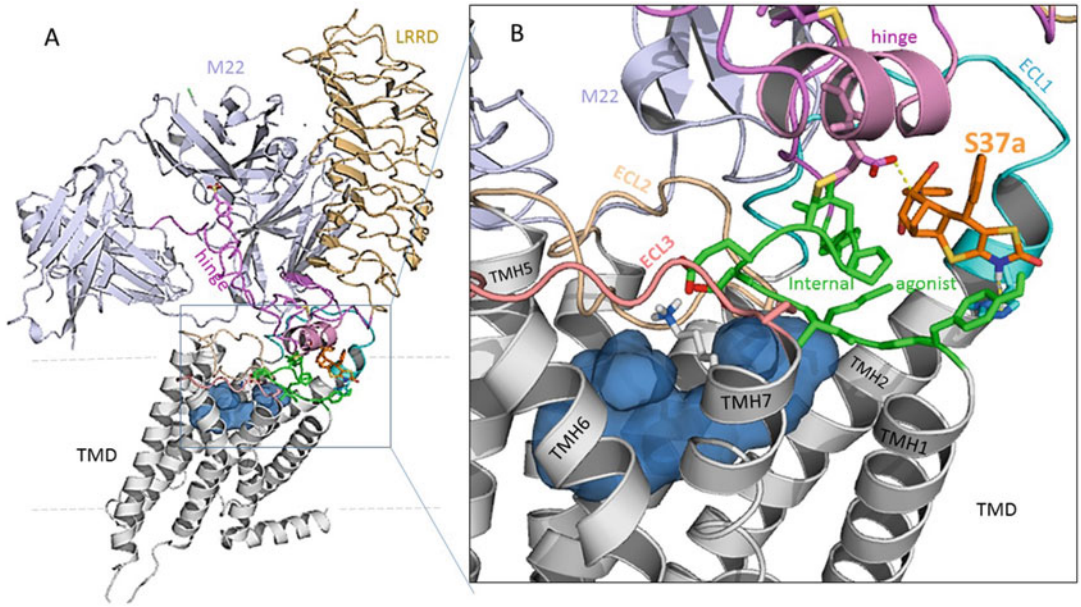
These crystal structures can be used as templates for various homologous receptor models. There are popular online servers for modelling GPCR structures, GPCR- ITASSER (https://zhanglab.ccmb.med.umich.edu/GPCR-I-TASSER/about.html), GPRM (http://gpcrm.biomodellab.eu/), and GPCR-Db (https://www.gpcrdb.org/) among others.

Most of them do not distinguish between the activation states or consider only inactive states of bound antagonists such as SSFE (http://www.ssfa-7tmr.de/ssfe2/). As with the selection of the force field, it is important that the selection of the template must be made according to the question to be investigated. The majority of the structure templates for GPCRs are in the inactive or intermediate state. For generating GPCR models in the active state, for example, to dock agonists, one should select such crystal structure as template, in which both an agonist and the corresponding G-protein or mimicking nanobodies are bound.

Incorporation of further experimental results like scanning accessible cysteines, cross linking, spin labelling, ligand binding, and site-directed mutations, etc. allowed molecular modelling to predict successfully ligand-binding sites and local activation mechanisms of diverse GPCR. In general the inactive conformation of GPCR is constrained by interaction of complementary residues in the interior side of the receptors which can be observed in the rhodopsin structure.

Activation mechanisms of homologous GPCRs can be revealed by molecular simulations and characterized by molecular experiments as in the following example.

The molecular activation mechanisms of the thyroid stimulating hormone receptor (TSHR), a homolog of glycoprotein hormone receptors (GPHR), LHR, and FSHR (web resource http://www.ssfa-gphr.de/) appear to be distinct from that of other GPCRs of family A, owing to its uniquely large N-terminal extracellular domain (ECD) in terms of overcoming its inhibitory function. The ECD of TSHR is comprised by a leucine-rich repeat domain (LRRD) and a hinge region. Molecular models of SHR indicate that the hormone TSH binds between the LRRD and hinge region and activates the transmembrane domain (TMD) indirectly via an internal agonist (Fig. 1a). TSHR-stimulating autoimmune antibodies (TSAb) such as M22 bind to TSHR at a similar site as TSH itself and cause deregulated TSHR hyper-activation in the thyroid and in the eye in pathogenic conditions of Graves' disease (GD) and Graves' orbitopathy (GO) respectively. Combined modelling and mutagenesis has shown that the intramolecular activation pathway is blocked by a recently identified highly TSHR-selective small molecule antagonist S37a (Marcinkowski et al. 2019) that binds allosterically between the hinge and the internal agonist (Fig. 1b). This binding site is distinct from a canonical transmembrane-binding site of GPCRs, wherein TSHR allosteric small molecule agonists are bound.

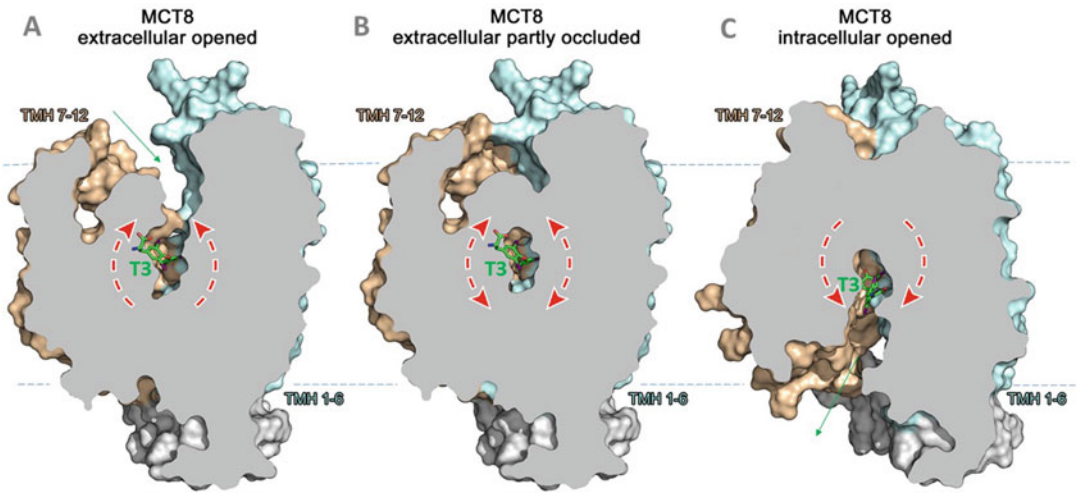


Molecular Modelling, Fig. 1 Homology model of the TSH-Receptor. (**a**) Autoimmune stimulating antibody M22 (light blue) binds to the extracellular leucine-rich repeat domain LRRD (beige) delocalizing the hinge region (magenta) that triggers the internal agonist sequence (green) to activate indirectly the transmembrane domain (TMD, grey), which contains a canonical allosteric binding pocket (blue surface), where also small molecule agonists can bind. (**b**) The antagonistic small molecule S37a (orange) binds into an alternative and distinct binding site between internal agonist (green), extracellular loop 1 (ECL1, cyan), and hinge region (magenta) and locks the inactive state of the TSHR (Marcinkowski et al. 2019)

Simulation of Functional Properties

With growing computer power, the abilities to simulate functional properties and dynamics of fast biological reactions are increasing. Today molecular dynamics (MD) can be traced over a time range of about 1 millisecond. Relevant dynamics of fast biological processes (vision and photosynthesis) like electron transfer reactions, proton translocation (e.g., bacteriorhodopsin, V-ATPase), ion transport (diverse ion channels), and hormone transport (e.g., thyroid hormone transporter) across the membrane via membrane proteins have been studied. MD simulations can provide a realistic molecular description of the actual reactive event. For example the dynamics and mechanisms of water permeation through biological membranes via pore proteins were studied using Aquaporin 1 (AQP1) and the homologous glycerol facilitator (GlpF). The selective pattern for transport of water at AQP1 and glycerol at GlpF could be identified. Also a fine-tuned water dipole rotation during the passage through the pores could be simulated by MD (de Groot and Grubmüller 2001).

Recently the molecular mechanisms of thyroid hormone (TH) transport across the plasma membrane via the transport protein Monocarboxylate transporter 8 (MCT8) were studied that comprises 12 transmembrane helices. Three new MCT8 homology models based on crystal structures of homologous sugar transporters XylE in the intracellular opened (PDB ID: 4aj4) and the extracellular partly occluded (PDB ID: 4gby) conformations as well as FucP (PDB ID: 3o7q) and GLUT3 (PDB ID: 4zwc) in the fully extracellular opened conformation allowed the identification of a membrane-traversing mechanism for THs such as T3 by MCT8 (Fig. 2). The N-terminal transmembrane six-helix-bundle undergoes a rigid body rotation around a central TH-interacting cavity with respect to the C-terminal transmembrane six-helix-bundle. This allows alternating extra- and intracellular accessibility of the TH recognition-sensitive residues His192, His415, Arg445, and Asp498,



Molecular Modelling, Fig. 2 Sliced MCT8 molecular models of (**a**) extracellular opened, (**b**) extracellular partly occluded, and (**c**) intracellular opened conformations are based on crystal structures of homologous sugar transporters in respective states (XylE, PDB:4aj4 & 4gby, GLUT3, PDB:4zwc and FucP, PDB:3o7q). The models indicate two revolving six–transmembrane helix (TMH)-bundles, one central substrate-recognition and membrane-traversing cavity, which is accessible by thyroid hormone (TH) T3 (green) from both sides. After extracellular TH recognition, T3 adheres to transport-sensitive residues during tilting (red arrows) of the N-terminal (TMH 1–6, cyan) and C-terminal (TMH 7–12, sand) six-TMH bundles around the central cavity and is thus lifted through the central traversing cavity and released towards the intracellular side and vice versa (Protze et al. 2017)

enabling MCT8 to transport TH into and out of the cell, depending on the concentration gradient (Protze et al. 2017).

Critical examinations of dynamic effects showed that they are rather unlikely contribute to processes with significant activation barriers. Even in cases of ion channels, it is found that the most important effects are associated with energies rather than dynamics.

The resulting insight of MD is crucial in studies of fast photo biological reactions and instructive in cases of slower processes. Very slow processes like folding of proteins can until now be traced only fragmentarily by MD, because folding takes a time range between 20 milliseconds and 1 h.

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Molecular Simulation

► Molecular Modelling

Monoamine Oxidases

▶ Monoamine Oxidases and Their Inhibitors

Monoamine Oxidases and Their Inhibitors

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Synonyms

Depressive illness; Inhibitors; Monoamine oxidases; Neuroprotection-neurorescue; Parkinson's disease

Definition

Monoamine oxidase (amine:oxygen oxidoreductase (deaminating); EC 1.4.3.4; MAO) exists in two forms, named type A and type B. They are responsible for oxidative deamination of primary, secondary, and some tertiary amines, including the neurotransmitters, noradrenaline, dopamine, and serotonin (5-hydroxytryotamine; 5-HT), the hormone adrenaline, and trace amines, such as tyramine, tryptamine, and 2-phenylethylamine. Their nonselective and selective inhibitors are employed for the treatment of depressive illness and Parkinson's disease.

Reaction Mechanism of Monoamine Oxidase

The role of monoamine oxidase in terminating the action of the aminergic neurotransmitters and dietary amines has been extensively studied; less attention has been paid to the functions of the products formed in the MAO reactions that include aldehyde and acidic metabolites.

$$RCH_2NR'R'' + O_2 + H_2O \rightarrow RCHO + NR'R'' + H_2O_2$$

Each of the products of this reaction may have important metabolic and signaling functions, but they are also potentially toxic at higher concentrations. In the presence of Fe(II), hydrogen peroxide may form highly toxic species, such as hydroxyl radicals, in the Fenton reaction. It has been suggested that the aldehydes derived from serotonin and noradrenaline deamination may be involved in the regulation of sleep. The aldehyde derived from dopamine, which is rapidly removed by aldehyde dehydrogenase and does not accumulate in normal brain, is thought to be highly toxic to dopamine neurons of substantia nigra in ▶ Parkinson's disease, since the levels of that enzyme are very low in parkinsonian substantia nigra pars compacta and also in the 6-hydroxydopamine model of that disease. Such aldehydes may also form adducts with amine groups, to yield compounds such as *N*-methyl-R-salsolinol and tetrahydropapaveroline as well as peptide adducts, which may have pathophysiological roles.

Basic Characteristics of Monoamine Oxidases

MAO is tightly bound to the mitochondrial outer membrane. The active site contains covalently bound FAD (flavin adenine dinucleotide) as cofactor. The existence of two forms of MAO was first identified by differences in sensitivity to some inhibitors in presence of different substrates. The irreversible inhibitor clorgyline inhibits serotonin oxidation at low concentrations, in a sigmoidal fashion, whereas it is a poor inhibitor of benzylamine and 2-phenylethylamine oxidation. However, with dopamine and tyramine, a double sigmoidal inhibition is observed. This was interpreted as indicating the involvement of two enzymes, termed MAO-A and MAO-B, where the

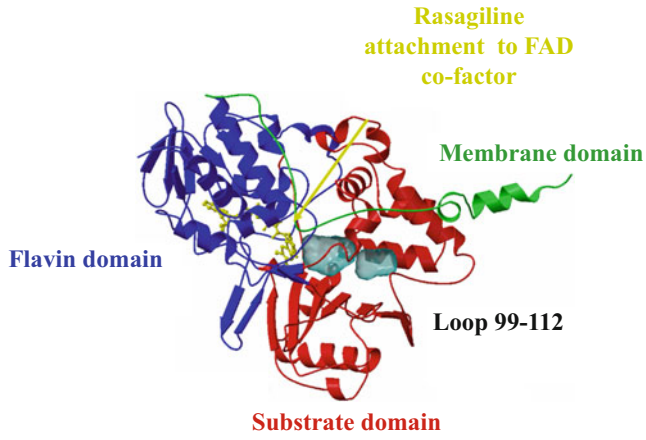
substrates for MAO-A include serotonin and clorgyline is a selective inhibitor of that enzyme. In contrast the irreversible inhibitor selegiline (*l*-deprenyl) is a potent inhibitor of MAO-B, which metabolizes benzylamine and 2-phenylethylamine. The responses to these inhibitors indicate that dopamine and tyramine are substrates for both enzymes (Tables 1 and 2). MAO-A and MAO-B have been purified and cloned. They have been shown to be different proteins, with MAO-A having about 70% identity to that of MAO-B form, with each enzyme containing FAD bound to a cysteine residue (Fig. 1). An important structural difference is the size of the entrance cavity that restricts the accessibility of bulkier substrates and inhibitors to the active site of MAO-B but allows them to access MAO-A. Comparisons of the three-dimensional structures of the enzymes from rat and human have shown the active site of the human MAO-A to comprise a single cavity, which is smaller than that of MAO-B from that

Monoamine Oxidases and Their Inhibitors, Table 1 Substrate specificity of the two forms of rat liver and brain monoamine oxidase

Rat liver and brain		
Substrates of the A form	Substrates of both forms	Substrates of the B form
A. Rat liver and brain		
Adrenaline	Dopamine	Benzylamine
Noradrenaline	Tyramine	2-Phenylethylamine
3-*O*-Methyladrenaline	3-Methoxytyramine	Phenylethenolamine
3-*O*-Methylnoradrenaline	p-Synephrine	O-Tyramine
Serotonin		1,4-Methylhistamine
		N-tele-Methylhistamine
		n-Pentylamine
B. Human brain		
	Adrenaline	Benzylamine
Serotonin	Noradrenaline	2-Phenylethylamine
	Dopamine	
	Tyramine	

Monoamine Oxidases and Their Inhibitors, Table 2 K_m values of the two forms of monoamine oxidase

Substrate	Source	Type A	Type B
		K_m (μM)	K_m (μM)
Serotonin	Rat liver	160	2000
Serotonin	Rat brain	180	1200
Serotonin	Human platelet	–	1800
Serotonin	Bovine chromaffin	–	2100
Noradrenaline	Bovine chromaffin	–	1750
2-Phenylethylamine	Human platelet	–	16
2-Phenylethylamine	Bovine chromaffin	–	21
Tyramine	Rat liver	110	580
2-Phenylethylamine	Rat liver	280	20
Dopamine	Human cerebral cortex	212	220
	Human striatum	180	210
Noradrenaline	Human cerebral cortex	284	238



Monoamine Oxidases and Their Inhibitors, Fig. 1 The crystal structure of human MAO-B. There are three functionally distinct domains, as shown. In red, the substrate domain contains two "cavities" shown in cyan. The outer space is the entrance cavity leading to the inner space, substrate binding cavity, closer to the flavin cofactor. The flavin-binding domain is shown in blue with the FAD molecule in yellow. In green, the C-terminal helical region which attaches the protein to the mitochondrial membrane. Rasagiline covalently links to the flavin via its propargylamine group (yellow arrow) and the indan ring and then extends into the substrate-binding cavity, blocking access for substrates

source but larger than that of rat MAO-A. Such differences suggest that the substrate specificities and sensitivities to inhibitors may show species differences. This structural difference may explain why adrenaline and noradrenaline have been reported to be a preferred substrate for MAO-A in rat liver and brain, but substrates for both enzymes in human brain.

The propargylamine-derived irreversible MAO-A and MAO-B inhibitors, clorgyline, *l*-deprenyl, and rasagiline, have been shown to form covalent adducts with the N-5 isoalloxazine moiety of covalently bound flavin (Fig. 1).

The separate genes for MAO-A and MAO-B are located on the X chromosome. Disruption of the MAO-A gene has been shown to result in a compulsive-aggressive phenotype, which is associated with some mental retardation. Similar responses have been observed in MAO-A knockout mice and in rats treated with clorgyline during the early postnatal period. The association with elevated serotonin levels in these subjects suggests the importance of maintaining low levels of this amine for normal brain development, a process which has been shown to be mediated by 5-HT-6 receptors. The effects of deletion of MAO-B are more modest but may be associated with reduced resistance to stress.

Drugs

The inhibitors clorgyline and deprenyl have been employed to identify the presence of each form in different tissues and species (Table 3) and also to determine the substrate specificities of MAO-A and MAO-B (Table 1) as well as their kinetic parameters (Table 2). A number of other inhibitors that are selective toward one or the other MAO have since been identified (Table 4, Fig. 2). The selective propargylamine inhibitors (clorgyline, selegiline, rasagiline, *N*-(2-propynyl)-2-(5-benzyloxy-indolyl) methylamine, aliphatic *N*-methyl propargylamine) may lose their selectivities at higher concentration in vitro and may do so after prolonged administrations in vivo. In contrast, the reversible inhibitors (Table 4) appear to retain their selectivity in vivo.

The effects of in vivo treatment with irreversible MAO inhibitors can be quite long-lasting, since regain of activity requires the synthesis of new enzymes. The rates of recovery from a single dose of an irreversible inhibitor show half-lives of about 2.5 and 3.5 days for MAO-A and MAO-B, respectively, in rat liver, 9 and 13 days in rat brain, and 2.2 and 7.7 days in rat intestine. A very much slower recovery rate has been reported for

Monoamine Oxidases and Their Inhibitors, Table 3 Distribution and proportions of the A and B forms of monoamine oxidases in adult rat and human tissues

% A and B form activity				
	Rat		Human	
	A	B	A	B
Kidney	70	30	57	40
Liver	55	45	54	50, 45
Spleen	95		34	70
Intestine	70	30	80	25
Lung	50			
Testis	90	10		
Vas deferens	50	50		
Brain	55	45		
Primate brain				
(a) Human basal ganglia			20	80
(b) Monkey striatum and basal ganglia			20	80
Superior cervical ganglion	90	10		
Pineal body	15			
Heart	98, 65		43	
Aorta			48	
Adrenal gland			60	
Adrenal chromaffin cells				
(a) Human			0	>90
(b) Bovine			0	>98
Ileum	78	22		80
Skeletal muscle			28	
Skin			31	
Human platelet			0	>98
Human placenta			~100	0

MAO-B in human brain, with a half-life of about 40 days.

Neuropharmacology of Monoamine Oxidases and Their Inhibitors

Acute treatment of nonselective MAO inhibitors (e.g., iproniazid, tranylcypromine, phenelzine) increases brain levels of all monoamines including the neurotransmitters dopamine, noradrenaline, and serotonin as well those of 2-phenylethylamine, tryptamine, and methylhistamine. In contrast, selective inhibitors of MAO-A (e.g., clorgyline) increase serotonin, whereas MAO-B inhibitors (e.g., selegiline, rasagiline) increase brain levels of 2-phenyethlamine and related amines. Neither classes of the selective inhibitors greatly affect brain levels of dopamine, since that neurotransmitter can be metabolized by either enzyme. Thus, when one form is inhibited, the other continues its degradation. A combination of MAO-A plus MAO-B inhibitors has the same effect as the nonselective inhibitors. As shown in Table 2, the substrate specificities of MAO-A and MAO-B are not absolute, in that MAO-B is able to oxidize higher concentrations of serotonin. Indeed, it has been shown, both in vitro and in vivo, that when the concentration of serotonin is high enough to satisfy the K_m of MAO-B, it can be metabolized by that enzyme. For this reason, selective MAO-A inhibitors do not increase the brain levels of serotonin to the same extent as nonselective inactivators. Although the changes in amine levels occur rapidly after treatment with nonselective inhibitors, the antidepressant effects are manifested more slowly, typically only after 2–3 weeks of chronic treatment. This has been attributed to system's responses to the elevated

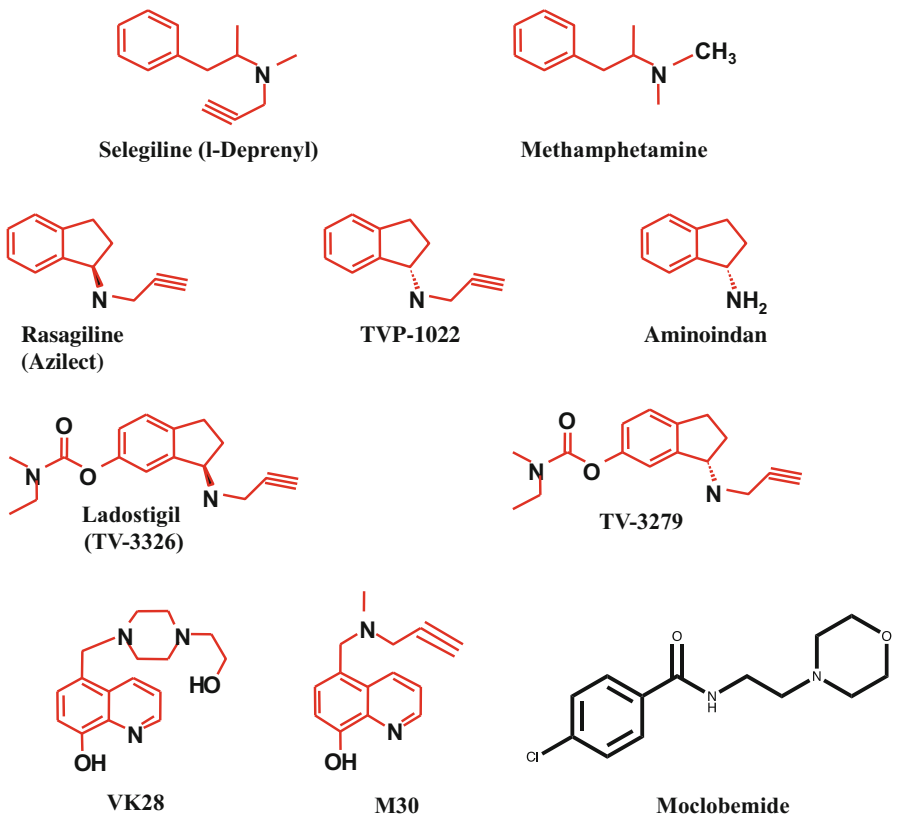
Monoamine Oxidases and Their Inhibitors, Table 4 Nonselective and selective monoamine oxidase inhibitors

Compound	MAO selectivity	Inhibitor type	Application
Iproniazid	A + B	Irreversible	Antidepressant
Phenelzine	A + B	Irreversible	Antidepressant
Isocarboxazid	A + B	Irreversible	Antidepressant
Tranylcypromine	A + B	Irreversible	Antidepressant
Nialamide	A + B	Irreversible	Antidepressant (antiparkinson?)
Pargyline∗	B	Ireversible	–
Clorgyline	A	Irreversible	–
l-Deprenyl (selegiline)	B	Ireversible	Antiparkinson
Rasagiline (Azilect)	B	Irreversible	Antiparkinson
Ladostigil (TV3326)	A + *Brain-Selective*B	Ireversible + *Acetylcholinesterase inhibitor*	Antidepressant, antiparkinson, anti-Alzheimer
M30	A + *Brain-electiveS*B	Irreversible *Iron chelator*	Antidepressant, Antiparkinson, anti-Alzheimer
R-HMP	B	Irreversible	Antiparkinson
Moclobemide	A	Reversible	Antidepressant
Brofaromine	A	Reversible	Antidepressant
Caroxazone	A	Reversible	Antidepressant
Toloxatone	A	Reversible	Antidepressant
BW 137OU87	A	Reversible	Antidepressant
Befloxatone	A	Reversible	Antidepressant
Lazabemide	B	Reversible	Antiparkinson
Safinamide	B	Reversible	Antiparkinson
Milacemide	B	Suicide substrate	Anticonvulsant

amine levels, including suppression of the synthetic enzymes, tyrosine hydroxylase, and tryptophan hydroxylase, as well as inhibition of aminergic neuronal firing, resulting from downregulation of presynaptic receptors alpha 2 receptors. Long-term postsynaptic adaptive changes include desensitization of postsynaptic beta receptors and subsensitivity of 5-HT1 receptors.

The Cheese Reaction

Iproniazid was among the first of the psychotropic antidepressants to be discovered and introduced into the clinic. Although this inhibitor and others that followed (tranylcypromine, phenelzine) demonstrated antidepressant activity, they also had a major side effect that became known as the "cheese reaction". This reaction (Fig. 3) is induced by tyramine and other indirectly acting sympathomimetic amines present in some foodstuffs (commonly in certain cheeses, hence the name) and fermented drink, such as beer and wine. Under normal circumstances, such dietary amines are extensively metabolized by MAO-A in the gut wall and in the liver and are, thus, prevented from entering the systemic circulation. If MAO is inhibited, this protective system is inactivated, and tyramine and other monoamines that are ingested are not metabolized and may enter the circulation, where they have access to, and induce a significant release of noradrenaline from, peripheral adrenergic neurons. This release results in a severe hypertensive response which, in some cases, can be fatal. This side effect stimulated a search for antidepressants that did not



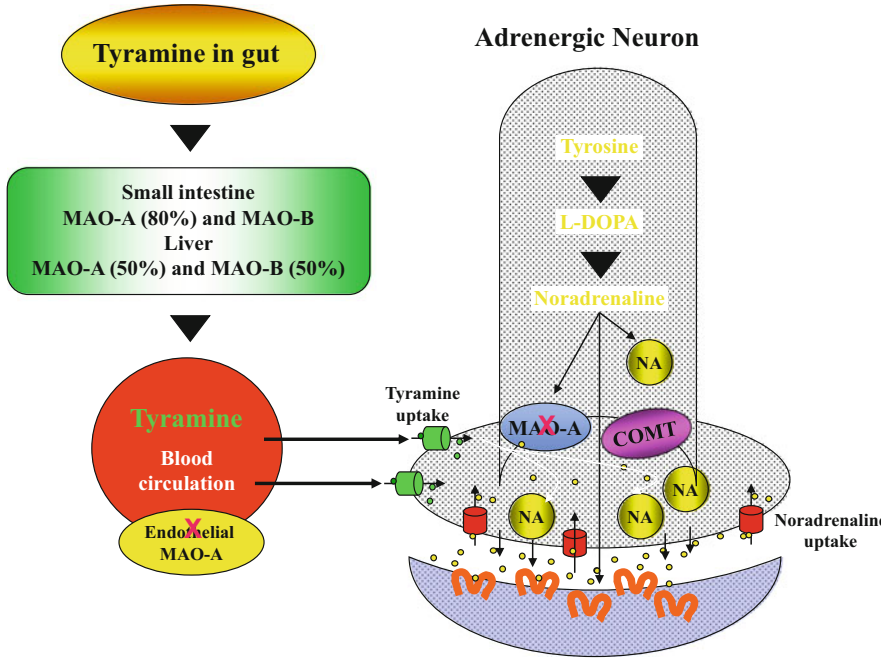
Monoamine Oxidases and Their Inhibitors, Fig. 2 Structures of some MAO inhibitors. In the top row, the structural similarity between selegiline/l-deprenyl and methamphetamine is shown. Below are the aminoindan series of propargylamine compounds such as rasagiline. Next, the bifunctional MAO and cholinesterase inhibitor (ladostigil) and the iron chelator-MAO inhibitors. The reversible MAO-A inhibitor moclobemide is shown in black

inhibit MAO. The amine uptake inhibitors, the tricyclic antidepressants, and, more recently, the serotonin selective reuptake inhibitors (SSRI), such as Prozac, have largely superseded MAO inhibitors as antidepressants. Better understanding of MAO-A and MAO-B distribution had led to the demonstration that the cheese reaction is the property of irreversible MAO-A inhibition in the gut and peripheral adrenergic neurons, where that enzyme resides. The irreversible MAO-B inhibitors, such as *l*-deprenyl (selegiline) and rasagiline (Azilect), do not induce a cheese effect and have limited cardiovascular potentiation at their MAO-B inhibitory dosage. However, at inhibitor concentrations where selectivity is lost and MAO-A is inhibited, the cheese reaction is prominent. Reversible and competitive MAO-A inhibitors (such as moclobemide) were developed as antidepressants that would not give a cheese reaction. The logic being that tyramine can compete with the reversible MAO-A inhibitor at the active site of MAO, allowing its metabolism before it can induce release of noradrenaline.

Monoamine Oxidase Inhibitors as Therapeutic Agents

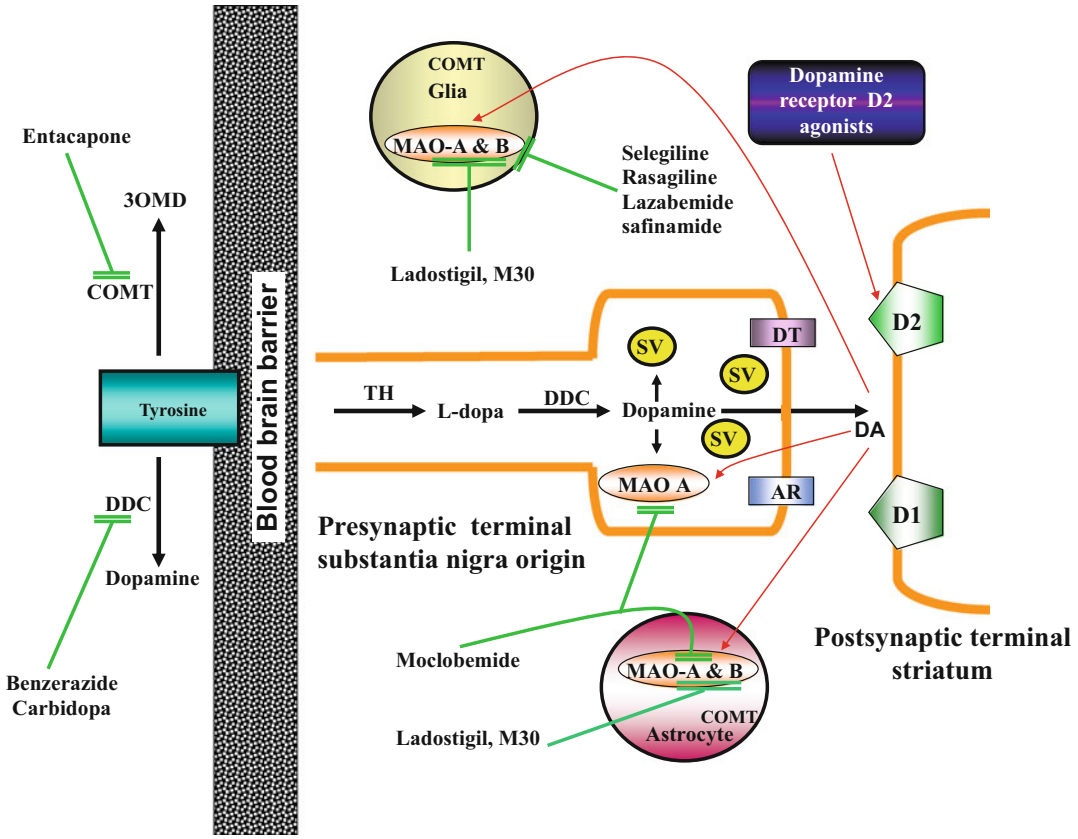
Parkinson's Disease

As early as 1961, the first generation of MAO inhibitors (iproniazid, isocarboxazid) was employed for the treatment of Parkinson's disease (PD). However, because of the severe side effects,



Monoamine Oxidases and Their Inhibitors, Fig. 3 The "cheese reaction" – potentiation of cardiovascular effects of tyramine (or other indirectly acting sympathomimetic amines) by irreversible inhibitors of MAO. Normally, dietary tyramine undergoes extensive "first pass" inactivation by the MAO-A and MAO-B types in gut wall and then in the liver. The tyramine that survives to enter the systemic circulation is further inactivated by the MAO in vascular endothelial cells and lung. At the adrenergic neuron, uptake of tyramine initiates the release of noradrenaline, which accounts for its sympathomimetic effects. Irreversible inhibition of MAO-A, the predominant form in the periphery adrenergic neurons, allows greatly increased tyramine entry in the systemic circulation and, from there, adrenergic neurons, consequently increasing noradrenaline release and effect. By contrast, reversible inhibitors of MAO-A (RIMAs) are displaced from the enzyme by tyramine which is then metabolized normally by the enzyme. Thus, circulating tyramine never attains the high levels resulting from irreversible inhibition of MAO

such as "cheese reaction," they were abandoned. The realization that the basal ganglia (extra pyramidal region) of human brain contained mostly MAO-B, which metabolized dopamine, and that the MAO-B inhibitor *l*-deprenyl did not potentiate the sympathomimetic actions of tyramine in vivo led to its employment as adjuvant to L-dopa therapy in 1975. Confirmation of these early results and the demonstration of its effectiveness in monotherapy led to its introduction into the clinic. The realization that MAO-B inhibitors had symptomatic activity and antiparkinson effects resulted in the development of other such MAO-B inhibitory drugs, many of which were subsequently abandoned because of toxicity or lack of effectiveness. A spur for such developments was the finding that *l*-deprenyl could be metabolized to *l*-amphetamine. The second-generation MAO-B inhibitor, rasagiline, which is not metabolized to amphetamine, is now in widespread use. The mechanism of the antiparkinson action of MAO-B inhibitors is not fully known but may be related to increased dopamine or to the ability of elevated 2-phenyethylamine to release dopamine. Since dopamine is metabolized both by MAO-A and MAO-B, it might be expected that MAO-A inhibitors and nonselective inhibitors would also have antiparkinson activity (Fig. 4), but these have not been employed because of the cheese reaction. However, some

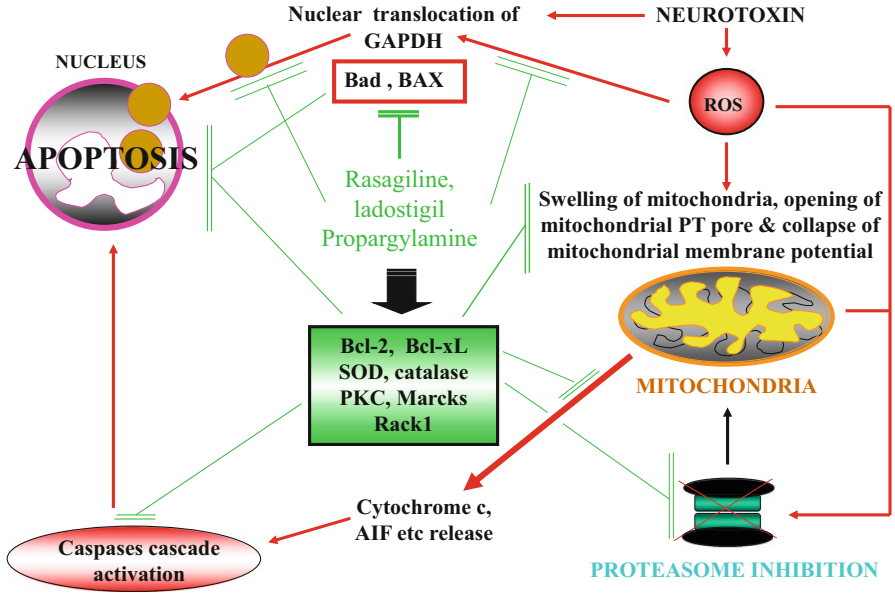


Monoamine Oxidases and Their Inhibitors, Fig. 4 Pathways of dopamine synthesis in dopaminergic neurons and metabolism by MAO-A and MAO-B in the brain. Tyrosine passes through the blood-brain barrier and is hydroxylated by tyrosine hydroxylase (TH) to DOPA and then decarboxylated by DOPA decarboxylase (DDC) to dopamine (DA) within the neuron. Dopamine is taken up into synaptic vesicles (SV) or metabolized by neuronal mitochondria MAO-A. After release from the terminal, extracellular dopamine is cleared by uptake into astrocytes and glia also containing MAO-A and MAO-B. Selective inhibition of one MAO isoform allows the other to metabolize dopamine effectively and does not greatly affect the steady-state levels of striatal dopamine. On the other hand, nonselective inhibition of MAOs induces highly significant increase in striatal dopamine and in other brain regions. D1 and D2, dopamine receptors. On the other hand, in adrenergic and serotonergic neurons, MAO-A and not MAO-B inhibition results in increased levels of these amines

clinical studies with the reversible MAO-A inhibitor, moclobemide, have shown that it does possess dopamine-sparing antiparkinson activity, without inducing a cheese reaction.

Neuroprotection in Neurodegenerative Diseases

The neurotoxin MPTP (*N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) can cause parkinsonism in humans, nonhuman primates, and rodents by its ability to cause degeneration of nigrostriatal dopamine neurons. MPTP is an inert tertiary amine which is oxidized by MAO-B to the neurotoxin MPP^+ (1-methyl-4-phenylpyridinium). MAO-B inhibitors prevent this and the neurotoxic effects of MPTP in animals and neuronal cell cultures. Since MPTP is a synthetic compound, it was suggested that PD could be a result of an environmental neurotoxin; however, no such neurotoxin has yet been discovered. Nevertheless, propargyl-derived

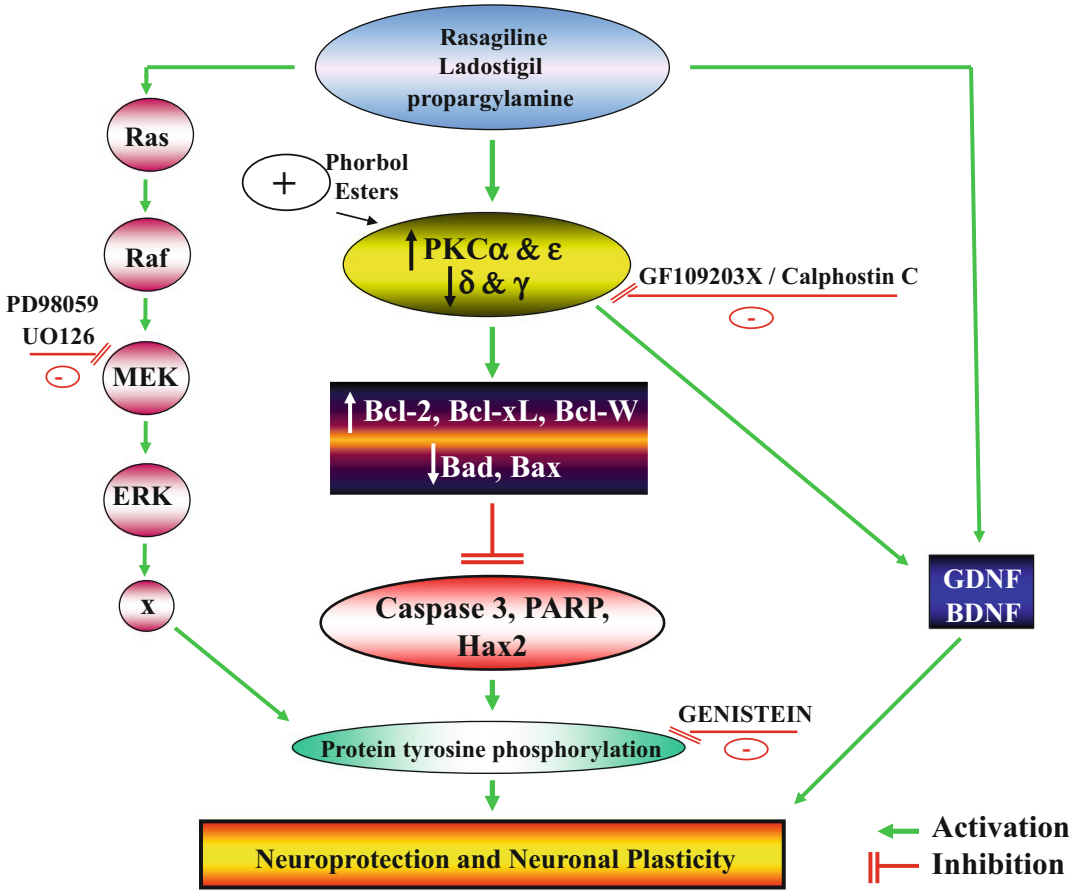


Monoamine Oxidases and Their Inhibitors, Fig. 5 The interactions of irreversible, propargylamine-based, MAO-B inhibitors with apoptotic pathways. Mitochondria are responsible for cell survival and death through the regulation of the B-cell leukemia/lymphoma 2 (BCL2) family anti-apoptotic (BCL2) and pro-apoptotic (BCL-associated death promoter, BAD, and BCL2-associated protein X, BAX) proteins. Rasagiline, selegiline, ladostigil, and propargylamine have been shown to induce cell survival in response to serum withdrawal or neurotoxins in neuronal cell cultures (SHSY-5Y and PC-12), through the activation of BCL2 and BCL-XL, and the downregulation of BAD and BAX. These propargylamines exert their neuroprotective activity by interacting with the mitochondrial outer membrane. The propargylamine moiety in these inhibitors prevents neurotoxin-induced collapse of mitochondrial membrane potential, mitochondrial permeability transition, and the opening of the voltage-dependent anion channel, as a consequence of the upregulation of anti-apoptotic BCL2 family proteins, which also leads to disinhibition of proteasome function. In addition, these propargylamines prevent the nuclear localization of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in response to neurotoxins and reactive oxygen species (ROS). *AIF* apoptosis-inducing factor, *PKC MARCKS* myristoylated alanine-rich protein kinase C (PKC) substrate, *RACK1* receptor of activated PKC, *SOD* superoxide dismutase

MAO-B inhibitors such as selegiline and rasagiline have received much attention as neuroprotective drugs. However, their neuroprotective activity appears to be independent from MAO-B inhibition, since several propargylamines, including propargylamine itself, that do not inhibit MAO also show neuroprotective properties. These drugs upregulate anti-apoptotic Bcl-2 while down-regulating pro-apoptotic Bcl-2 family proteins (Fig. 5) and activate PKC-MAP kinase pathways (Fig. 6). The result is prevention of mitochondrial impairment and cell survival. Some propargylamines have also been shown to be capable of causing regeneration of dying neurons (neurorescue). A delayed-start study where rasagiline was given to two groups of parkinsonian subjects, 6 months apart, has shown that subjects treated with rasagiline for 12 months showed less functional decline than subjects whose treatment was delayed for 6 months. This was interpreted as indicating a disease-modifying action.

Depressive Illness

Although the cheese reaction seen with non-selective and irreversible MAO inhibitors curtailed their use, two of them, phenelzine and tranylcypromine, are still prescribed, with patients being instructed about foods and beverages that



Monoamine Oxidases and Their Inhibitors, Fig. 6 The mechanism of neuroprotection and/or neurorescue by propargylamine MAO inhibitors. The propargylamine monoamine oxidase (MAO) inhibitors (such as rasagiline and ladostigil) activate the protein kinase C (PKC)-dependent mitogen-activated protein (MAP) kinase pathway 185,187 and mitochondrial B-cell leukemia/lymphoma 2 (BCL2) family cytoprotective proteins and downregulate pro-apoptotic BCL-associated death promoter (BAD), BCL2-associated protein X (BAX), caspase 3, poly (ADP-ribose) polymerase 1 (PARP1), and H2AX (a phosphorylated mammalian histone H2A) 141. One direct consequence is the processing of amyloid precursor protein (APP) through the activation of α-secretase, resulting in increased release of the neuroprotective/neurotrophic soluble APPα (sAPPα). The activation of PKCα and PKCε might explain the ability of these propargylamines to induce expression of glia cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) mRNAs and stimulate the release of GDNF. Inhibitors of PKC (such as GF109203X and calphostin C), extracellular signal-regulated kinase 1/2 (ERK1/2) (e.g., PD98059 and UO126), and tyrosine kinase (for instance, genistein) prevent APP processing and release of GDNF and block BCL2-dependent neuroprotective pathways, supporting a mechanism by which propargylamines operate at different levels and suggesting a direct link between PKC and BCL2 family protein expression. MEK, MAP kinase kinase; Raf, Ras, small G proteins; X represents a phosphorylated protein that activates tyrosine kinase

they should avoid. In comparison with the tricyclic antidepressants, it has been reported that monoamine oxidase inhibitors were less effective in endogenous depressive conditions involving melancholia whereas they were particularly effective in the so-called atypical depressions. With the reversible MAO-A inhibitor, moclobemide (Figs. 2 and 4), dietary restriction is unnecessary

since it has highly limited capacity to induce the cheese reaction. Because of its mild antidepressant activity, it is generally considered to be more suitable for elder subjects, who react poorly to standard antidepressants.

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Monokines

► Cytokines

Mood Elevators

► Antidepressant Drugs

Mood Stabilizers

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Definition

Bipolar disorder (BD) is a common, chronic, heritable, recurring mood disorder that involves manic, depressive, and mixed episodes. It is often comorbid with other medical conditions and is also associated with high morbidity and mortality. Without adequate maintenance treatment with mood stabilizers, the frequency and severity of BD episodes typically increases over time.

Mood stabilizers are highly effective for preventing mood episodes and improving manic and/or depressive symptoms; these medications are used as long-term prophylactic treatment based on their ability to prevent the recurrence of depression and mania. Mood stabilizers are also expected to prevent and treat switches between depression and mania, a feature that distinguishes these agents from other pharmacological approaches in mood disorders, such as antidepressants (Manji et al. 2009; Machado-Vieira et al. 2017; Goodwin 2015).

No mood stabilizer has ever been developed based on the known molecular pathophysiology of BD (Manji et al. 2009; Machado-Vieira et al. 2017; Goodwin 2015). To date, all FDA-approved mood stabilizers were either serendipitous discoveries or medicines initially developed for other clinical indications. These agents include lithium and several anticonvulsants, namely, valproate,

lamotrigine, and carbamazepine. Atypical antipsychotics have also been approved to treat mood episodes and for maintenance therapy (alone or in combination); these include aripiprazole, asenapine, cariprazine, lurasidone, olanzapine (+ fluoxetine), paliperidone, quetiapine, risperidone, and ziprasidone. More detailed information on these second-generation antipsychotics is provided in a different chapter of this series.

Mechanism of Action

With regard to the biological effects potentially related to their clinical efficacy in BD, mood stabilizers have been shown to reverse impaired cellular resilience and neuroplasticity pathways across biological systems in both clinical studies and preclinical models. Numerous studies exploring these effects have been conducted in humans – including in postmortem brain tissue, neurons, and other brain cells derived from human pluripotent stem cells – as well as in experimental animal models to explore the underlying pathophysiology of BD (Manji et al. 2009; Machado-Vieira et al. 2017; Goodwin 2015).

Despite a wide range of studies, neither the precise biological underpinnings of this disorder nor the mechanisms of action underlying the mood stabilizing effects of available agents are fully understood. Nevertheless, numerous direct targets of these agents have been identified. In this context, lithium has been and continues to be the gold standard and proof of concept agent used to study neurobiological targets and pathways related to the therapeutics of mood episodes in BD. Mood stabilizers in general target inflammation, neurotrophic factors, mitochondrial function, calcium metabolism, neurotransmitter systems, and HPA axis/stress pathways, all of which are factors closely implicated in BD (Teixeira et al. 2016). For example, lithium is known to inhibit: (1) inositol monophosphatase, (2) inositol polyphosphate 1-phosphatase, (3) glycogen synthase kinase-3 (GSK-3), (4) fructose 1,6-bisphosphatase, (5) bisphosphate nucleotidase, (6) phosphoglucomutase, and (7) neuronal calcium sensor-1 (NCN-1) (Manji et al. 2009; Teixeira et al. 2016). Another widely used mood stabilizer is valproate. This agent targets different proteins, enzymes, and neurotransmitters, including: (1) succinate semialdehyde dehydrogenase, (2) succinate semi-aldehyde reductase, (3) gamma aminobutyric acid (GABA) transaminase, and (4) histone deacetylase (HDAC) (Manji et al. 2009). With regard to other mood stabilizers, the known targets of carbamazepine include: sodium/calcium channels, adenosine receptors, and adenylate cyclase (Manji et al. 2009), and lamotrigine blocks voltage-sensitive sodium channels as well as L-, N-, and P-type calcium channels (Manji et al. 2009; Machado-Vieira and Zarate 2011).

Given the polygenic basis of BD and its complex neurobiological basis, it is likely that additional targets and systems that underlie mood stabilization will be identified. Indeed, the breadth of targets listed above underscores a major problem central to mechanistic research, namely the difficulty of attributing therapeutic relevance to any observed biological finding. One fruitful approach is to identify convergent mechanisms and targets in the brain produced by drugs that possess distinct chemical structures but belong to the same therapeutic class. As a relevant example, lithium is a monovalent cation and valproate is an eight-carbon branched fatty acid, but both are mood stabilizing agents that yield similar effects when administered in a "therapeutically relevant" paradigm (that is, the same plasma drug levels and treatment duration in human and experimental animals). This approach has led to the identification of several key signaling and downstream pathways regulated by different classes of mood stabilizers. These pathways include: (1) phosphoinositol/protein kinase C (PKC); (2) cyclic adenosine monophosphate (cAMP) and cAMP response element binding protein (CREB); (3) arachidonic acid (AA); (4) neurotrophic factor signaling (such as brain-derived neurotrophic factor (BDNF)) signaling; (5) glycogen synthase kinase 3 (GSK-3); and (6) Wnt signaling (Machado-Vieira et al. 2017; Manji et al. 2009). Through their effects on these pathways and downstream, mood stabilizers stabilize intracellular calcium homeostasis, protect and enhance mitochondrial function,

stimulate outgrowth, and regenerate neuronal processes (Machado-Vieira et al. 2017; Manji et al. 2009).

Overall, mood stabilizers target a wide range of biological systems in the limbic, striatal, and fronto-cortical neurotransmitter neuronal circuits that directly affect synaptic function, neurotransmitter activity, and related downstream targets at the cellular and molecular level. These targets and systems regulated by mood stabilizers play a key role in regulating mood states, cognition, and other functions such as autonomic and circadian systems (Machado-Vieira et al. 2017). Recent efforts to develop personalized medicine approaches in BD also hold considerable promise for identifying specific biomarkers that may help define the most effective therapeutic agent for each patient (Henter et al. 2017).

Clinical Use, Efficacy, and Side Effects

Maintenance therapy with mood stabilizers is recommended for all individuals with BD, preferably early in the course of the illness. Early intervention with mood stabilizers prevents the recurrence of new mood episodes, reduces the risk of suicide and violent behaviors, prevents substance abuse, and improves psychosocial functioning (Fazel et al. 2014).

With regard to choice of mood stabilizer, the ability to effectively treat different types of mood episodes is key. For instance, five mood stabilizers are recommended for the treatment of acute bipolar depression, alone or in combination with monotherapy: lamotrigine, lurasidone, lithium, quetiapine, and cariprazine (Grunze et al. 2018). In acute and long-term treatment of mixed states in BD, manic symptoms seem to respond to therapy with several atypical antipsychotics, with the best evidence supporting the use of olanzapine. Besides olanzapine and quetiapine, valproate and lithium should also be considered for preventing recurrences (Grunze et al. 2018).

During ongoing treatment, monitoring individuals with BD using standardized rating scales may identify outcomes such as suicidal ideation, self-harm behavior, and mood switches, as well as help identify nonresponders earlier and adjust pharmacotherapy. While monotherapy usually increases adherence and tolerability and also minimizes adverse effects, combination treatments are commonly required due to high rates of partial response as well as the emergence of undesirable side effects or comorbidities (Grunze et al. 2013; Post 2019). Below, we briefly review the most commonly prescribed mood stabilizers.

Lithium

Lithium is the best studied of the mood stabilizers and the only one whose initial FDA approval was specifically for BD (all other mood stabilizers were first tested as anticonvulsants or antipsychotics). Lithium is also the most studied and effective add-on therapy (at lower doses) for unipolar depression and is sometimes used in combination with one of the anticonvulsant mood stabilizers (e.g., valproate or lamotrigine) (Machado-Vieira 2018). Often referred to as the "classic" treatment for BD, lithium can be used alone or in combination with antipsychotics to treat mania, alone or in combination with antidepressants to treat bipolar depression, and as monotherapy for maintenance treatment (American Psychiatric Association 2002), where it has been shown to prevent relapse significantly better than placebo (Machado-Vieira 2018). Lithium has also been found to have significant anti-suicidal ideation/self-harm effects (Grunze et al. 2013). For adults with BD, a standard lithium serum level ranges from 0.60 to 0.75 mmol/L, with the option to reduce it to 0.40–0.60 mmol/L in case of good response but poor tolerance or to increase it to 0.80–1.00 mmol/L in case of insufficient response and good tolerance (Nolen et al. 2019). However, marked clinical improvement to lithium only appears after weeks of treatment (American Psychiatric Association 2002).

The side effects of lithium can include polyuria, polydipsia, weight gain, edema, gastrointestinal distress, hair loss, acne, benign leukocytosis, cognitive problems, tremor, sedation or lethargy, thyroid function alterations, and impaired coordination (American Psychiatric Association 2002). These side effects can typically be alleviated by changing lithium dose or treatment schedule. Most patients experience varied toxic effects with lithium levels above 1.5 meq/L, and levels above 2.0 meq/L are associated with life-threatening

side effects (American Psychiatric Association 2002). To minimize toxicity at higher lithium levels, hemodialysis may be needed. Lithium levels are regularly monitored after treatment has begun, as well as after every dosage change (American Psychiatric Association 2002).

Valproate

The anticonvulsant valproate is used alone or in combination with antipsychotics to treat bipolar mania as well as mixed episodes. When used as a maintenance treatment, valproate also significantly reduces the number of recurrent mood episodes in individuals with BD, especially depressive episodes (Grunze et al. 2013). Therapeutic serum levels are between 45 and 100 ug/ml (Grunze et al. 2013). As with lithium, significant clinical improvement only appears after weeks of treatment (American Psychiatric Association 2002).

Common side effects associated with valproate include gastrointestinal distress, benign hepatic transaminase elevations, osteoporosis, tremor, and sedation (American Psychiatric Association 2002). Some patients may also experience hair loss, increased appetite, and weight gain (Cipriani et al. 2013; American Psychiatric Association 2002). There is increased risk for hepatotoxicity in patients taking valproate with past or current hepatic disease. As with lithium, monitoring blood levels of valproate helps maximize efficacy and minimize side effects. Finally, valproate inhibits the metabolism of lamotrigine; therefore, lamotrigine must start from less than half of its usual dose when used in combination.

Lamotrigine

The anticonvulsant lamotrigine is typically used during depressive episodes and thus is often recommended for use as an initial or add-on treatment for bipolar depression (American Psychiatric Association 2002); it appears to be particularly effective in a subset of patients with bipolar depression (Prabhavalkar et al. 2015). However, it has shown no efficacy in treating acute mania (Osser 2019). Advantages to lamotrigine monotherapy include that the drug is superior to placebo in improving symptoms in both unipolar and bipolar depression, is generally well-tolerated, is not associated with weight gain, and is less likely to cause sedation (Osser 2019). For the treatment of bipolar depression, lamotrigine appears to be as effective as lithium, olanzapine+fluoxetine, citalopram, or inositol (Solmi et al. 2016). It has also been shown to be effective as a maintenance treatment, though its ability to reduce risk of relapse is modest compared to lithium and valproate (American Psychiatric Association 2002; Shelton and Bobo 2019).

Lamotrigine is usually well tolerated and has a benign metabolic profile. Common side effects include headache, nausea, and diarrhea. One key disadvantage to lamotrigine monotherapy, however, is that initial doses need to be titrated slowly in order to reduce the risk of life-threatening skin rashes. The starting dose is usually 25 mg once per day for 2 weeks; this is then increased to 25 mg twice per day (Shelton and Bobo 2019). Rashes occur in approximately 0.3% of adults and in 1% in children at any time during treatment but are more likely early in treatment (American Psychiatric Association 2002). Lamotrigine should be discontinued if rashes are accompanied by fever or sore throat, are diffuse and widespread, or have prominent facial and mucosal involvement (American Psychiatric Association 2002). Notably, case reports show that concomitant use of lamotrigine with valproate – which delays the clearance of lamotrigine (Zaccara and Perruca 2014) – increases the risk of rashes, including Stevens–Johnson syndrome and toxic epidermal necrolysis.

Quetiapine

An option for treating mania and for maintenance therapy in BD is the atypical antipsychotic quetiapine. Multiple randomized trials found that time to recurrence of mood symptoms was longer with quetiapine than lithium (Weisler et al. 2011). Furthermore, quetiapine is often used in combination with lithium or valproate as a first-line treatment and for successful maintenance therapy (Yatham et al. 2018). Quetiapine's efficacy in double-blind, randomized, controlled trials was the basis for its FDA approval in treating bipolar mania and bipolar depression, as well as its use as a maintenance treatment for BD and as either monotherapy or as an add-on treatment for major depressive disorder (Sanford 2011). As a result, quetiapine has become one of the most commonly

prescribed mood stabilizers in BD for all phases of the disorder (Plosker 2012) and is sometimes recommended if polarity is predominantly depressive or if there is no predominant polarity (Fountoulakis et al. 2017). Common adverse effects include constipation, dizziness, drowsiness, headache, sedation, increased serum cholesterol/triglycerides, increased thyroid stimulating hormone level, and weight gain.

Other Treatment Options

Many national and international guidelines suggest that lithium should continue to be the first-line choice for maintenance therapy in BD, and most recommend atypical antipsychotics to rapidly control severe manic symptoms and to treat psychotic symptoms (National Institute for Health and Care Excellence 2020; Goodwin et al. 2016). However, other treatment options for BD include the atypical antipsychotics aripiprazole, asenapine, cariprazine, lurasidone, olanzapine, paliperidone, risperidone, and ziprasidone, as well as the anticonvulsants carbamazepine and oxcarbazepine. The reader may wish to refer to treatment guidelines for more information regarding the circumstances under which any of these agents might ideally be prescribed (American Psychiatric Association 2002; Fountoulakis et al. 2017; Goodwin et al. 2016; Grunze et al. 2013, 2018; National Institute for Health and Care Excellence 2020; Yatham et al. 2018). In general, however, the clinical choice of prophylactic agents is considered on an individual basis rather than on explicit guidelines and can depend on efficacy and tolerability. For instance, aripiprazole, paliperidone, and risperidone are recommended for predominantly manic polarity, while aripiprazole plus a mood stabilizer might be recommended for frequent mixed episodes (Fountoulakis et al. 2017). Likewise, for depressive symptoms, adding ziprasidone to treatment as usual may provide additional benefits; however, the evidence is much more limited than for manic symptoms (Grunze et al. 2018).

Aripiprazole, which is also FDA-approved for the treatment of unipolar depression (Keck et al. 2007), has been shown to be an effective maintenance treatment for BD; it is also used adjunctively to treat bipolar mania. Long-acting injectable (depot) aripiprazole has increasingly been used but does not necessarily improve compliance. For BD, the FDA approved a new once-monthly, long-acting injectable form of aripiprazole for maintenance monotherapy treatment of BD-I in adults.

Similarly, several studies have shown that olanzapine is a more effective treatment than placebo for BD and prevents relapses more effectively than placebo. It is sometimes recommended if polarity is predominantly depressive or if there is no predominant polarity (Fountoulakis et al. 2017). Despite its robust results, it is typically considered a second-line therapy because of its metabolic effects (Yatham et al. 2018).

The atypical antipsychotic cariprazine has also been approved to treat BD, including mania with or without depressive symptoms as well as depression with or without manic symptoms. Specifically, post-hoc analyses of individuals with BD found that those with and without manic features improved following treatment with cariprazine (Stahl et al. 2020). Maintenance studies in BD are ongoing.

Based on long-term studies (Lindstrom et al. 2017), the long-acting injectable (depot) form of risperidone was FDA-approved for maintenance treatment in BD; however, no randomized trial has evaluated oral risperidone for this indication. Other atypical antipsychotic treatment options include ziprasidone, asenapine, and paliperidone, all of which are FDA-approved for maintenance therapy in BD.

Finally, the anticonvulsant carbamazepine and its analogue oxcarbazepine are also recommended for maintenance therapy in BD as alternatives to lithium and valproate (American Psychiatric Association 2002). The most common side effects associated with these agents include fatigue, nausea, diplopia, blurred vision, and ataxia. Skin rashes, mild leukopenia, mild liver enzyme elevations, mild thrombocytopenia, hyponatremia, and (less commonly) hypoosmolality can also occur. In addition, agranulocytosis, aplastic anemia, thrombocytopenia, hepatic failure, exfoliative dermatitis (e.g., Stevens–Johnson syndrome), and pancreatitis are rare and idiosyncratic but serious and potentially fatal side effects associated with their use. Oxcarbazepine has a superior side effect profile to carbamazepine.

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Morbus Alzheimer

▶ Alzheimer's Disease

MS

▶ Multiple Sclerosis

MSU

▶ Physiology and Treatment of Hyperuricemia and Gout

mTOR Signaling Pathways

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Synonyms

Mechanistic/mammalian target of rapamycin complex 1 signaling network

Definition

mTOR: A master regulator protein kinase hub which controls a variety of anabolic or catabolic cellular processes, such as protein synthesis, ribosomal biosynthesis, and autophagy, among others.

Basic Characteristics

*M*echanistic (or *m*ammalian) *t*arget *o*f *r*apamycin (mTOR) is a highly conserved Ser/Thr protein kinase that governs versatile cellular signaling networks to regulate a wide range of anabolic and catabolic processes. It plays central roles in maintaining normal cellular functions and, when dysregulated, in diverse diseases. mTOR forms two quite distinct types of multiprotein complexes (mTOR complexes 1 and 2). The complexes are activated by different signals and exert their physiological effects via the distinct downstream substrates which it phosphorylates (Xie and Proud 2014).

Both mTORC1 and mTORC2 contain the core component mTOR, plus mLST8 (*m*ammalian ortholog of *l*ethal with *s*ec *t*hirteen) and DEPTOR (*DEP* domain-containing m*TOR* interacting protein). The specific components of mTORC1 include the proteins RAPTOR (regulatory-associated protein of mTOR) and PRAS40 (*P*ro-*r*ich *A*kt *s*ubstrate of *40* kDa). mTORC2-specific components are RICTOR (rapamycin-insensitive companion of TOR), mSIN1 (*m*ammalian *s*tress-activated protein kinase *in*teracting protein 1) and PROTOR (protein observed with RICTOR) [reviewed in (Xie and Proud 2014; Xie et al. 2018), also see Fig. 1].

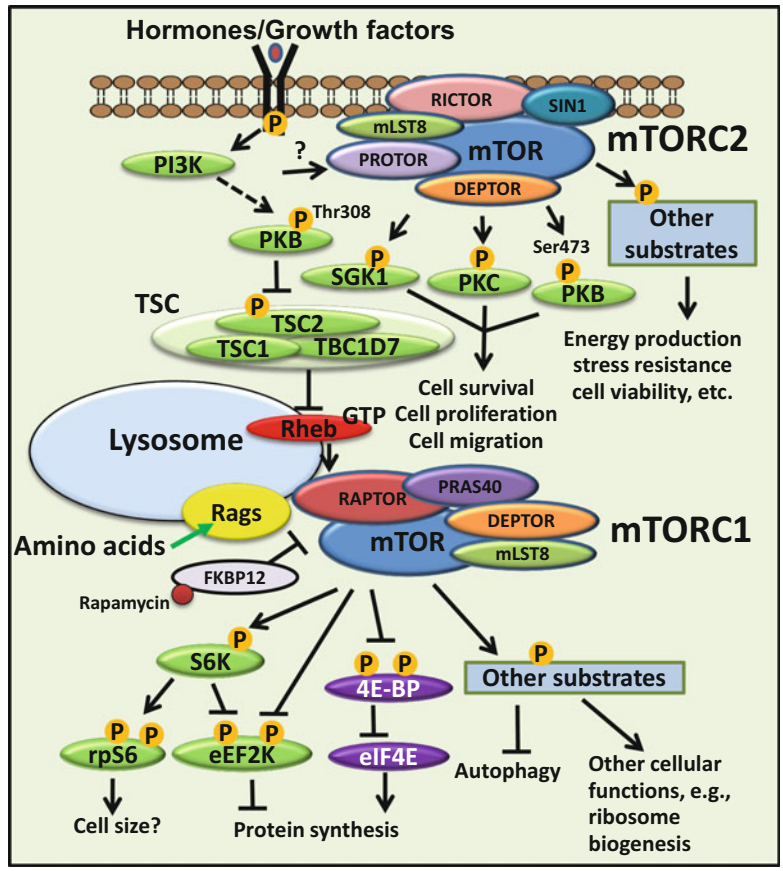
Upstream of mTORCs

mTORC1 indirectly senses a variety of environmental and other cues and integrates these signals to regulate a range of anabolic or catabolic processes. The small G-protein Rheb (*R*as *h*omolog *e*nriched in *b*rain) mediates the activation of mTORC1 by hormones and growth factors. When in its GTP-bound (Rheb-GTP) state, Rheb activates mTORC1. Rheb is regulated through the activity of its GTPase-activating protein TSC (*t*uberous *s*clerosis *c*omplex; comprising TSC1 or hamartin; TSC2 or tuberin, and TBC1D7), which negatively regulates mTORC1 by promoting the formation of inactive GDP-bound Rheb. Several upstream signaling pathways converge on TSC1/2 through downstream kinases that phosphorylate TSC1/2, thereby affecting its activity. Examples include the PI 3-kinase or PI3K (*p*hospho*i*nositide 3-*k*inase)/PKB (*p*rotein *k*inase *B*, also known as Akt) and classical MAP kinase pathways. These pathways are activated by many hormones and growth factors, and by phosphorylating and inactivating TSC1/2, bring about the activation of mTORC1 [reviewed in (Xie and Proud 2014)]. On the other hand, AMP-activated protein kinase (AMPK), which is activated when cellular energy [ATP] levels are low, phosphorylates TSC1/2 or other proteins to switch off mTORC1.

Lysosomes are the cellular sites of autophagy, a degradative process that, among other substrates, breaks down faulty or misfolded proteins thereby generating free amino acids. The surface of the lysosome is a major location where mTORC1 is activated; when phosphorylated via pathways that promote activation of mTORC1, TSC1/2 moves away from the lysosome, allowing Rheb to remain in its active GTP-bound state. Once it has been activated on the surface of the lysosome, mTORC1 is able to dissociate from the lysosome surface, remain active, and reach substrates at other locations in the cell (Fig. 1).

Certain amino acids are essential for the activation of mTORC1, which makes physiological sense since mTORC1 drives protein synthesis, which requires a constant supply of amino acids. Activation of mTORC1 by amino acids, including leucine and arginine, occurs on the surface of the lysosome, and involves the Rag GTPases, which are controlled by a rather complex set of proteins, including the Ragulator and GATOR multiprotein complexes [reviewed in Kim and Guan (2019)].

In contrast to mTORC1, the mechanism by which mTORC2 is regulated is much less well understood. The activity of mTORC2 towards one of its best-known substrates, PKB, is also acutely stimulated by insulin and serum, via PI 3-kinase. This is at least partially due to the fact that both PKB and mSIN1, an essential component of mTORC2, contain a PH (*P*leckstrin *H*omology) domain, that allows them to be recruited to the plasma membrane by interacting with phosphatidylinositol-3,4,5-trisphosphate (Xie et al. 2018), the product of Class I PI 3-kinases.



mTOR Signaling Pathways, Fig. 1 mTOR complexes and their substrate networks. Schematic summary representation of the pathways leading to mTOR activation, and of the main established targets and physiological processes specifically associated with each mTOR complex. The components of mTOR complex are shown. Rapamycin inhibits mTORC1 by binding to FKBP12. mTORC1 is activated by hormones and growth factors via the PKB-TSC pathway. mTORC2 phosphorylates PKB and PKC. Note that although mTORC2 is responsible for the phosphorylation of PKB (at Ser473), this does not contribute to the activation of mTORC1 (Frias et al. 2006; Jacinto et al. 2006). PKB phosphorylates and inhibits the TSC complex which acts as GAP for Rheb, while Rheb-GTP activates mTORC1 at the surface of lysosomes. Amino acids also activate mTORC1 by recruiting it to the surface of lysosomes via the Rag GTPases. S6K-rpS6 and 4E-BP1 are the best-characterized substrates for mTORC1; other substrates are responsible for controlling several anabolic effects driven by mTORC1 or for suppressing autophagy

Downstream of mTORCs

As protein kinases, mTORC1 and mTORC2 regulate cell function by phosphorylating a number of substrate proteins. They phosphorylate distinct substrates, because of the presence of different "substrate-targeting" subunits within mTORC1 and mTORC2, i.e., RAPTOR and RICTOR, respectively. In general, two main types of sites are phosphorylated by mTOR: some are followed by a proline residue, as in 4E-BP1 [eIF*4E* (*e*ukaryotic *i*nitiation *f*actor *4E*)-*b*inding *p*rotein 1, also referred to as PHAS-I], while others have hydrophobic residues at positions −4, −1, and +1 relative to the residue that is phosphorylated; such sites are typified by Thr389 in S6K1 (ribosomal protein *S6 k*inase *1*) or Ser473 in PKB/Akt.

RAPTOR, encoded by *Rptor*, is essential for the integrity of mTORC1 and is responsible for recruiting mTOR substrates, such as S6Ks and 4E-BPs, to mTOR. RAPTOR interacts with their TOS (*TO*R *s*ignaling) motifs, which comprise five amino acids including an N-terminal

phenylalanine (Schalm and Blenis 2002). Both S6Ks and 4E-BPs contain a TOS motif.

mTORC2 phosphorylated and promotes the activation of several members of the so-called "AGC" family of protein kinases, including PKB, SGK1 (*s*erum- and *g*lucocorticoid-induced protein *k*inase 1), conventional PKC (*p*rotein *k*inase *C*) isoforms (including α, βI, βII, and γ); ε, a novel PKC; and ζ, an atypical PKC (Fig. 1). Two types of phosphorylation sites ("turn" and "hydrophobic" motifs, both towards the C-termini of these enzymes) within these enzymes are targets for mTORC2 [reviewed in (Xie et al. 2018)]. These kinases play diverse roles in cellular regulation, placing mTORC2 at the center of a web of intracellular control processes.

Drugs

Rapamycin and "First Generation" mTOR Inhibitors

Given the role of mTORC1 in cell growth and proliferation, processes that are important in diseases such as cancers, there have been extensive efforts to develop compounds that inhibit mTORC1 function. Rapamycin was first isolated from the bacterium *Streptomyces hygroscopicus* found on Easter Island. The local name for this island is Rapa Nui, hence "rapamycin." Rapamycin was first studied as an antifungal agent and was later shown to block immune cell activation. It was subsequently found to impair the activity of mTORC1. Rapamycin forms a complex with the immunophilin protein, FKBP12 (*FK*506-*b*inding *p*rotein of *12* kDa), which interacts with mTOR through the FRB (*F*KBP12-*r*apamycin *b*inding) domain.

The binding of the rapamycin-FKBP12 complex to mTORC1 restricts access of substrates to its active site. Rapamycin inhibits the phosphorylation of some mTORC1 substrates, such as S6K, but not all of them (Thoreen and Sabatini 2009). In general, rapamycin prevents mTORC1 from phosphorylating "weak" substrates but not "strong" ones, although the sensitivity of different substrates to rapamycin seems to differ between cell types (Kang et al. 2013).

Rapamycin does not affect mTORC2 in the short term, as a consequence of occlusion of the FKBP12-binding site by RICTOR and mSIN1 (Chen et al. 2018). However, rapamycin can interfere with mTORC2 over longer time periods (after 24 h) in certain cell types, by preventing the formation of new mTORC2 complexes (as a consequence of the competition between rapamycin-FKBP12 and RAPTOR/Sin1 for binding to mTOR). Thus at least some of the long-term effects of rapamycin probably reflect inhibition of mTORC2.

The pharmacological properties of rapamycin itself are not ideal, leading to the development and application of rapamycin analogs (rapalogs, e.g., sirolimus, temsirolimus, everolimus; Table 1) with superior characteristics. Several such compounds have been developed and evaluated for their efficacy in treating diseases, including cancers (Table 1). These are semisynthetic rapamycin analogues which have typically been derivatized at the C-43 position on the cyclohexane outside the macrolide ring in order to improve aqueous solubility and permit oral administration. This also provides a more advantageous intellectual property position than for rapamycin itself. Notably, rapalogs have been successfully used in the clinic against acute renal allograft rejection and in preventing restenosis after angioplasty. Since 1999, rapalogs have also been approved by the FDA (U.S. Food and Drug Administration) against several types of cancer including renal cell carcinoma, tuberous sclerosis, pancreatic neuroendocrine tumors, and neurofibromatosis.

However, rapalogs are ineffective against other cancers, potentially for two major reasons. Firstly, rapalogs only partially inhibit mTORC1, and secondly, the inhibition of mTORCs triggers several feedback loops potentially (re)activating upstream oncogenic signaling pathways such as PI 3-kinase (Xie and Proud 2013, 2014). Furthermore, inhibition of mTORC1 promotes autophagy, which can aid the survival of cells within poorly vascularized solid tumors.

In addition, and probably not surprisingly given the key role of mTORC1 in cell physiology, rapalogs do exert a number of adverse "side-effects," as do mTOR kinase inhibitors. In the

mTOR Signaling Pathways, Table 1 Examples of inhibitors to the mTOR pathway and their effects on human diseases

Generation	Compound name	Approved year/ current phase	Developer	Examples of indications in completed clinical trials and other features
1st	Rapamycin (sirolimus)	1999	Wyeth Ayerst	Acute renal allograft rejection/ restenosis
1st	RAD001 (everolimus)	2003 – 2011	Novartis	Allograft rejection/advanced kidney cancer/ tuberous sclerosis/ RCC/pNET/neurofibromatosis
1st	CCI-779 (temsirolimus)	2007 – 2008	Wyeth Ayerst/Pfizer	RCC/mantle cell lymphoma
DI	NVP-BEZ235 (dactolisib)	Phase I/II	Novartis	MBC/pNET
DI	GSK2126458	Phase I/II	GlaxoSmithKline	Advanced solid tumors, lymphoma
DI	XL765	Phase I/II	Sanofi-Aventis, Exelixis	Glioblastoma multiforme/ NSCLC/MBC
2nd	AZD8055	Phase I/II	AstraZeneca	Advanced solid tumors/glioma/ HCC
2nd	INK128/ MLN0128	Phase I/II	Intellikine	Advanced solid tumors/multiple myeloma/Waldenstrom macroglobulinemia
2nd	OSI027	Phase I/II	OSI pharmaceuticals	Advanced solid tumors/ lymphoma
2nd	Compound 7c	Developed in 2021	Xu T *et al.*	Induce autophagic death and apoptosis in breast cancer cell lines
3rd	RapaLink	Developed in 2016	Rodrik-Outmezguine VS *et al.*	Tested in rapamycin- and AZD8055-resistant cell lines and mouse xenografts
mTORC1-specific	DL001	Developed in 2019	Schreiber KH *et al.*	Highly selective to mTORC1 *in vivo*
mTORC1-specific	NV-5440	Developed in 2019	Kang SA *et al.*	Selectively inhibits mTORC1 via modulation of GLUT1 transporters
Rheb inhibitor	NR1	Developed in 2018	Mahoney SJ *et al.*	Selective to mTORC1, does not affect mTORC2 even upon prolonged treatment
mTORC2-specific	CID613034	Developed in 2017	Benavides-Serrato A *et al.*	Selective to mTORC2, does not affect the phosphorylation of mTORC1 substrates

Adapted from (Xie et al., 2016)
CRPC = castration-resistant prostate cancer; *DI* = dual mTOR/PI3K inhibitors; *HCC* = hepatocellular carcinoma; *MBC* = metastatic breast cancer; *NSCLC* = non-small cell lung cancer; *pNET* = pancreatic neuroendocrine tumors; *RCC* = advanced renal cell carcinoma

case of treating restenosis, the use of drug-eluting stents ensures local rather than systemic delivery of the rapalog, thus reducing unwanted effects.

Dual PI3K/mTOR Inhibitors

The kinase domain of mTOR (Fig. 1) is widely conserved in a group of protein kinases which display prominent structural similarities to PI 3-kinase-related kinases. As a result, it has been discovered that several PI 3-kinase inhibitors (including derivatives of the early inhibitors LY294002 and wortmannin) developed during drug discovery programs can also effectively suppress the activation of both mTOR

complexes. These are consequently classified as dual PI 3-kinase/mTOR inhibitors (Table 1). Unlike the rapalogs, this class of catalytic site-directed inhibitors blocks the activities of both mTOR complexes. Also, as anticancer agents, they offer superior benefits in comparison to the first class of mTOR inhibitors, because of inhibiting simultaneously both PI3K and mTOR, two crucial signaling hubs that promote cancer cell growth. Arguably the best successful example in clinical trials from this class of inhibitors is the imidazoquinoline derivative NVP-BEZ235 (Maira et al. 2008), developed by Novartis. NVP-BEZ235 exerts potent anti-tumor activity in vivo, and its effect can be further enhanced by combining it with inhibitors of other mitogenic pathways.

Second-Generation mTOR Inhibitors

Given the inability of rapamycin to affect all functions of mTORC1, and its frequent inefficacy in anticancer therapy, several academic and pharmaceutical laboratories have developed compounds that inhibit the catalytic activity of mTOR itself (which include the compounds described in the previous section). This yielded a second generation of mTOR inhibitors which are designed to act as ATP-competitive inhibitors of mTOR. This means they can potentially inhibit all phosphorylation events catalyzed by mTORC1 but will also affect mTORC2. These inhibitors (Table 1) exhibit a much lower IC_{50} against mTORCs than against PI3K.

The first such compound to be reported was PP242. INK128 (later renamed as MLN0128) is a PP242-derivative developed by Intellikine and has or is being tested in 42 clinical trials according to clinicaltrials.gov (searched on 19/20/20). Torin 1 and Torin 2 were synthesized by Nathanael Gray's lab and developed by AstraZeneca. Ku-0063794 and Ku-0068650, developed by KuDOS Pharmaceuticals (now AstraZeneca), are also examples of early ATP-competitive mTOR inhibitors which exhibit strong antiproliferative potential against cancer cells in vitro. AZD8055 and AZD2014 are two orally bioavailable compounds derived from the Ku compounds.

Third Generation of mTOR Inhibitors

The efficacy of mTOR inhibitors can be severely compromised by a wide range of clinical-relevant mutations in mTOR that can increase in catalytic activity of mTOR. To try to tackle this issue, careful analysis of the molecular model of mTOR revealed a juxtaposition of the rapamycin and AZD8055 binding sites, prompting to creation of a powerful bivalent mTOR inhibitor, named as RapaLink (1 and 2; Table 1, (Rodrik-Outmezguine et al. 2016)). RapaLink contains structural elements of both rapamycin and an mTOR kinase inhibitor within the same molecule, connected by a cunningly designed non-perturbing, strain-free cross-linker with optimum length, which allows the compound to interact with the FRB domain of mTOR through binding to FKBP12, as well as reaching the kinase domain of mTOR so that it can act as an ATP-competitive inhibitor at the same time (Rodrik-Outmezguine et al. 2016). RapaLink prevented growth of breast cancer xenografts bearing rapamycin and/or AZD8055-resistant mTOR mutations (Rodrik-Outmezguine et al. 2016). This landmark study gave thus birth to a new generation of mTOR inhibitors.

mTOR Complex-Specific Inhibitors

Most of abovementioned mTOR inhibitors unavoidably inhibit both mTOR complexes, at least in some types of cells (in the case of rapalogs). As noted, this would promote a number of feedback loops that (re)activate upstream signaling pathways (Xie and Proud 2013) and which may result in undesirable secondary effects. There have therefore been attempts to develop mTORC1/2-specific inhibitors that only affect one, but not both, mTOR complexes (Table 1).

In 2016, a small molecule called NR1 was discovered, which specifically targets Rheb (Mahoney et al. 2018). In particular, NR1 bound to the switch II domain of Rheb, which is crucial for binding to, and hence activating, mTORC1.

The following year, Gera's group discovered a compound named CID613034 which interferes with the mTOR-RICTOR interaction. It therefore selectively inhibits mTORC2 activity in glioblastoma xenografts while leaving mTORC1 activity intact (Benavides-Serrato et al. 2017). Dudley Lamming's group reported a rapalog named DL001 which is highly specific for mTORC1 (40 times more selective for mTORC1 than rapamycin) (Schreiber et al. 2019). DL001 exhibited remarkably reduced side effects in mice relative to rapamycin, i.e., it did not affect, or had smaller effects, on lipid metabolism, glucose homeostasis, and the immune system. Nevertheless, it is yet to be determined whether NR1, DL001, or CID613034 represent safer and more effective clinical options compared to other aforementioned mTOR inhibitors.

Given the manifold roles of mTOR signaling in human diseases, one can expect further intensive research will identify, develop, and test novel inhibitors of the mTOR complexes and/or their downstream effectors.

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Multidrug-Resistant

▶ Peptides and Peptidomimetics as Foundations for Drug Discovery

Multiple Sclerosis

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Synonyms

MS

Definition

Multiple sclerosis (MS) is the most frequent chronic neuroinflammatory disorder of the central nervous system and a cause of severe disability in adults. Globally, MS affects about two million people and two to three times more females than males. Its prevalence varies markedly between geographic regions with an overall tendency to increase with latitude. A hallmark of the disease is the dissemination of inflammatory lesions, both in time and space. In most patients (about 85%), the disease follows a relapsing-remitting course. Typical symptoms of attacks include visual disturbance of one eye (optic neuritis), double vision, ataxia, sensory disturbance (tingling, numbness), paresis, and sphincter dysfunction. First, symptoms typically remit after weeks to months. Over time, some residual disability remains after attacks and the disease follows a secondary progressive course. In the primary progressive variant of the disease affecting about 15% of patients, the disability slowly deteriorates from the beginning. Eventually, many MS patients suffer from impaired mobility or cognition. However, the clinical course of MS is highly variable, and a considerable proportion of patients do not experience severe disability.

Diagnosis relies on evidence for the spatial and temporal dissemination of the inflammatory process. As magnetic resonance imaging (MRI) provides a sensitive technique to detect acute and chronic lesions, including asymptomatic ones, it has become a cornerstone for diagnosis and follow-up studies. With the help of MRI, it is nowadays possible to diagnose MS after the first attack. Typically, oligoclonal IgG bands are found in CSF but not in blood. Other blood tests are normal in MS.

Basic Mechanisms

The cause of MS is still unknown. There is a polygenetic component as shown by a concordance rate in monozygtic twins of about 25% (Baecher-Allan et al. 2018). Genome-wide association studies have identified numerous loci that overlap with other autoimmune diseases (Farh et al. 2015). The genetic evidence points toward immune mechanisms, involving T and B lymphocytes, NK cells, and microglia, but provides little information on the neural response to the immune attack (International Multiple Sclerosis Genetics Consortium 2019). In addition to genetics, environmental factors influence the disease risk as illustrated by the observation that migrants acquire the risk of their new home after a while. Specific factors include UV irradiation, vitamin D supply, and Epstein-Barr virus infections.

Lesions often surround blood vessels and are characterized by inflammatory cell infiltrates, demyelination, axonal loss, and glial activation. In acute lesions, the blood-brain barrier is disrupted as shown by the contrast enhancement in MRI. With ongoing disease, atrophy of the cortex, optic nerve, spinal cord, or other parts of the CNS becomes evident. Detailed neuropathological investigations have defined subtypes of lesions that differ in respect to immunoglobulin and complement deposition as well as oligodendrocyte apoptosis (Reich et al. 2018). Interestingly, individual patients tend to have lesions of the same subtype, but, because of a lack of clinical biomarkers, this information does not yet influence therapeutic decisions.

According to current concepts, autoreactive T cells migrate into the CNS. Upon reactivation by antigen-presenting cells, they produce various cytokines that attract additional T cells and macrophages. Essential are $CD4^+$ Th1 and Th17 cells releasing IFNγ and IL-17. In addition to $CD4^+$ helper T cells, human MS lesions contain numerous $CD8^+$ effector T cells that also release IFNγ

and IL-17 and may induce death of oligodendrocytes and axonal damage. The suppressive capacity of regulatory Treg and Tr1 cells or $CD56^{bright}$ NK cells seems to be impaired in MS patients.

Recent studies highlighted the importance of B cells, especially $CD20^{+}$ memory B cells, in MS (Baecher-Allan et al. 2018). In addition to their role in autoantibody production, they present antigens to T cells and release inflammatory factors. The cellular network activated in MS encompasses myeloid cells, such as neutrophils (Liu et al. 2010). Monocyte-derived and dendritic cells present antigens to lymphocytes, while microglia seem to synthesize inflammatory factors and remove debris (Jordao et al. 2019).

The animal model experimental autoimmune encephalomyelitis (EAE) has greatly influenced understanding of the basic mechanisms and drug development of MS. Although this disease model deviates from the human disease in several aspects (Lassmann et al. 2012), it has allowed the discovery of several clinically effective drugs, such as glatiramer acetate and natalizumab.

Pharmacological Intervention/ Pharmacotherapy

Since the early 1990s, numerous drugs that ameliorate the course of relapsing-remitting MS have been introduced in routine clinical practice. Therapeutic options for primary or secondary progressive MS are more restricted. The following drugs allow for a disease-modifying therapy (DMT) but do not cure MS.

Parenteral Drugs

Interferon β (IFNβ)-1a and IFNβ-1b were the first drugs in the 1990s that became available as DMTs. The two variants of human IFNβ differ slightly in the amino acid sequence, in the pharmaceutical production mode, and consequentially in their glycosylation. IFNβ-1a is administered i.m. or s.c. and IFNβ-1b s.c. Being the standard of care for long time, they served as active comparator against which newer drugs were tested in trials. These studies confirmed that IFNβs are less effective in reducing the relapse rate than ocrelizumab, fingolimod, or alemtuzumab.

As a type-I interferon, IFNβ binds to a hetero- or homodimeric membrane receptor consisting of the subunits IFNAR1 and IFNAR2 that are present on virtually all cell types. IFNβ effects are mediated by several intracellular pathways that include tyrosine kinases JAK1 and TYK2, the transcription factors STAT1 and STAT2 (Chow and Gale 2015), the small G protein RAC1, and the inflammasome. Subsequently, it stimulates a large set of genes, so-called INFβ-stimulated genes (ISGs). Immunomodulation in EAE and multiple sclerosis involves several mechanisms. One of the ISGs is CD69 that inhibits S1P1 and the egress of lymphocytes from lymph nodes, mimicking the effect of pharmacological S1P inhibitors (Shiow et al. 2006). In patients with MS, IFNβ treatment elevates plasma levels of soluble VCAM-1 that may interfere with the penetration of immune cells across the blood-brain barrier (Calabresi et al. 1997). Moreover, IFNβ inhibits Th1 and promotes Th2 polarization; it counteracts the disruption of the blood-brain barrier in EAE and downregulates the induction of MHC II molecules. Which of these effects are essential for the disease-modifying activity in patients is still unclear. In experimental EAE, myeloid cells mediate the protective effect of endogenous IFNβ (Prinz et al. 2008). In some patients, IFNβ is inactive. This may be due to the formation of neutralizing antibodies (Rommer et al. 2019) or to a form of MS that is resistant to IFNβ treatment (Inoue et al. 2016).

Glatiramer acetate was discovered during attempts to induce EAE in animals with copolymers of amino acids that frequently occur in myelin basic protein. In contrast to expectations, Cop-1, one of the copolymer preparations, reduced the incidence and severity of EAE (Arnon and Aharoni 2019). It turned out that glatiramer acetate binds to various MHC II molecules on antigen-presenting cells and modulates the response of dendritic cells, monocytes/macrophages, and the polarization of B and T lymphocytes into an anti-inflammatory and neuroprotective direction. Glatiramer acetate is effective against relapsing-remitting multiple sclerosis. Its safety

profile is favorable and it is the only DMT that is licensed during pregnancy.

Natalizumab is a humanized antibody against integrin α4 on leukocytes. By inhibiting the interaction of α4 integrin with the endothelial adhesion factor VCAM-1, natalizumab restricts the access of T lymphocytes and other immune cells to the brain. This mechanism translates into a high efficacy against relapsing-remitting MS. However, its widespread use is limited by its safety profile. Natalizumab is responsible for progressive multifocal leukoencephalopathy in up to 1 out of 1,000 patients treated with the antibody (Major et al. 2018). This potentially lethal demyelinating disease in the brain is caused by an insufficient immune control of a JC virus infection. In patients receiving natalizumab, the risk and the prognosis of the infection can be mitigated by monitoring anti-JC virus titers and MRI.

Alemtuzumab is a humanized antibody targeted against CD52, a surface protein on T and B lymphocytes, monocytes, and eosinophiles. The drug is administered in a 3- to 5-day course once a year leading to the depletion of circulating T and B cells and monocytes. Despite a subsequent repopulation, lymphocyte counts remain low for a considerable time. Moreover, the nadir is followed by a change in lymphocyte subpopulations, including an increase in Treg cells. Alemtuzumab is more effective than INFβ-1 in reducing relapses and other activity markers of MS. However, its use is limited by a considerable rate of autoimmune diseases, such as Graves' disease or idiopathic thrombocytopenic purpura (Katsavos and Coles 2018).

Ocrelizumab is a humanized antibody targeting CD20 on B cells. It depletes B cells, both immature and mature, but not CD20-negative plasma cells or hematopoietic stem cells by complement-dependent cytotoxicity and antibody-mediated cellular cytotoxicity (Sellebjerg et al. 2020). Depletion is most efficient in blood but less so in lymphoid tissues or the brain. Ocrelizumab is effective in relapsing-remitting MS, and, importantly, ocrelizumab is the only drug that has been approved for primary progressive MS following the placebo-controlled phase III trial ORATORIO (Montalban et al. 2017).

Mitoxantrone was discovered as an analogue of the antibiotic doxorubicin with enhanced cytotoxic effects and less cardiotoxicity. Like the parent compound, mitoxantrone intercalates into DNA and inhibits topoisomerase II. Currently, mitoxantrone is used for the pharmacotherapy of acute myeloid leukemia, prostate cancer, and for MS therapy. The death of lymphocytes explains its immunosuppressive effects. Beyond cytotoxicity, immunomodulatory effects of mitoxantrone have been reported, such as inhibiting the migration of mononuclear cells (Kopadze et al. 2006). Although cardiotoxicity is less than with doxorubicin, it limits the application of mitoxantrone to a fixed cumulative lifetime dose. Mitoxantrone was the first drug to be approved for secondary progressive MS. However, with the advent of other effective drugs, it has become second choice.

Oral Drugs

Dimethyl fumarate is a prodrug that is converted into monomethyl fumarate in the gut after oral ingestion. The latter is the active compound and has three molecular targets that may potentially explain its clinical activity. It is an agonist of the G protein-coupled membrane receptor HCA2 on neutrophils, monocytes/macrophages, and microglia. By activating HCA2, monomethyl fumarate inhibits the migration and modulates the function of myeloid cells and microglia. HCA2 is required for the protective effect of dimethyl fumarate in EAE (Chen et al. 2014). In addition, monomethyl fumarate is able to react with cysteine residues in GAPDH and KEAP1, thereby inhibiting glycolysis and activating the anti-oxidative transcription factor NRF2 (Kornberg et al. 2018; Linker et al. 2011). Both mechanisms are able to modulate immunity. Dimethyl fumarate is used for the treatment of relapsing-remitting MS. Adverse effects include transient gastrointestinal effects and flushing. Rarely, it causes progressive multifocal leukoencephalopathy.

S1P receptor modulators antagonize the effects of sphingosine-1-phosphate (S1P) receptors. S1P receptors consist of five subtype (S1P1–S1P5) and are expressed on lymphocytes, on neural cells, and on atrial myocytes. As a prodrug,

fingolimod is converted to fingolimod-phosphate that binds to S1P1, S1P3, S1P4, and S1P5 receptors. It stimulates the internalization and degradation of S1P1. S1P1 mediates a key signal for T and B lymphocytes to egress from thymus and lymph nodes. Blocking the S1P phosphate effect interferes with the migration of lymphocytes to the CNS. As a side effect, fingolimod lowers heart rate and can cause atrioventricular block. Siponimod is a more selective modulator of S1P1 and S1P5 receptors, but it also transiently induces bradycardia (Gergely et al. 2012). S1P receptor modulators are highly efficacious in MS. Fingolimod has market authorization in relapsing-remitting MS and siponimod in the relapsing-remitting and secondary progressive phase of the disease.

Teriflunomide is the active metabolite of leflunomide, a drug that is approved for the treatment of rheumatoid arthritis. Teriflunomide reversibly inhibits dihydro-orotate dehydrogenase, a key mitochondrial enzyme for pyrimidine synthesis. Pyrimidines are required for the rapid proliferation of T and B lymphocytes. Notably, inhibition of dihydro-orotate dehydrogenase by teriflunomide specifically targets the differentiation and polarization of Th1 cells, apparently by interfering with oxidative phosphorylation and glycolysis (Klotz et al. 2019). In relapsing-remitting MS, teriflunomide is equally effective as IFNβ-1. The pharmacokinetic profile of teriflunomide is characterized by its long elimination half-life (about 19 days).

Cladribine is a purine analogue that enters cells by nucleoside transporters. The rate of phosphorylation to cladribine-triphosphate determines the cellular sensitivity to the drug. Cladribine-triphosphate is incorporated into DNA during replication or repair and inhibits cell proliferation and gene expression. It is able to induce apoptosis (Jacobs et al. 2018). These mechanisms lead to a depletion of both B and T lymphocyte as well as innate immune cells including NK cells and monocytes. Beyond depletion, cladribine inhibits chemokine release and chemotaxis. Cladribine is effective in relapsing-remitting MS. It is teratogenic and possibly increases the malignancy rate.

Future Directions

In the last decades, efforts to prevent attacks in relapsing-remitting MS have been successful. There is a now an impressive therapeutic armamentarium to treat this phase of MS. Future research will deal with the questions which drug (s) to use first and how to escalate therapy if the disease progresses. To answer these questions, controlled clinical trials that compare the long-term effect of different DMTs or of different treatment strategies are required. Such studies are currently underway (Ontaneda et al. 2019). Even more difficult will be attempts to reverse disability by enhancing remyelination or halting axonal loss. Existing therapies target the immune system and not the neural response to the immune challenge. However, there are promising approaches at the preclinical level, and clinical trials have started investigating remyelinating and neuroprotective principles (Faissner et al. 2019).

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Muscarinic Acetylcholine Receptors

▶ Muscarinic Receptors

Muscarinic Receptors

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Synonyms

Muscarinic acetylcholine receptors

Definition

Muscarinic acetylcholine receptors (mAChRs) form a class of cell surface receptors that are activated upon binding of the neurotransmitter, acetylcholine. Structurally and functionally, mAChRs are prototypical members of the superfamily of G protein-coupled receptors. Following acetylcholine binding, the activated mAChRs interact with distinct classes of heterotrimeric G proteins, resulting in the activation or inhibition of distinct downstream signaling cascades.

Basic Characteristics

The neurotransmitter acetylcholine (ACh) exerts its diverse pharmacological actions via binding to and subsequent activation of two general classes of cell surface receptors, the nicotinic and the mAChRs. These two classes of ACh receptors have distinct structural and functional properties. The *nicotinic receptors*, which can be selectively activated by the alkaloid nicotine, represent ACh-gated ion channels. In contrast, the mAChRs, which can be selectively activated by the alkaloid muscarine, are members of the *G protein-coupled receptor* (GPCR) superfamily.

General Structural Features

Molecular cloning studies have revealed the existence of five molecularly distinct mammalian mAChRs (M_1–M_5; Table 1). All five receptor subtypes are members of the so-called class A GPCR subfamily (rhodopsin-like receptors) with which they share about 20 highly conserved amino acids (Wess 1996). These highly conserved residues play important roles in proper receptor folding and receptor activation (Wess 1996). The structural hallmark of mAChRs (and GPCRs in general) is the presence of seven α-helically arranged transmembrane domains (TM1–7) which form a tightly packed transmembrane core. The N-terminal portion of the receptor protein is located extracellularly, whereas the C-terminal segment protrudes into the cytoplasm.

During the past decade, high-resolution structures have been obtained for four of the five mAChR subtypes (M_1–M_4) (Haga et al. 2012; Kruse et al. 2012, 2013; Thal et al. 2016). These structures show that the classical (*orthosteric*) binding site for muscarinic agonists and antagonists is highly conserved among the different mAChR subtypes (Kruse et al. 2014; Thal et al. 2016).

Distribution and Physiological Functions of mAChRs

mAChRs are found in virtually all organs, tissues, and cell types. All five mAChRs are expressed in both the central nervous system (CNS) and the body periphery. Whereas the M_2 and M_3 receptor subtypes are the predominant mAChRs found in

Muscarinic Receptors, Table 1 Summary of key features of the five human mAChRs (M_1–M_5)

Receptor subtype	M_1	M_2	M_3	M_4	M_5
Amino acids (number)	460	466	590	479	532
Chromosomal localization	11q12	7q35–36	1q43–44	11p12–11.2	15q26
GenBank/EMBL accession number	X15263	X15264	X15266	X15265	M80333
		M16404		M16405	
G protein-coupling selectivity	$G_{q/11}$	$G_{i/o}$	$G_{q/11}$	$G_{i/o}$	$G_{q/11}$
Subtype-preferring antagonists (examples)[a]	VU0255035	Tripitramine	Darifenacin	PD102807	ML381
	MT7[b]		MT3[b]		

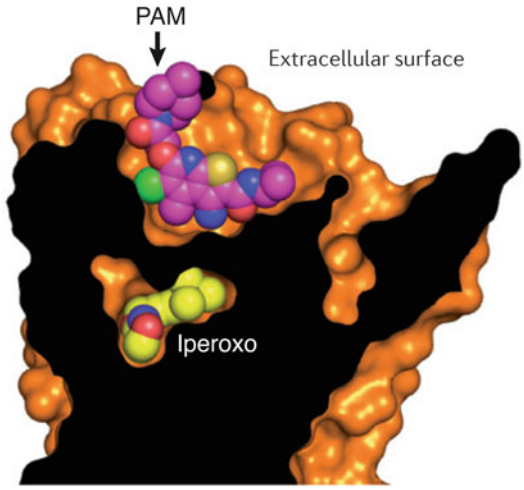
[a]Except for certain snake toxins, such as MT3 or MT7, the degree of subtype preference of so-called "subtype-selective" muscarinic antagonists is usually rather limited (for more details, see https://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=2)
[b]MT3 and MT7 are toxins isolated from the venom of the green mamba and act as allosteric inhibitors

peripheral tissues, the M_1 and M_4 mAChRs are abundantly expressed in the CNS, specifically in higher brain regions. The M_5 mAChR is expressed at rather low levels in various central and peripheral tissues. Characteristically, most tissues or organs express multiple mAChR subtypes. Peripheral mAChRs are known to mediate the well-documented actions of ACh at *parasympathetically* innervated effector tissues (organs) including the heart, endocrine and exocrine glands, and smooth muscle tissues (Caulfield and Birdsall 1998; Wess 2004). The most prominent peripheral actions mediated by activation of these receptors are reduced heart rate and cardiac contractility, contraction of smooth muscle tissues (e.g., smooth muscles of the eye, gastrointestinal system, lung, and urinary bladder), and stimulation of glandular secretion (e.g., lachrymal, salivary, and gastrointestinal glands). Whereas the cardiac muscarinic actions of ACh are mediated by M_2 receptors, the M_3 receptor subtype plays a major role in mediating ACh-dependent stimulation of glandular secretion and smooth muscle contraction (Caulfield and Birdsall 1998; Wess 2004).

Central mAChRs are involved in modulating a very large number of behavioral, autonomic, sensory, and motor functions. For example, central muscarinic mechanisms play important roles in the control of body temperature, cardiovascular and pulmonary functions, learning and memory, emotional responses, arousal, attention, rapid eye movement (REM) sleep, and stress modulation. Moreover, increased or decreased muscarinic cholinergic neurotransmission has been implicated in the pathophysiology of several important disorders of the brain, including *Alzheimer's* and *Parkinson's disease*, depression, schizophrenia, and epilepsy (Kruse et al. 2014; Wess et al. 2007). The roles of the individual mAChRs in mediating the diverse central muscarinic functions of ACh are not well understood at present, primarily due to the lack of muscarinic ligands endowed with a high degree of receptor subtype selectivity (see below). However, recent studies analyzing newly developed mutant mouse strains deficient in specific mAChR subtypes have led to many new insights into the physiological roles of the M_1–M_5 receptors (Kruse et al. 2014; Wess et al. 2007).

Ligands and Mechanisms Involved in Ligand Binding and mAChR Activation

ACh is predicted to bind to the M_1–M_5 receptors within a cleft enclosed by the ringlike arrangement of TM1–7, about 10–15 Å away from the membrane surface (Fig. 1) (Kruse et al. 2014). This orthosteric muscarinic binding site consists of a cavity lined by amino acid chains located on several of the seven transmembrane (TM) helices (TM1–7) (Kruse et al. 2014; Thal et al. 2016). A key ligand/receptor interaction involves a charge-charge interaction between the tertiary or quaternary nitrogen of the orthosteric muscarinic



Muscarinic Receptors, Fig. 1 Agonist-induced conformational changes on the cytoplasmic surface of the M_2 mAChR. After agonist binding to the M_2 mAChR, the cytoplasmic end of TM6 rotates outward in the active M_2 receptor structure (orange), relative to the inactive state of the receptor (blue). This conformational change allows the activated receptor to bind to and activate heterotrimeric G proteins. (This figure has been modified based on recently published images Kruse et al. 2013)

ligands (agonists and antagonists) and a conserved TM3 aspartic acid chain (Kruse et al. 2014). This ion pair is surrounded by a cluster of tyrosine residues, thus creating a charge-stabilized aromatic cage. A TM6 asparagine residue that is characteristic for the mAChR family is also important for the binding of orthosteric muscarinic ligands (Kruse et al. 2014). Structural studies with the M_2 mAChR have shown that agonist binding leads to a contraction of the ligand-binding pocket, an effect that is not observed with muscarinic antagonists which are usually larger in size (Kruse et al. 2013; Thal et al. 2016).

The agonist binding pocket partially overlaps with that of competitive muscarinic antagonists such as tiotropium or quinuclidinyl benzilate (Kruse et al. 2014; Thal et al. 2016). However, antagonists form additional interactions with hydrophobic receptor residues, thus stabilizing the inactive state of the receptor (Kruse et al. 2014; Thal et al. 2016).

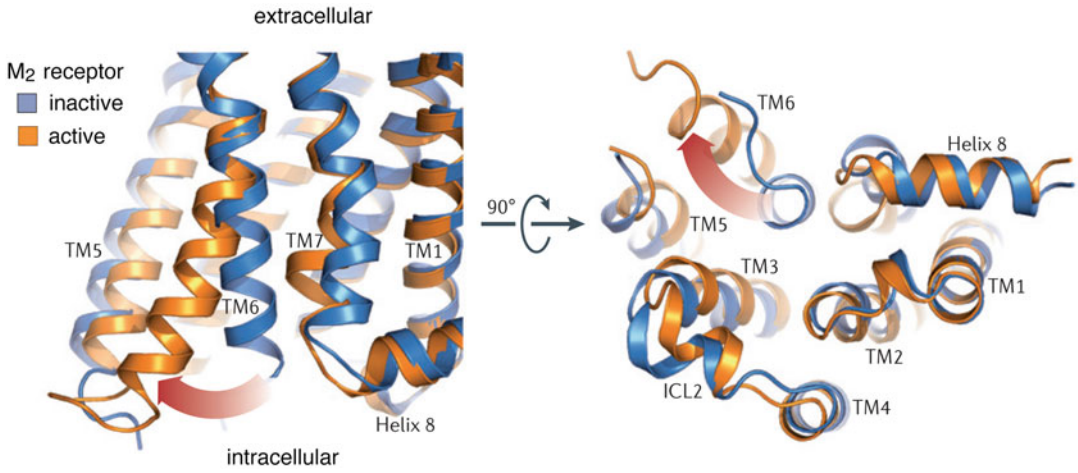
The amino acids lining the ligand-binding cavity are highly conserved among the M_1–M_5 mAChRs (Kruse et al. 2014; Thal et al. 2016). For this reason, the development of agonist or antagonist ligands that are able to interact with individual mAChR subtypes with a high degree of selectivity has proven to be a very difficult task. Except for so-called allosteric activators of M_1 mAChRs (see below), agonists that display a high degree of selectivity for a particular mAChR subtype are not available. Moreover, the degree of receptor subtype selectivity of so-called "selective" muscarinic antagonists, which are often used to distinguish between different mAChR subtypes, is generally rather modest. Representative mAChR subtype-preferring muscarinic antagonists are listed in Table 1.

However, several snake toxins have been identified that display an unprecedented degree of mAChR subtype selectivity. For example, the MT7 and MT3 toxins (Table 1) are highly selective antagonists for M_1 and M_4 mAChRs, respectively (Caulfield and Birdsall 1998). The binding of these polypeptide ligands is predicted to involve interactions with less well-conserved amino acids present on the extracellular surface of the mAChRs.

Structural studies with the M_1 and M_2 mAChRs have shown that agonist binding induces changes in the arrangement of individual TM helices that are transmitted to the intracellular surface of the mAChR proteins, enabling the receptors to productively interact with specific classes of *heterotrimeric G proteins* (Kruse et al. 2013; Maeda et al. 2019). The key feature of the active-state mAChRs is an outward rotation of TM6 that creates a G protein-binding cavity on the cytoplasmic receptor surface (Fig. 2). The active-state structures of the M_1 and M_2 receptors are similar to those of most other GPCRs for which high-resolution structural information is available.

Allosteric Modulators of mAChR Function

mAChRs possess one or more topographically distinct allosteric binding sites formed by amino acid side chains located within the extracellular loops or the outer segments of different TM



Muscarinic Receptors, Fig. 2 Ligand binding sites on the M_2 mAChR. This figure shows that the orthosteric muscarinic agonist (iperoxo) binds to a cavity within the transmembrane receptor core (black). Shown in magenta is a mAChR PAM (LY2119620) that binds to the extracellular receptor surface, thus interfering with the dissociation of the orthosteric agonist from its binding pocket. (This figure has been modified based on recently published images Kruse et al. 2013)

helices (Burger et al. 2018; Kruse et al. 2014). Since these regions are not highly conserved among the M_1–M_5 receptors, considerable progress has been in developing so-called allosteric modulators with a high selectivity for distinct mAChR subtypes (Burger et al. 2018; Conn et al. 2009; Kruse et al. 2014). These agents act by altering the affinity or efficacy of orthosteric muscarinic ligands. While positive allosteric modulators (PAMs) enhance the activity of orthosteric agonists, negative allosteric modulators (NAMs) exert the opposite effect. Allosteric agents that can directly activate mAChRs are referred to as allosteric agonists. It is likely that the development of subtype-selective muscarinic allosteric agents will lead to novel, clinically useful drugs that are endowed with increased efficacy and an improved side effect profile. Currently, a major focus in the field is to develop allosteric muscarinic drugs for the treatment of several severe disorders of the CNS, including Alzheimer's disease and schizophrenia (Conn et al. 2009; Kruse et al. 2014).

An X-ray structure of the M_2 mAChR has revealed the molecular details of a PAM-mAChR complex, confirming that the binding pocket for allosteric muscarinic agents is located just above the orthosteric binding cavity (Kruse et al. 2013; Fig. 1). This binding mode enables the PAM to interfere with the dissociation of the bound orthosteric agonist from the receptor (Kruse et al. 2013). The development of bitopic orthosteric/allosteric ligands, which can interact with both orthosteric and allosteric sites, also represents a promising approach to obtain high-affinity muscarinic ligands endowed with a high degree of receptor subtype selectivity (Lane et al. 2013).

G Protein-Coupling Properties of mAChRs

Based on their G protein-coupling properties, the M_1–M_5 mAChRs can been subdivided into two major functional subclasses (Caulfield and Birdsall 1998; Wess 2004). The M_1, M_3, and M_5 mAChRs are preferentially coupled to G proteins of the $G_{q/11}$ family, which mediate the activation of different isoforms of *phospholipase Cβ* resulting in the breakdown of phosphatidylinositol and the generation of the second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. In contrast, the M_2 and M_4 mAChRs are selectively linked to

G proteins of the $G_{i/o}$ class, which, at a biochemical level, reduce the accumulation of intracellular cAMP via inhibition of *adenylyl cyclase*. However, the G protein-coupling selectivity of the individual mAChRs is relative rather than absolute, as has been observed with most other GPCRs. Both mutagenesis and structural studies have shown that amino acids located within the i2 loop and the membrane-proximal portions of the i3 loop play key roles in determining the G protein-coupling profile of the individual mAChRs (Maeda et al. 2019; Wess 1996).

Recent cryo-electron microscopic studies carried out with M_1 and M_2 receptors complexed with their respective G proteins have revealed the structural details underlying the G protein-coupling selectivity of individual mAChR subtypes (Maeda et al. 2019). These new structures show that the C-terminal tail of the activated M_1 receptor, but not the active M_2 receptor, can also interact with heterotrimeric G proteins (Maeda et al. 2019).

Signaling Pathways Activated by mAChR Subtypes

At a cellular level, the activation of mAChRs leads to a wide spectrum of biochemical and electrophysiological responses (Lanzafame et al. 2003; Wess 1996). The precise pattern of responses that can be observed depends on the nature of the activated G proteins (receptor subtypes) and the specific components of different signaling cascades (e.g., effector enzymes or ion channels) that are expressed in a particular cell type or tissue. The observed effects can be caused by direct interactions of the activated G protein(s) with effector enzymes or ion channels or may be mediated by second messengers (Ca^{2+}, IP_3, etc.) generated upon mAChR stimulation. Activation of M_1, M_3, and M_5 mAChRs does not only lead to the generation of IP_3 followed by the mobilization of intracellular Ca^{2+} but also results in the stimulation of phospholipase A_2, phospholipase D, and various tyrosine kinases. Similarly, M_2 and M_4 receptor activation does not only mediate the inhibition of adenylyl cyclase but also induces other biochemical responses including the augmentation of phospholipase A_2 activity. Moreover, the stimulation of different mAChR subtypes is also linked to the activation of different classes of mitogen-activated protein kinases (MAP kinases), resulting in specific effects on gene expression and cell growth or differentiation. Stimulation of mAChRs also results in the activation or inhibition of a large number of ion channels (Lanzafame et al. 2003). For example, stimulation of M_1 receptors leads to the suppression of the so-called M current, a voltage-dependent K^+current found in various neuronal tissues. M_2 receptors, on the other hand, mediate the opening of cardiac $I_{K(ACh)}$ channels, and both M_2 and M_4 receptors are linked to the inhibition of voltage-sensitive calcium channels (Lanzafame et al. 2003).

Regulation of mAChR Activity

Like most other GPCRs, mAChRs are subject to desensitization, which is defined as diminished responsiveness of the receptor/effector signaling pathway upon prolonged exposure of the receptor to an activating ligand. The phenomenon of GPCR desensitization involves a complex series of events, including G protein uncoupling, receptor sequestration/internalization (removal of receptors from the cell surface), and receptor downregulation associated with the net loss of receptor protein (van Koppen and Kaiser 2003). Many of these processes are regulated by receptor phosphorylation catalyzed by various protein kinases including different members of the family of *GPCR kinases (GRKs)*, casein kinase 1α, or second messenger-dependent protein kinases. Phosphorylation occurs on threonine and serine residues located within the i3 loop and the C-terminal tail of the mAChRs. The phosphorylated mAChRs can interact with beta-arrestins that play a key role in mediating receptor desensitization and internalization. The individual mAChR subtypes differ in their ability to serve as substrates for phosphorylation by the kinases mentioned above. The rapid removal of mAChRs from the cell surface following agonist stimulation (referred to as receptor internalization/sequestration) occurs through multiple pathways, one of

which involves the targeting of receptors to *clathrin-coated pits* (van Koppen and Kaiser 2003). Long-term treatment of mAChRs with muscarinic agonists results in receptor downregulation, the extent of which depends on receptor subtype and cell type (van Koppen and Kaiser 2003).

Drugs

Current Clinical Uses of Muscarinic Drugs

Muscarinic agonists and antagonists are used for the treatment of a variety of pathophysiological conditions. For example, muscarinic agonists (pilocarpine, carbachol, or aceclidine) reduce intraocular pressure when applied locally to the eye and are therefore widely used for the treatment of glaucoma. Muscarinic agonists (carbachol or bethanechol) are also employed in certain cases of atonia of the stomach, bowel, or urinary bladder. The agonists pilocarpine and cevimeline are used to stimulate salivation under conditions where the function of the salivary glands is impaired. Methacholine, an ACh analogue, is administered by inhalation for the diagnosis of bronchial airway hyperreactivity.

Centrally acting muscarinic antagonists (e.g., trihexyphenidyl, procyclidine, or biperiden) are useful for the treatment of Parkinson's disease or Parkinson-like symptoms caused by the administration of antipsychotic drugs, probably due to their ability to reduce excessive striatal muscarinic neurotransmission resulting from the lack of striatal dopamine. Muscarinic antagonists are also of considerable value in the treatment of clinical disorders characterized by an increased tone or motility of the gastrointestinal and urogenital tract (e.g., overactive bladder) and in the local therapy of chronic obstructive pulmonary disease. Antimuscarinic agents are widely used in ophthalmology to produce mydriasis and/or cycloplegia, are effective in the treatment of peptic ulcer disease (e.g., pirenzepine) and certain forms of cardiac arrhythmias, and can be used as part of routine preoperative medication, primarily to reduce reflex bradycardia and excessive bronchial secretion. A transdermal preparation of scopolamine is highly effective in preventing motion sickness.

Potential Clinical Uses of Muscarinic Drugs

A major problem associated with the use of classical muscarinic drugs is the rather common occurrence of bothersome side effects, primarily due to the simulation or inhibition of cardiac, glandular, smooth muscle, or central mAChRs. It is likely that the development of muscarinic agonists and antagonists that can interact with individual mAChRs with a high degree of selectivity will lead to novel muscarinic drugs with reduced side effects and increased efficacy. For example, several studies suggest that selective activation of central M_1 receptors may prove useful for the treatment of Alzheimer's disease (Kruse et al. 2014). Selective M_5 receptor antagonists are predicted to have potential for treating drug abuse (Kruse et al. 2014). Moreover, accumulating evidence indicates that agents that can selectively activate central M_1 and/or M_4 receptors may prove beneficial as novel antipsychotic drugs (Kruse et al. 2014).

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Myocardial Infarct

▶ Myocardial Infarction

Myocardial Infarction

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Synonyms

Cardiac arrest; Cardiac infarction; Heart attack; Myocardial infarct

Definition

Myocardial infarction (MI) is defined as the death of myocardial cells due to a prolonged time of ischemia (Thygesen et al. 2018). Clinically, the term should be used when acute myocardial injury is detected by abnormal cardiac biomarkers (such as troponins, myoglobin, creatine kinase-MB isoform) in the setting of evidence of acute myocardial ischemia (Thygesen et al. 2018). If there is no conclusive evidence to support the theory of myocardial ischemia but elevated cardiac biomarkers are present, the term myocardial injury should be used according to the latest universal definition of myocardial infarction published in 2018 as a consensus statement of many major cardiovascular societies (Thygesen et al. 2018).

Historically, MI is one of the most severe and furthermore most feared complication of progressive coronary artery disease (CAD). CAD refers to an atherosclerotic process taking place in the coronary vessels and represents a localized subset of the generalized process of atherosclerosis (Nabel and Braunwald 2012). Due to its widespread prevalence, the disease bears an enormous socioeconomic burden (Fryar et al. 2012). In the USA alone, over 1 million coronary events occurred in 2019 alone, meaning that every 42 s an US American will have a myocardial infarction (Mozaffarian et al. 2016). Worldwide, CAD causes over 7.4 million deaths annually making it the leading cause of death (WHO 2018). However, since the mid-1990s, there has been a steady decline in mortality from coronary heart disease and most notably in ST-segment elevation myocardial infarction (STEMI), owed substantially to refined evidence-based therapies especially due to advances in pharmacological, reperfusion, and preventive strategies (Nichols et al. 2013).

Pathophysiologic Mechanisms

General

MI is generally grouped into two major entities: ST segment elevation myocardial infarction (STEMI) and non-ST segment elevation myocardial infarction (NSTEMI). Unstable angina, meaning typical chest pain without exertion and negative cardiac biomarkers, is usually also considered an acute coronary syndrome (ACS), because it is believed to occur imminently before myocardial infarction. Since NSTEMI and

unstable angina share a similar pathophysiological principle, they are grouped together as so-called non-ST-segment elevation ACS (NSTE-ACS) to help clinicians streamline management pathways. In our current age, about 60–75% of all patient presenting with ACS are classified as NSTEMI whereas the proportion of STEMI is declining in the past decades (Yeh et al. 2010; McManus et al. 2011).

Furthermore, MI can be classified into five types depending on pathophysiological characteristics, prognosis, as well as clinical presentation (Thygesen et al. 2018). *Type I MI*, or spontaneous MI, is defined as myocyte necrosis due to atherothrombotic coronary artery disease (CAD) and usually precipitation by atherosclerotic plaque disruption (rupture or erosion). *Type II MI* is defined by an imbalance of myocardial oxygen supply and demand. The presence of CAD is specifically not required (but can be present) in type II MI. From a pathophysiological view, type II MI is a very heterogeneous group with many possible underlying causes such as coronary artery spasm, coronary microvascular dysfunction, severe bradyarrhythmia, respiratory failure with severe hypoxemia, severe anemia, and hypotension/shock, increased myocardial oxygen demand due to sustained tachyarrhythmia or severe hypertension. Notably, it has been shown that type II MI can present with (mostly transient) ST segment elevation in 3–24% of cases. The diagnosis of *type III MI* is reserved for patients who suffer cardiac death with symptoms suggestive of myocardial ischemia but who decease before cardiac biomarkers can be evaluated or MI is detected postmortem by autopsy. *Type IV and V MI* is designated for patients suffering from procedure-related myocardial infarctions in a PCI setting (IVa), related to acute stent thrombosis (IVb), related in-stent restenosis or after coronary artery bypass grafting (V). The diagnostic criteria for type IV MI is arbitrarily defined by an elevation of cardiac specific biomarkers values more than five times the 99th percentile upper reference limit in patients with normal baseline values up to 48 h after the index PCI procedure. Additionally, either new ischemic ECG changes, development of new pathological Q waves, imaging evidence of new loss of viable myocardium, or angiographic evidence of flow limiting procedural complications (such as coronary dissection) must be present. In the setting of coronary artery bypass grafting (Type V MI), the elevation of cardiac specific biomarkers must be larger than ten times the upper reference limit in addition to the development of new pathological Q waves, angiographic documentation of new graft or native coronary artery occlusion, or imaging evidence of loss of new loss of viable myocardium. For both type IV and V MI, the development of new pathological Q waves regardless of biomarker elevation meet the criteria for the respective MI. For an overview of MI Types, see Table 1.

Coronary Artery Disease

Coronary artery disease (CAD) is the underlying condition precipitating ischemic heart disease and thereby most cases (or all cases within MI type I) of myocardial infarction. CAD itself is only a subset of the generalized process of atherosclerosis localized in the coronary vasculature. Plaques in CAD usually consist of a fibrous cap covering a lipid rich core. Since the fundamental work of R Ross, the process of plaque development is understood as a systemic inflammatory process rather than a bland lipid storage in the artery walls (Ross 1999). For details on plaque composition, development, and formation, see the corresponding chapter on atherosclerosis. The risk factors of developing CAD are subsequently also shared with arteriosclerosis and include high blood pressure, smoking, diabetes, lack of exercise, adipositas, genetic factors, systemic rheumatologic diseases, high blood cholesterol, and high levels of lipoprotein (a) (Wilson 1994).

Over time, atherosclerotic plaques can develop further and grow to a size that the lumen of a coronary artery is reduced beyond a critical threshold (usually >80–90% considered hemodynamically relevant). This in turn limits the blood flow to the dependent myocardial cells which causes ischemia and exposing the cells to an ischemic environment with anaerobic metabolic homeostasis (Olivier 2011).

Myocardial Infarction, Table 1 Classification of Types of Myocardial Infarction

Type	Classification	Characterization
I	Spontaneous MI	Primary event caused by atherothrombotic coronary artery disease and usually precipitated by atherosclerotic plaque disruption (rupture or erosion)
II	Supply/demand mismatch	Myocardial infarction secondary to ischemia other than coronary artery disease due to a mismatch between oxygen supply and demand.
III	Death suspected to be related to MI	Cardiac death suggestive of an ischemic process without definitive biomarker evidence of myocardial infarction
IVa	MI associated with percutaneous coronary intervention	Rise in cardiac biomarkers (>5 times URL) up to 48 h after index PCI with evidence of new myocardial ischemia
IVb	MI associated with stent/scaffold thrombosis	Myocardial infarction fulfilling the same criteria as type I MI but with evidence of stent or scaffold thrombosis after index PCI
IVc	MI associated with (in-stent) restenosis	Myocardial infarction due focal or diffuse restenosis, or a complex lesion after index PCI associated with a rise or fall of cardiac biomarker values above the 99th percentile URL applying the same criteria utilized for type I MI
V	MI associated to coronary artery bypass grafting	Rise in cardiac biomarkers (>10 times URL) up to 48 h after index coronary artery bypass grafting with evidence of new myocardial ischemia
Injury	Myocardial injury	Acute or chronic elevation in cardiac biomarkers (above 99th percentile URL) without overt ischemia due to multifactorial etiology but associated with averse outcome

Overview of MI types and myocardial injury according to the Fourth Universal Definition of Myocardial Infarction; MI: Myocardial infarction; URL: Upper reference limit; PCI: Percutaneous coronary intervention

Since oxygen demand is higher during exercise (as coronary flow can rise up to fourfold under exercise), stable angina pectoris develops in these patients under exertion. However, not all plaques resulting in a certain degree of stenosis inevitably cause limiting blood flow and thereby symptoms of stable angina. The severity of flow obstruction is determined by a multitude of factors including plaques size, vasospasms, arterial remodeling, and ultimately also collateralization.

With progression of CAD (also called stable CAD), whether symptomatic or not, the process of plaque progression can be complicated by plaque rupture. In case of a resulting total occlusion of the coronary artery, a type I MI occurs. However, more often a plaque rupture is clinically silent and does not result in occlusive thrombus formation or is removed naturally by a process of endogenous lysis and following migration of smooth muscle cells that migrate to the area and cover the surface with connective tissue. During this process, plaques can increase in size in a phasic manner rather than linear and produce complicated plaques with several possible rupture sites.

Stable Coronary Plaques

A severe stenosis is usually required to cause angina. It was shown that these types of lesions less commonly cause type I myocardial infarction. This is believed to be caused by plaques composition as these lesions often tend to have a dense fibrotic cap that covers a rather small lipid pool and is in turn less likely to rupture and thereby allows for formation of collateral circulation over time. Stable plaques hereby promote a "barrier" usually composed of collagen fibers and smooth muscle cells and in comparison low amount of inflammatory cells (especially macrophages and T-lymphocytes). This keeps the blood flow separated from the highly thrombogenic lipid core of the plaque, preventing the initiation of the thrombotic cascade and subsequently formation of an occlusive (or non-occlusive) thrombus.

Vulnerable Coronary Plaques

In contrast to stable coronary plaques, vulnerable plaques often tend to be only 30–50% in absolute luminal stenosis and a thus not flow limiting eluding diagnosis in conventional coronary angiography. These plaques often show thin fibrous caps and a high burden of inflammatory cells (especially macrophages). Additionally, these lesions often have a large lipid-rich (and often necrotic) core. Additional features can be positive signs of vascular remodeling (vessel enlargement), increased vasa-vasorum neovascularization, and intra-plaque hemorrhage. This characteristic lesion profile leads to a zone especially vulnerable to mechanical stress (for example, high blood pressure) and ultimately plaque rupture. Even though plaque rupture is believed to be the most common entity that causes ACS, several modes of action including superficial plaque erosion, erosion of calcium nodules, and intra-plaque hemorrhage have been studied over the recent years (Libby 2011).

In case of plaque rupture, the often-thin fibrous cap beaks up and exposes its thrombogenic contents to the blood stream. The fibrous cap itself can uphold its structural integrity if the smooth muscle cells (SMC) can shift the balance between collagen synthesis and degradation (mostly by inflammatory cells) in their favor. By infiltration of dendritic cells mostly in the shoulder region of the plaque and following secretion of T-lymphocyte recruiting chemokines, T-lymphocytes migrate to the site and begin to secrete interferon-γ and TNF-α. This not only reduces the ability of SMCs to uphold the fibrous cap but also activates macrophages. The resulting presentation of pro-inflammatory cytokines can promote apoptosis of SMCs. Furthermore, matrix metallopeptidases (MMP) are overexpressed in these lesions and produced by the attracted macrophages leading to further collagen degradation and thinning of the fibrous cap (Libby et al. 2019).

Acute Coronary Syndrome

Acutely decreased blood flow in the coronary arteries causes a set of symptoms (syndrome) commonly termed acute coronary syndrome (ACS). The most prevalent clinical symptom is chest pain, often with radiation to the left shoulder, jaw or left arm and commonly associated with nausea and sweating. The sensation is often subjectively felt as crushing or weighing in the chest area in addition to a feeling of acute illness. However, many patients with ACS can present differently. Particularly, women, patients with diabetes mellitus or older patients often show a plethora of symptoms (often called "atypical") that can delay diagnosis in the clinical setting. In the case of suspected ACS and normal cardiac biomarker values (<99th percentile upper reference limit), an alternative diagnosis or unstable angina should be considered.

Electrocardiogram

The electrocardiogram (ECG) with its rapid application and widespread availability plays a crucial role in the diagnostic workup of patients with suspected MI and should be acquired at best immediately and not later than 10 min after first medical contact. Acute myocardial ischemia is often associated with dynamic changes in ECG waveform and thus serial acquisition is recommended. Suspect of myocardial ischemia are new ST-elevation at the J-point in two contiguous leads with the cut-point: >1 mm in all leads other than leads V2–V3 (>2 mm in men >40 years; >2.5 mm in men <40 years, or >1.5 mm in women regardless of age). Furthermore, new horizontal or downsloping ST-depression >0.5 mm in two contiguous leads and/or T-wave inversion >1 mm in two contiguous leads with prominent R wave or R/S ratio >1 should be considered a correlate of myocardial ischemia. Additionally, a newly diagnosed complete left branch block can be a sign of myocardial ischemia.

On a pathophysiological level, when a complete blockage of a coronary artery occurs, ischemia will affect all three layers of the myocardium (transmural). The ECG leads facing the corresponding area of cardiac muscle cells will show an action potential as ST elevation during systole, while during diastole, there is a depression of the PR segment and the PT segment. Since those intervals are regarded as baseline when evaluation ECG waveforms, ST segment

elevation is regarded as a sign of myocardial ischemia and damage.

Cardiac Biomarkers

Over the last decade, the isoforms T and I of cardiac troponins (cTnT and cTnI) have emerged as the preferred diagnostic tool, owing this status to their high sensitivity and specificity for myocardial cell damage. Both enzymes are components of the contractile apparatus of myocardial cells and are expressed almost exclusively in the cardiac muscle (Vasile and Jaffe 2017).

Today, high sensitivity essays (hscTnT or hscTnI) are recommended in the first line for routine clinical use. Since the introduction of high-sensitive essays, there has been a significant reduction in the diagnosis of unstable angina corresponding to an increase in diagnosis of NSTEMI. In general, myocardial injury is defined as being present when blood levels of a cardiac troponin are increased above the 99th percentile of the upper reference limit (URL) of the corresponding essay. This may be described as acute, evident by a newly detected dynamic rising and/or falling pattern or chronic in case of persistently elevated levels of cardiac troponins. Despite having a high sensitivity and specificity, elevated levels of troponin do not reflect the underlying pathophysiological mechanism, and it is not yet possible for clinicians to distinguish from the blood work alone whether myocardial injury is ischemic or not. However, per definition, a rising and/or falling pattern of cardiac troponin values with at least one value above the 99th percentile URL and suspected myocardial ischemia by ECG changes, clinical presentation, or imaging is designated as an acute MI.

Other available biomarkers that were used historically to detect MI such as myoglobin, creatine kinase, or the myocardial isoform creatine kinase MB have fallen out of favor since they cannot produce comparable levels of specificity and sensitivity as high sensitive cardiac troponin.

Myocardial Cell Death

When myocardial ischemia occurs, a large number of cardiomyocytes die rapidly due to factors including acidosis, oxidative stress, and of course hypoxia with resulting lack of aerobic energy production. The myocardium is usually not affected equally with irreversible injury starting in the endocardium as this area is the furthest away from the vessels and gradually continuing to the epicardium. After about 30 s of non-perfusion, diastolic and systolic dysfunction can begin to occur followed shortly after by noticeable ECG changes that begin with large peaked and sometimes widened T waves followed later by the defining ST segment elevation. First evidence of necrosis can be seen as soon as 30 min after flow discontinuation. From this point forward, the cell death becomes exponentially rather than linear, heightening the importance of swift revascularization to prevent extensive myocardial damage ("time is muscle"). After about 6 h of no perfusion, all affected myocardium will become necrotic.

On the cellular level, myocardial cells are very prone to suffer from ischemia due to their lack of anaerobic metabolic alternative pathways. Adenosine triphosphate (ATP) depletion occurs comparatively fast. As a result, ATP gets degraded and produces hydrons (H^+) which in turn lower the intracellular pH levels (intracellular acidosis). This now causes inactivation of troponin C which disables contraction and an increased influx of sodium ions (Na^+) causing oncotic swelling of the cells. Furthermore, since ATP gets scarce, inactivation of ATP-dependent proteins such as calcium ATPases lead to increased intracellular levels of calcium. The death of cardiomyocytes can be finally caused by oncotic swelling, necrosis, or controlled apoptotic pathways which are believed to occur mainly in the border zones of the infarcted myocardium and are believed to benefit the most from swift reperfusion. Cell death itself leads to a massive inflammatory process with invasions of neutrophils and monocytes causing further swelling and cytokine production that is believed to further promote apoptosis. Anti-inflammatory treatment in the acute phase of myocardial infarction has therefore been the aim of pharmacological intervention in clinical trials to improve outcome. As part of the normal healing process inflammatory cells promote the invasion of fibroblasts and endothelial cells that in turn

create primarily vascularized granulation tissue that over time matures into noncontractile acellular scar tissue.

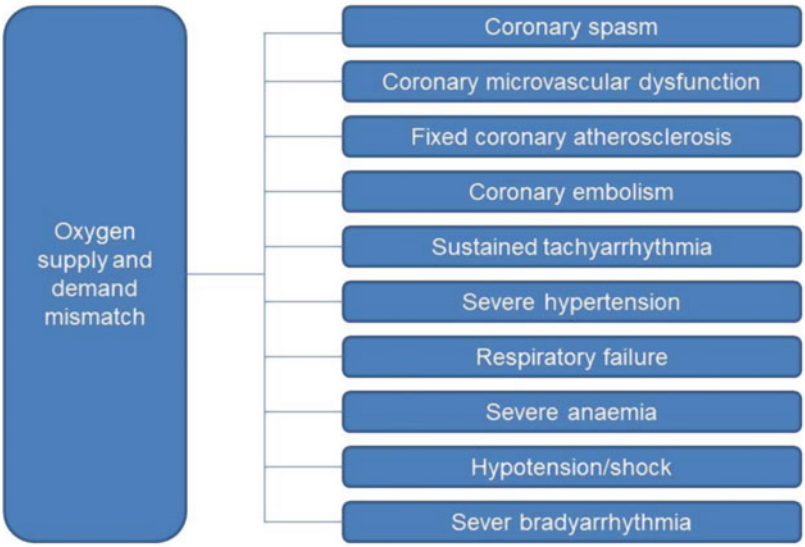
Reperfusion Injury

Even though swift reperfusion is the therapy of choice and is correlated with improved outcomes in patients with STEMI, the sudden reperfusion of the infarcted can prove to be problematic for the affected cells. With restoration of epicardial coronary flow, the extracellular pH is rapidly normalized, causing a significant H^+ gradient across the cell membrane. This in turn promotes further Na^+ influx via the Na^+/H^+ exchange protein and subsequently further calcium overload caused by activation of the NA^+/Ca^{2+} exchanger. In addition, the sudden shift to an again aerobic environment causes a high activity of the respiratory chain in the mitochondria resulting ultimately in high levels of reactive oxygen species (ROS) causing further oxidative damage. This process is usually called reperfusion injury and remains a field of inconclusive research concerning strategies (pre- and post-conditioning) to improve outcome beyond the lone revascularization of the epicardial coronary arteries.

Type II Myocardial Infarction

With an aging population and an increased prevalence of multimorbidity especially in developed countries, patients often show signs of myocardial injury as seen in elevated levels of cardiac troponins without signs of actual myocardial ischemia caused by acute atherosclerotic plaque disruption. Myocardial injury is in this case usually caused by a mismatch between oxygen supply and demand of the myocardium. From an epidemiological standpoint, type II MI might be even more common than type I MI. Such findings are common in several US studies where 57–75% of MIs were classified as type II MI. This subset of MI is a very heterogeneous entity which is also reflected in a highly variable clinical presentation of patients that often have less chest pain and more often have dyspnea or other atypical symptoms. Since patients can also often have CAD (obstructive or non-obstructive) distinction between type I MI, type II MI and acute myocardial injury can prove to be challenging especially when taking multiple comorbidities into account. Table 2 shows a list of possible clinical entities that can cause type II MI such as coronary spasm, coronary embolism, coronary, coronary endothelial

Myocardial Infarction, Table 2 Pathophysiology of Type II Myocardial Infarction



dysfunction, spontaneous coronary artery dissection, tachyarrhythmia, severe hypertension, bradyarrhythmia, severe hypoxia, severe anemia, or severe hypotension. Unfortunately, patients suffering from type II MI have similar all-cause mortality rates compared to patients with type I MI and are at high risk for cardiovascular mortality.

Pharmacological Intervention

After diagnosis of MI, the focus of clinicians should be on hemodynamic stabilization, pain control, decreasing myocardial oxygen consumption, increasing myocardial oxygen supply, and initiating antithrombotic therapy.

Following the immediate management of patients, coronary catheterization is indicated. The timing of the invasive strategy depends on multiple factors and also on the estimated complication risk. In patients with STEMI and very high risk NSTEMI, reperfusion should be initiated as soon as possible in less than 120 min if the time from symptom onset is less than 12 h. Reperfusion should be achieved by percutaneous coronary intervention (PCI), with a prompt initiation of fibrinolysis, or with a combination of both. Direct transport to the PCI center is indicated when PCI can be performed within 60–90 min. Patients presenting to a center without PCI capabilities with STEMI should be immediately transported to a PCI center, provided that reperfusion can be achieved in less than 120 min. If transport times are higher than 30–60 min, prehospital fibrinolysis is commended but must be followed by PCI within 3–24 h. High-risk NSTE-ACS patients require early PCI in the first 24 h. Intermediate-risk patients and patients with positive noninvasive ischemia tests should be referred to coronary angiography in a 72-h window.

Fibrinolysis

Even though primary PCI is the preferred method of reperfusion therapy, systemic fibrinolytic therapy by administering recombinant tissue plasminogen activators (rtPA) such as alteplase, reteplase, and tenecteplase can be performed in certain circumstances especially if PCI is not readily available. If the so-called door-to-balloon time exceeds 120 min, the benefit of primary PCI over primary fibrinolysis is lost and fibrinolysis should be administered within 30 min of first medical contact given that there are no contraindications. However, this is only applicable for patients with STEMI as bleeding risk outweighs the benefits in patients with NSTEMI.

Antiplatelet Agents

Patients should receive 150–325 mg acetylsalicylic acid to inhibit further platelet aggregation on first presentation. Additional inhibition can be achieved by blocking the P2Y12 receptor on the thrombocyte surface. Three substances with clopidogrel, ticagrelor, and prasugrel are readily available for oral administration. Whereas clopidogrel and prasugrel inhibit P2Y12 irreversible, ticagrelor is a reversible inhibitor.

Current guidelines recommend the use of the more potent prasugrel or ticagrelor in patients with acute MI; however, bleeding risk, reperfusion strategy, and local guidelines must be taken into account, as well as the results of emerging trials changing the landscape of antiplatelet therapy rapidly. The role of the only intravenously available reversible ADP receptor antagonist cangrelor is not yet well defined but might be useful in patients not adequately loaded with a P2Y12 inhibitor.

Glycoprotein IIb/IIIa inhibitors such as abciximab, tirofiban, and eptifibatide can provide additional very potent inhibition of platelet aggregation but confer a significant increased bleeding risk. In the area of a potent dual antiplatelet therapy, administration of glycoprotein IIb/IIIa seems to be reserved for patients with high-risk ACS or high thrombus burden shown in the initial coronary angiography.

Anticoagulant Agents

Inhibition of the thrombotic cascade is essential in initial management of ACS regardless if an invasive or conservative strategy is planned for the patient. Historically, unfractionated heparin is widely used to reduce thrombus formation and promote thrombolysis especially as a first-line treatment in a prehospital setting. In the past, trials could also show that the low-molecular-

weight heparin enoxaparin is non-inferior to unfractionated heparin if there is no immediate PCI planned. The use of direct thrombin inhibitors such as bivalirudin as the probably most widely studied substance remains controversial in acute myocardial infarction as pooled data suggest that bivalirudin monotherapy might increases the risk of acute stent thrombosis but on the other hand might reduce major bleeding when compared with unfractionated heparin or enoxaparin-based regimens depending on whether a glycoprotein IIb/IIIa inhibitor is given or not (Capodanno et al. 2016; Cavender and Sabatine 2014).

Secondary Prevention

As with all forms of degenerative vascular diseases, secondary prevention of patients suffered from myocardial infarction is as important if not more important than primary treatment itself. Next to lifestyle modification and specifically smoking cessation, a strict medical therapy is essential for lowering long-term mortality. Depending on bleeding risk, stent use, and type of myocardial infarction, a dual antiplatelet therapy should be continued for up to 12 months followed by lifelong single antiplatelet therapy with acetylsalicylic acid. Furthermore, recent studies suggest that selected high-risk patients might benefit from additional therapy with low dose directly acting oral anticoagulants such as rivaroxaban where the reduction in cardiovascular events outweighs the increased bleeding risk. Additionally, beta-blockers, angiotensin-converting enzyme inhibitors, and aldosterone antagonists in case of a left ventricular ejection fraction below 40% have been shown to improve long-term outcomes in selected patients after myocardial infarction.

Also, it is recommended that all patients after MI should be treated with lipid lowering agents namely statins to reduce events. Recent data suggest that even more aggressive control of LDL cholesterol with high-intensity statin therapy to LDL-C targets below 1.4 mmol/l improves cardiovascular outcomes in patients after ACS.

Cross-References

- ▶ Antiplatelet Drugs
- ▶ Atherosclerosis

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Myorelaxation

▶ GABAergic System

N

Na $^{+}$/K $^{+}$-ATPase: Na $^{+}$/K $^{+}$ Exchanging ATPase

► Na^+/K^+-ATPase

Na^+/Ca^{2+} Antiporter

► Na^+/Ca^{2+} Exchangers

Na^+/Ca^{2+} Exchanger

► Na^+/Ca^{2+} Exchangers

Na^+/Ca^{2+} Exchangers

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Synonyms

Na^+/Ca^{2+} antiporter; Na^+/Ca^{2+} exchanger; NCX; NCX1; NCX2; NCX3; Sodium calcium exchanger

Definition

The plasma membrane Na^+/Ca^{2+} exchanger is a high-capacity and low-affinity ionic transporter that exchanges three Na^+ ions for one Ca^{2+} ion. When intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) rise and the cells require the return to resting levels, this exchanger couples the uphill extrusion of Ca^{2+} ions to the influx of Na^+ ions into the cells down their electrochemical gradient. This mode of operation, defined as forward mode, or Ca^{2+} efflux mechanism, keeps the 10^4-fold difference in $[Ca^{2+}]_i$ across the cell membrane. Under other physiological or pathophysiological conditions when the intracellular Na^+ concentrations ($[Na^+]_i$) rise, or membrane depolarization occurs and reduces the transmembrane Na^+ electrochemical gradient, the Na^+/Ca^{2+} exchanger mediates the extrusion of Na^+ and the influx of Ca^{2+} ions. This last mode of operation is defined as reverse mode or Ca^{2+} influx mechanism. The mode of operation of the antiporter depends on (i) the Na^+ gradient, (ii) the Ca^{2+} gradient, and (iii) the membrane potential. Therefore, NCX exerts a role in the maintenance of $[Ca^{2+}]_i$ and $[Na^+]_i$ homeostasis and plays a role in the regulation of physiological responses to increases of $[Ca^{2+}]_i$ and $[Na^+]_i$ (Annunziato et al. 2004; Philipson and Nicoll 2000).

Basic Characteristics

Molecular Biology and Topology

Three genes coding for the three different NCX1, NCX2, and NCX3 proteins are present in mammals. These genes appear to be dispersed, since *ncx1*, *ncx2*, and *ncx3* are localized in human

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chromosomes 2, 19, and 14, respectively. At the posttranscriptional level, at least 15 NCX1 and 5 NCX3 proteins are generated through an alternative splicing of the primary nuclear transcripts. No alternative splicing was found in NCX2 transcript.

NCX1 mRNA encodes for 970 amino acids; however, the initial hydrophobic segment of 32 amino acids is a signal peptide cleaved during the initial processing in the endoplasmic reticulum. The role of this cleavage is still unknown. The mature protein of NCX1 on plasma membrane is composed of 938 amino acids. Similarly, NCX2 and NCX3 mRNA encodes for 921 and 927 amino acids, respectively, but because of the cleavage, the mature proteins NCX2 and NCX3 contain 899 and 896 amino acids, respectively. The most studied isoform of NCX is NCX1 with a theoretical molecular mass of 120 kDa. Regarding the topology of this exchanger, preliminary biochemical analyses have indicated the presence of nine transmembrane segments (TMS), but recent data strongly support a model of ten TMS and with both the amino- and carboxyl-terminus located in the extracellular space (Ren and Philipson 2013). The ten TMS can be divided into an N-terminal hydrophobic domain, composed of the first five, TMS 1–5, and into a C-terminal hydrophobic domain, composed of the last five, TMS 6–10. Two amino acid sequences, named α_1 and α_2 repeats, are located on the opposite site of the membrane where they form reentrant loops that interact within the protein. The α_1 repeat (106–145) comprises part of the TMS2 and TMS3 amino acid sequences and their connecting loop. The α_2 repeat (807–844) comprises part of the TMS7 and part of the connecting loop with TMS8. Since the putative α-helices of the α-repeats are amphipathic, the hydrophilic faces of these helices may form a portion of the ion translocation pathway. Indeed, these two hydrophobic domains are important for the binding and the transport of ions. Interestingly, just after the α_2 repeat sequence, there is a GIG amino acid sequence similar to the GYG sequence present in the P loop of K^+ channels (Philipson and Nicoll 2000). The first five TMS 1–5 are separated from the last five TMS 6–10 through a large hydrophilic intracellular loop of 550 amino acids, named f loop (Fig. 1).

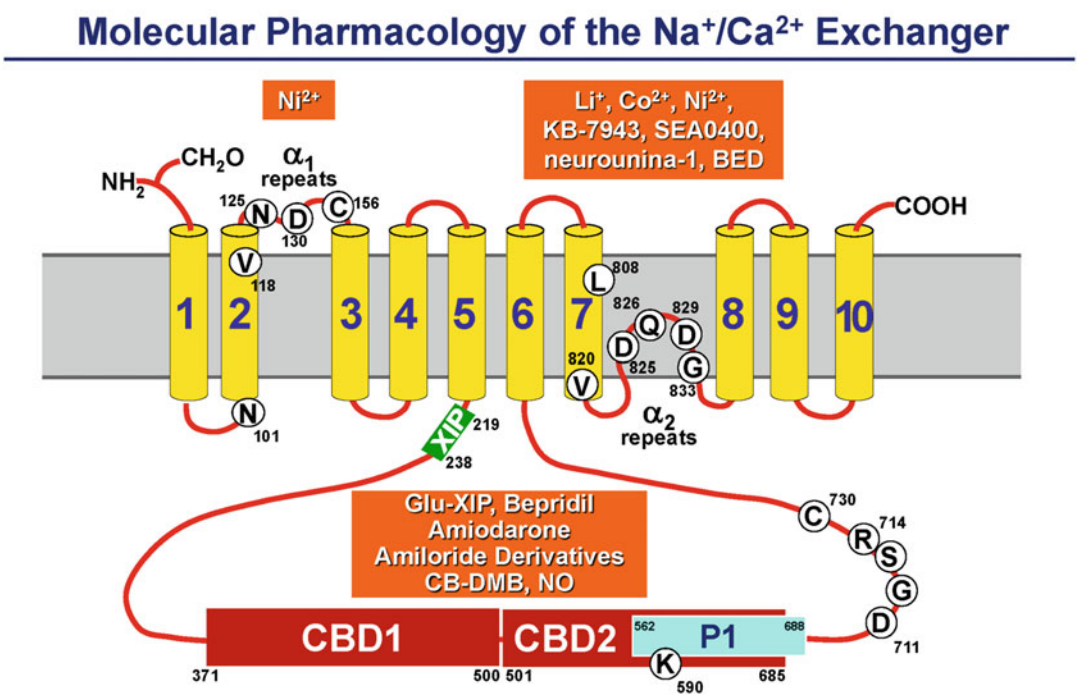
Although the f loop is not implicated in Na^+ and Ca^{2+} translocation, it is responsible for the regulation of NCX activity elicited by several cytoplasmic messengers and transductional mechanisms, such as Ca^{2+}, Na^+, and H^+ ions, NO (nitrergic systems, NO-synthases), sumoylation, phosphatidylinositol 4,5 bisphosphate (PIP_2), protein kinase C (PKC), protein kinase A (PKA), and ATP. Furthermore, in the f loop, there are two Ca^{2+} binding domains named Ca^{2+} binding domain 1 and 2 (CBD1 and CBD2, respectively) (Hilge et al. 2006; Ottolia et al. 2009). CBD1 is the primary Ca^{2+} sensor (371–500 aa) and detects slightest increases in cytosolic Ca^{2+}, and the associated large structural changes activate the exchanger. In contrast, CBD2 (571–685 aa) undergoes comparably modest structural alterations and binds Ca^{2+} only at elevated concentrations. These two different sensitivity thresholds may enable NCX to function dynamically over a wide range of Ca^{2+} concentrations and permit high Ca^{2+} fluxes in excitable cells.

At the N-terminal end of the f loop, in proximity with the membrane lipid interface, there is an autoinhibitory domain, rich in both basic and hydrophobic residues and consisting of a 20-amino acid sequence (219–238). This amino acid sequence, named exchange inhibitory peptide (XIP), is involved in the Na^+-dependent inhibitory effect on NCX activity through the binding to the amino acid sequence named P1 (562–688) near the C-terminal of the f loop (Molinaro et al. 2015).

The other two isoforms NCX2 and NCX3 are characterized by molecular masses of 102 and 105 kDa, respectively. NCX2 displays a 65% sequence identity with NCX1, whereas NCX3 possesses a 73% sequence identity with NCX1 and 75% sequence identity with NCX2. The high amino acid sequence homology indicates that all these three NCX gene products share the same membrane topology (Fig. 1).

Distribution

Na^+/Ca^{2+} exchanger activity is present in virtually every cell type examined. The NCX1 gene is almost ubiquitously expressed since it is found in many tissues, including brain, heart, skeletal



Na^+/Ca^{2+} Exchangers, Fig. 1 Molecular pharmacology of the Na^+/Ca^{2+} exchanger. The first five domains, TMS 1–5, are separated from the last five domains, TMS 6–10, by the f loop provided with regulatory functions. α_1 and α_2 regions are responsible for ion translocation. (Modified from Annunziato et al. (2004))

muscle, smooth muscle, kidney, eye, secretory, immune system, and blood cells. By contrast, NCX2 expression is found exclusively in neurons and glia, whereas NCX3 has been found in neuronal, glial, and skeletal muscle tissues. In addition, NCX1 and NCX3 give rise to several splicing variants that appear to be selectively expressed in different regions and cellular populations of the brain.

At the cellular level, all the three NCX proteins are present on the cell membrane; however, more recently, it has been reported that nuclear-encoded NCX3 isoform is located on the outer membrane of neuronal mitochondria and co-localizes with the protein kinase A anchoring protein (AKAP-121) (Scorziello et al. 2013). In addition, evidence has been provided on the presence of NCX1 in the nucleus at the level of the inner membrane where it contributes to the neuronal differentiation (Secondo et al. 2018). NCX2 was also found on neuronal neurotransmitter vesicles where it possibly participates in Ca^{2+} clearance and neurotransmitter release (Jeon et al. 2003; Takamori et al. 2006).

Regulatory Factors

Several factors are involved in the regulation of Na^+/Ca^{2+} exchanger activity: (i) the two transported cations, Na^+ and Ca^{2+}; (ii) the intracellular pH; (iii) the metabolic related compounds, ATP, PIP_2, PKA, and PKC; and (iv) reactive oxygen species, including NO.

The domains CBD1 and CBD2 are situated in the center of the intracellular f loop, and they regulate NCX activity but do not participate in Ca^{2+} transport. Indeed, nanomolar concentrations (20–50 nM) of intracellular Ca^{2+} are needed to activate the antiporter through the first Ca^{2+} binding site, CBD1, whereas submicromolar concentrations (0.1–0.3 μM) of intracellular Ca^{2+} are needed to increase the antiporter activity with the second Ca^{2+} binding site, CBD2 (Table 1).

An increase in $[Na^+]_i$ leads to an inactivation process of the exchanger. This inactivation

Na^+/Ca^{2+} Exchangers, Table 1 Regulatory mechanisms of Na^+/Ca^{2+} exchanger activity

Regulatory factors	Effect
$[Ca^{2+}]_i$	Increase Na^+/Ca^+ exchanger activity (low affinity, 0.1–0.3 μM; high affinity, 20–50 nM)
$[Na^+]_i$	$[Na^+]_i$-dependent inactivation of outward Ca^{2+} currents
NO	Stimulates NCX1 and NCX2 activity via a cGMP pathway Inhibits NCX3 activity via a direct nitrosylation
Sumoylation	Increases NCX3 protein half-life
Redox status	Oxidizing agents stimulate activity
PIP_2	Elimination of the $[Na^+]_i$-dependent inactivation
ATP	Increases $[Na^+]_o$-dependent Ca^{2+} efflux
PKC	Increases the affinity of $[Ca^{2+}]_i$ and $[Na^+]_o$ for the Na^+/Ca^{2+} exchanger
PKA	Increases exchange activity in splicing isoforms of NCX1 containing the exon 3 (namely, exon A)
PKT	Increases expression and activity of NCX1 and NCX3

process, very similar to the phenomenon occurring in voltage-dependent ionic channels, is named Na^+-dependent inactivation. In particular, this autoinhibition process seems to be mediated by the XIP domain that, in turn, might interact with a sequence of f loop including a portion of CBD2 (562–688), named P1. In accordance with this view, a synthetic peptide provided with the same sequence of XIP region binds the P1 region on the f loop and inhibits NCX activity. By contrast, a synthetic peptide provided with the same sequence of P1 domain binds the XIP sequence on the f loop and removes this latter from the inhibitory interaction with the P1 domain 562–688 on the f loop.

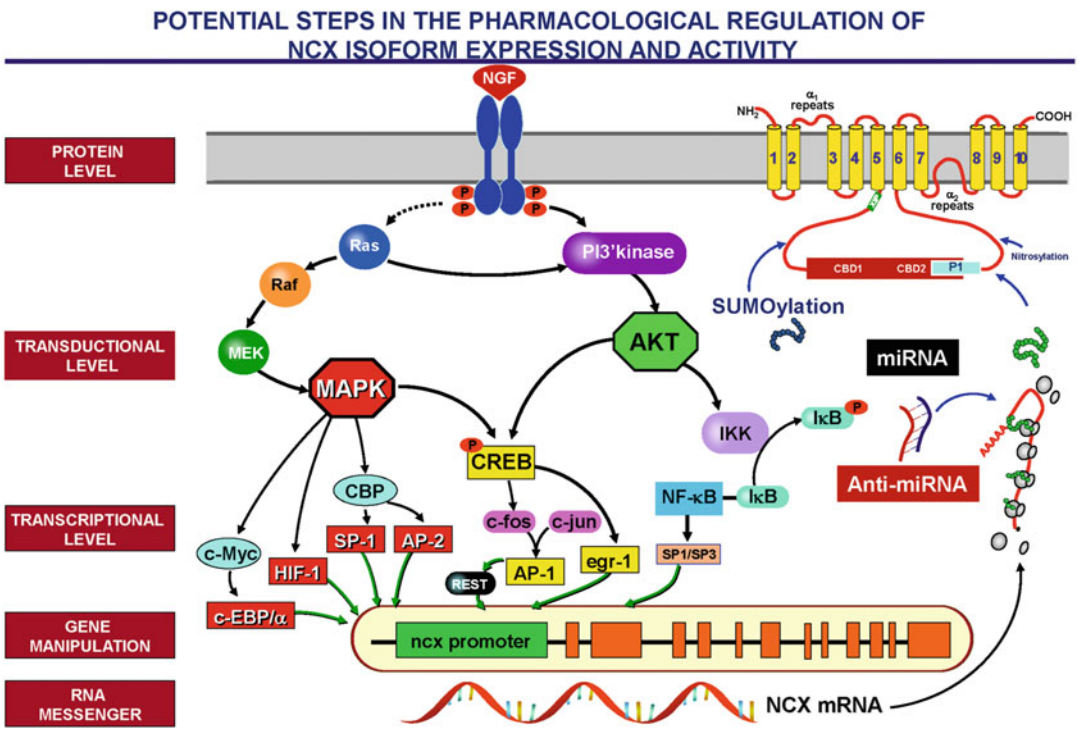
The intracellular pH can also regulate the exchanger. Indeed, reduction in $[pH]_i$ values, as little as 0.4, can induce a 90% inhibition of NCX activity under steady-state conditions. Such inhibitory action depends on the presence of intracellular Na^+ ions; hence, the action exerted by H^+ ions is pathophysiologically relevant with regard to brain and heart ischemia, two pathological conditions characterized by pH lowering.

ATP, acting as a phosphoryl donor molecule, may increase the activity of the exchanger in a number of ways. Firstly, ATP directly participates in the NCX molecule phosphorylation process by PKA and PKC. Secondly, it increases PIP_2 production. Finally, by activating G-protein-coupled receptors, via endogenous and exogenous ligands, ATP can stimulate the activity of the Na^+/Ca^{2+} exchanger through the pathway involving PKC or PKA activation. The mechanism underlying the phosphorylating effect on the exchanger is related to an increase in its affinity for both internal Ca^{2+} and external Na^+ and to a decrease in its inhibition by internal Na^+. Each of the NCX isoforms has distinctive putative phosphorylation sites; ATP cellular depletion inhibits NCX1 and NCX2 but does not affect NCX3 activity (Secondo et al. 2007; Linck et al. 1998). The other mechanism by which ATP can activate NCX occurs through the production of the lipid PIP_2. In fact, this lipid, binding the XIP region of the f loop, eliminates NCX inactivation and stimulates NCX function. In addition, Na^+/Ca^{2+} exchanger can be sensitive to reactive oxygen species, since modifications of the cellular redox state can cause an increase of NCX activity.

Transcriptional Factors Regulating NCX Expression

Several factors were identified to regulate the expression of NCX1 and NCX3 genes (Fig. 2), whereas no studies were reported on the transcriptional regulation of NCX2 gene.

Ncx1 gene expression appears to be directed by three alternative regulatory regions, named heart (Ht), kidney (Kd), and brain (Br) promoters. In cardiomyocytes, the expression of NCX1 is controlled by the Ht promoter that includes serum response factor (SRF), GATA-4, AP-1, and Nkx2.5 binding sites. In kidney, *ncx1* expression is controlled by the Kd promoter region that includes a typical TATA box preceded by two tandem GATA elements, NF-Y binding site, and two CAAT/enhancer binding protein-like (C/EBP-like) sites. Br *ncx1* promoter is a



Na^+/Ca^{2+} Exchangers, Fig. 2 Potential steps in the pharmacological regulation of NCX isoform expression and activity. This scheme reproduces the potential levels at which drugs can interfere with the transduction, transcription, translation, and activity of NCX

ubiquitous GC-rich TATA-less promoter, giving rise to the majority of *ncx1* transcripts in the CNS and also drives the expression of NCX1 in other tissues at lower levels. This region contains several putative binding sites for transcriptional factors including Sp1, AP1, AP2, nuclear factor kB (NF-kB), CREB, hypoxia-inducible factor 1 (HIF-1), and RE1-silencing transcription factor (REST). Recent data showed that NF-kB increases NCX1 protein expression in cortical neurons under hypoxic conditions (Sirabella et al. 2009). In addition, two epigenetic complexes also regulate NCX1 expression in stroke. The first complex comprises Sp3/REST/HDAC1/HDAC2 proteins and downregulates the expression of NCX1 in stroke. The second complex is composed of Sp1/HIF-1/p300 proteins and upregulates the expression of NCX1 in the stroke preconditioning. In addition, a number of microRNA were found to regulate NCX1 expression in several tissues (Table 2).

To date, the promoter of *ncx2* gene has not yet been identified, and moreover, no extensive data are available for its transcriptional regulation. The promoter of *ncx3* is located immediately upstream of the first exon and includes binding sites for AP-1, AP2, CREB, downstream regulatory element antagonist modulator (DREAM), Egr-1, KROX24, MyoD, GATA 2/3, and Sp1. Two of these transcription factors, CREB and DREAM, seem to be the most important regulators of *ncx3* gene transcription in response to changes in the intracellular concentrations of Ca^{2+} and cAMP.

Physiological Roles

Because of the limitations of pharmacological tools acting on NCX, several genetic modified mice were generated to identify the role of each isoform under physiological and pathophysiological conditions. Results showed that the genetic ablation of NCX1 is lethal, since it is essential for embryo development. By contrast, NCX2 and

Na^+/Ca^{2+} Exchangers, Table 2 Drug families inhibiting the Na^+/Ca^{2+} exchanger

Pharmacological class	Drugs
Amiloride derivatives	CB-DMB, DCB, DMB
Substituted pyrrolidines	Bepridil
Isothiourea derivatives	KB-R7943
Ethoxyanilines	SEA0400
Benzofuran derivatives	Amiodarone
Quinazolinone derivatives	SM-15811
Thiazolidine derivatives	SN-6
Phenoxypyridine derivatives	JP11092454
Nicotinamide derivatives	YM-244769
Piperidine derivatives	YM-252077
Ylacetamide derivatives	YM-270951
Benzamide derivatives	BED
Peptides	XIP, Glu-XIP, FMRFa, FRCRCF
Small interference RNA	siRNA-NCX1, siRNA-NCX1, siRNA-NCX3
microRNA	miR-103-1, miR-132, miR-135a, miR-206, miR-212-5p
Antisense oligodeoxynucleotides	AS-NCX1, AS-NCX2, AS-NCX3
Inorganic cations	Ni^{2+}, La^{2+}, Gd^{2+}

NCX3 knockout mice survive until the adulthood, and both mainly regulate neuronal Ca^{2+} clearance and synaptic plasticity with consequences on learning and memory processes (Jeon et al. 2003; Molinaro et al. 2011).

Pathophysiological Implications

In consideration of the relevant role played by the forward and reverse mode operation of NCX in the maintenance of $[Na^+]_i$ and $[Ca^{2+}]_i$ homeostasis in cells of the cardiovascular and central nervous system, alterations in gene structure, gene activation, protein expression, stability, and function may take part in the pathophysiology of several diseases. As matter of fact, homozygous overexpression of NCX1 in the heart results in mild cardiac hypertrophy and in a higher susceptibility to ischemia-reperfusion injury, whereas NCX1 gene deletion confers protection. In addition, NCX is widely recognized as a contributing factor to both impaired contractile performance and electrical instability like arrhythmia in human and experimental heart failure. It is also hypothesized that in salt-dependent hypertension, NCX1, operating in the reverse mode, may be involved in Ca^{2+} entry, thus resulting in vasoconstriction.

Recently, relevant roles have been attributed to NCX in brain disorders. In fact, several studies showed that NCX activation is desirable in numerous neurological disorders such as Alzheimer (Pannaccione et al. 2012; Sokolow et al. 2011), Parkinson (Sirabella et al. 2018), multiple sclerosis (Boscia et al. 2012; Casamassa et al. 2016), and stroke (Molinaro et al. 2008; Pignataro et al. 2004; Vinciguerra et al. 2014; Annunziato et al. 2007; Molinaro et al. 2016).

In particular, rats treated with NCX1 or NCX3 antisense and genetic modified mice lacking *ncx1* or *ncx3* display a remarkable enlargement of the infarct volume when subjected to stroke (Molinaro et al. 2016; Molinaro et al. 2008; Pignataro et al. 2004), whereas genetically induced NCX1 overexpression determines an amelioration of stroke (Molinaro et al. 2016). This evidence suggests that a pharmacological intervention aimed at increasing NCX expression and activity might be a promising perspective to reduce cerebral damage after ischemic insult.

More recently, the activity of NCX3 has been linked to myelination/demyelination processes occurring in multiple sclerosis. Indeed, silencing or knocking out NCX3 impairs the differentiation of oligodendrocyte precursor cells in mature oligodendrocytes producing myelin (Boscia et al. 2012), and the activation of NCX3 attenuates myelin damage and stimulates myelin repair (de Rosa et al. 2019).

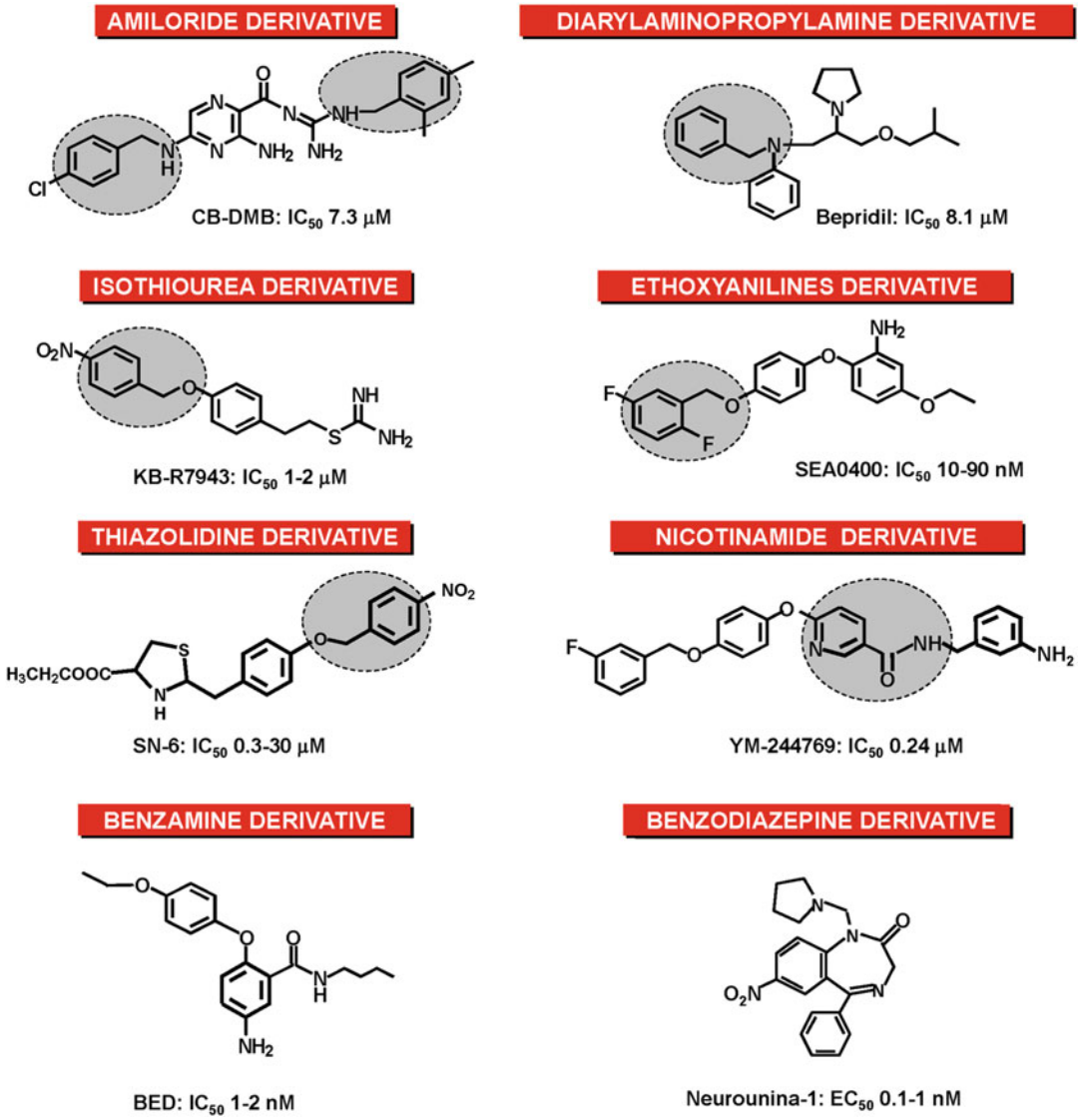
Drugs

Molecular Pharmacology of Na^+/Ca^{2+} Exchanger

The use of genetic-modified mice helped to discriminate the role played by each NCX gene product in several animal models mimicking human

pathological conditions. On the basis of results obtained, a great deal of interest has been devoted to find clinically effective drugs for those pathophysiological conditions, including cerebral ischemic disease, arrhythmias, heart failure, and hypertension, in which a stimulation or an inhibition of NCX might exert beneficial effects. To this aim, in the last 40 years, several inorganic and organic compounds have been reported to activate or to block NCX activity (Figure 3, Tables 2 and 3).

However, these compounds besides inhibiting NCX may also interfere with other cellular ion transporting mechanisms, i.e., Na^+ epithelial channels; K^+ channels; plasma membrane store-operated Ca^{2+} channels; VGCC-, NMDA-, and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate receptor-operated channels; Na^+/H^+ exchanger; Na^+/K^+ ATPase; and plasma membrane Ca^{2+} ATPase. In addition, other aspects that should be taken in consideration in the



Na^+/Ca^{2+} Exchangers, Fig. 3 Chemical structures of some NCX inhibitors. IC_{50}/EC_{50} values for NCX inhibition of drugs belonging to different chemical families. (Modified from Annunziato et al. (2004))

pharmacological modulation of NCX activity are (i) the existence of three different gene products NCX1, NCX2, and NCX3 that, although all share a high degree of amino acid sequence homology, display a different pharmacological sensitivity; (ii) the existence of at least 15 splicing variants for NCX1 and 5 for NCX3, each of them might be selectivity modulated by various compounds; and (iii) the double mode of operations of the exchanger, forward or reverse, that can be modulated in a differential manner by different drugs (Table 4). Thanks to site direct mutagenesis, a great advancement has been achieved in the last few years in the molecular pharmacology characterization of the domains, of the transmembrane segments, of the connecting loops, and finally of the single amino acids responsible of drug action (Fig. 1).

Na^+/Ca^{2+} Exchangers, Table 3 Compounds stimulating the Na^+/Ca^{2+} exchanger

Pharmacological class	Drugs
Redox agents	DTT, GSH, Fe^{2+}, O_2^- and of Fe^{3+}, H_2O_2
Heterocyclic compounds	Neurounina-1, CNPYB2
Inorganic cations	Li^+
Agonists of GPCRs	α, β agonists, histamine, 5HT $_{2c}$ agonists, endothelin-1, angiotensin-II
Peptides	Insulin, concanavalin A, tat-P1
Neurotrophic factors	NGF

Among the organic derivates, amiloride analogs were the first to be described as powerful inhibitors of NCX activity when the antiporter operates either in the forward or the reverse modes of operation. These compounds are reversible blockers and the inhibition is competitive with respect to Na^+ ions. The compounds of the amiloride family bearing substituents on the terminal guanidino hydrogen atom behave as a specific NCX inhibitor but are devoid of any effect on other antiporters such as Na^+/H^+ exchanger. Later, Shigekawa's and Iwamoto's groups identified an isothiourea derivative named KB-R7943 that is able to block the reverse mode operation of the antiporter in the low micromolar range, whereas concentrations 30 times higher are needed to

Na^+/Ca^{2+} Exchangers, Table 4 Molecular properties of drugs interacting with NCX

Drug	IC_{50}/ EC_{50} (μM)	Mode selectivity	Site of action	Site of action at amino acid level	Isoform specificity
Inhibitors					
Bepridil	8.1	Forw > Rev	f loop	–	–
BED	0.001–0.002	Rev > Forw	α_1/α_2 repeats	–	NCX3 > NCX2
CB-DMB	7.3	Forw = Rev	Na^+ binding site	–	NCX1 = NCX3 >NCX2
KB-R7943	1–2.5 >30	Rev > Forw	α_2 repeat	Ala809, Val820, Gln826, Gly833, Asn839	NCX3 > NCX2 > NCX1
SEA0400	0.05–0.09	Rev > Forw	TMS5, XIP α_2 repeat	Phe213, Gly833, Tyr224, Tyr226, Tyr228, Tyr231	NCX1 > NCX2
SN-6	0.3–30	Rev	TMS5, XIP α_2 repeat	Phe213, Gly833, Asn839, Val227, Val238	NCX1 > NCX3 > NCX2
Stimulators					
Neurounina-1	0.001	Forw > Rev	α_1/α_2 repeats	Val118, Asn125, Leu808	NCX1 > NCX2
GLU-XIP	0.1–1.0	Forw = Rev	f loop	652–688	NCX1, NCX2, NCX3
CN-PYB2	0.001	Rev > Forw	–	–	NCX1

inhibit the forward mode. Another peculiarity of this isothiourea compound is the different ability to block NCX activity depending on the gene product involved. In fact, NCX3 inhibition requires concentrations that are threefold lower than those necessary to inhibit NCX2 and NCX1. KB-R7943 interacts with α_2 repeat of the exchanger molecule at the level of Ala809, Val820, Gln826, Gly833, and Asn839. In light of these peculiar pharmacological properties, KB-R7943 has aroused a great deal of interest among investigators working on NCX activity. However, an increasing number of reports have shown that KB-R7943 also exerts an inhibitory effect on several other transport mechanisms such as L-type voltage-gated Ca^{2+} channels and receptor-operated NMDA channels.

In 2001, a new compound belonging to the ethoxyaniline family, SEA0400, was reported as being the most potent NCX inhibitor available at the time, IC_{50} = 5–92 nM, with a predominant activity on NCX1, a lower affinity for NCX2, and no effect on NCX3. However, the specificity of SEA0400 on NCX activity has recently been questioned, since it can also interfere with Ca^{2+} movement across the cell membrane. In 2002, by screening benzyloxyphenyl derivatives, Iwamoto's group discovered the new compound SN-6, which differs from KB-R7943 only in the substituent of phenyl moiety. The presence of this phenyl group confers to this antiporter inhibitor a more selective action on NCX1 rather than NCX3. Using chimeric analysis and subsequent site-directed mutagenesis, some critical amino acid residues responsible for SN-6 inhibition in the XIP region of the antiporter have been identified. Interestingly, SN-6 preferentially acts on the exchanger under ATP-depleted conditions (Iwamoto 2004).

In addition, the further screening of new benzyloxyphenyl derivatives revealed a highly potent NCX inhibitor, named YM-244769. This orally bioavailable compound is more potent inhibiting NCX3 than NCX1 and NCX2 in the reverse mode, but it is not active on the forward mode of operation of the three antiporter isoforms (Table 4).

Finally, two new compounds have been synthesized, 7-nitro-5-phenyl-1-(pyrrolidin-1-ylmethyl)-1H-bezo[e][1,4]diazepin-2(3H)-one (neurounina-1) that increases both NCX1 and NCX2 activity in a concentration-dependent manner (Molinaro et al. 2013), and 5-(2-chlorophenyl)-7-nitro-1-(pyrrolidin-1-ylmethyl)-1H-benzo[e][1,4]diazepin-2(3H)-one 2,2,2-trifluoroacetate (CN-PYB2), that selectively stimulates NCX1 activity in a concentration-dependent manner (Natale et al. 2020). More important, since NCX activity is involved in stroke pathophysiology, neurounina-1 has been reported as neuroprotectant agent in in vitro and in vivo model of adult and neonatal brain ischemia (Molinaro et al. 2013; Cerullo et al. 2018).

In addition to the conventional pharmacological strategies, new techniques were used to selectively inhibit each NCX gene product. These strategies consist in the antisense oligodeoxynucleotide, small interference RNA, and microRNA directed against mRNA encoding for NCX1, NCX2, or NCX3. On the contrary, when it is required to increase NCX expression and/or activity, it may be useful to use antimiRNA strategy to prevent NCX downregulation. For instance, miR-103-1 downregulates NCX1 in stroke; thus the antimiR-103-1 was proposed to reduce brain damage and neurological deficits in a preclinical model of stroke (Vinciguerra et al. 2014). This strategy, coupled with new nanotechnologies for drug delivery and release in CNS, might offer an additional opportunity to develop new effective therapies for stroke.

Glossary

CBD1 and CBD2 Calcium binding domain 1 and 2 of the f loop

f loop Intracellular domain between transmembrane segments 5 and 6 of the sodium calcium exchanger

Forward or Ca^{2+} efflux mode Mode of operation of NCX extruding calcium and intruding sodium ions

Na^+-dependent Ca^{2+} uptake Calcium uptake elicited by reduced concentrations of extracellular sodium

PIP_2 Phosphatidylinositol 4,5 bisphosphate

Reverse or Ca^{2+} influx mode Mode of operation of NCX extruding sodium and intruding calcium ions

XIP 20 amino acid region of the f loop of the sodium calcium exchanger

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Na^+/H^+ Exchangers

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Synonyms

Sodium/proton exchanger; Sodium/proton antiporter; Alkali cation/proton exchanger; Alkali cation/proton antiporter; Solute carrier 9 family

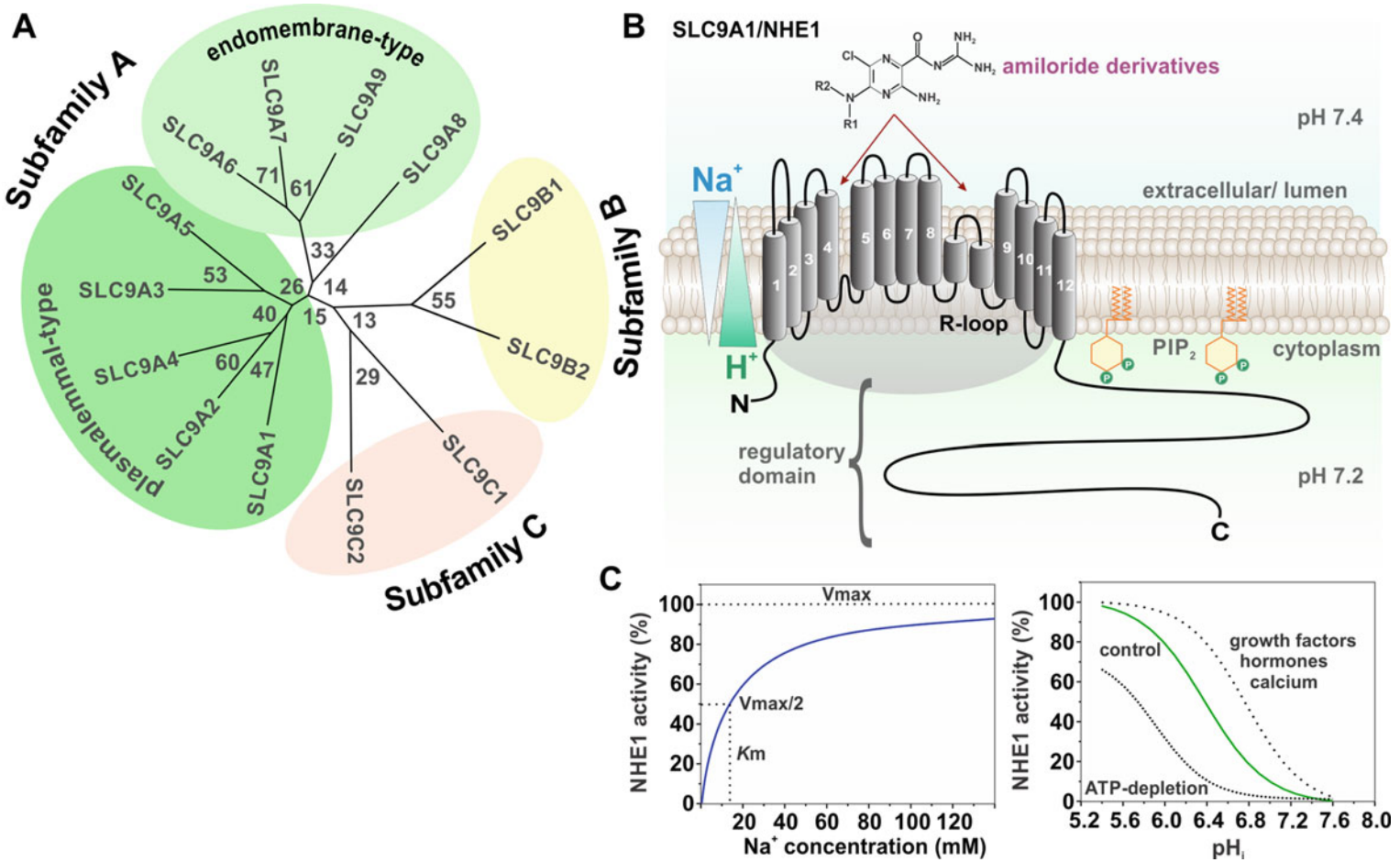
Definition

Sodium (Na^+)/proton (H^+) exchangers or antiporters (NHE or NHA) are a structurally diverse group of integral membrane proteins that mediate the counterion movement of Na^+ in exchange for H^+ across biological membranes of virtually all living organisms. Certain NHEs/NHAs also

transport other alkali metals or monovalent cations such as lithium (Li^+), potassium (K^+), and ammonium (NH_4^+). As such, this class of transporters is more aptly referred to as alkali or monovalent cation/proton exchangers or antiporters. Mammals encode 13 distinct *NHE/NHA* genes (classified as the solute carrier 9 (*SLC9*) family by the Human Genome Organization, HUGO), several of which undergo alternative splicing of their precursor mRNAs that has the potential to expand their functional diversity. These transporters are expressed in cell- and subcellular-specific patterns and exhibit unique substrate specificities, drug sensitivities, and regulatory properties. They are instrumental in regulating not only cellular and systemic pH, electrolyte and fluid volume homeostasis, but also in supporting other vital biological processes that range from organellar biogenesis, trafficking, and function to the control of cell differentiation, shape, adhesion, migration, proliferation, survival, and apoptosis. Given their involvement in such wide-ranging activities, it is not surprising that disruptions of their normal operations have been linked to various pathophysiologic conditions, including heart and brain ischemia/reperfusion injuries, diabetes, hypertension, congenital secretory diarrhea, infertility, tumor progression, and neurological disorders. Thus, there is considerable interest in developing therapeutic strategies to countermand these afflictions.

Basic Characteristics

The *SLC9* gene family consists of three subgroups: the *SLC9A* (encoding SLC9A1–9/NHE1–9), *SLC9B* (encoding SLC9B1–2/NHA1–2), and *SLC9C* (encoding SLC9C1–2/NHE10–11) subfamilies that share approximately 13–71% amino acid identity (Fig. 1a) (Brett et al. 2005; Casey et al. 2010; Pedersen and Counillon 2019). Several of the *SLC9* genes undergo alternative cis-splicing of their precursor mRNAs which in principal increases their protein diversity (Table 1), but evidence supporting unique functional properties for the different splice-variants is wanting. Much of our basic understanding of these transporters comes from studies of the SLC9A (NHE1–9) subfamily. NHE1 is present in most tissues whereas NHE2–5 are expressed in a tissue-specific manner (Table 2). Irrespective of their tissue distribution, all are primarily active at the plasma membrane where they preferentially exchange extracellular Na^+ (and to a lesser extent Li^+ and NH_4^+) for cytoplasmic H^+ (i.e., Na^+-selective NHEs) (Table 3). However, NHE3 and NHE5 are also present in intracellular vesicles that are thought to serve as reservoirs of functional transporters that can be rapidly mobilized to the plasma membrane (or conversely internalized from the cell surface) in response to various hormonal, metabolic, or physical stimuli. By contrast, NHE6–9 are widely expressed and reside predominately in organelles of the biosynthetic (i.e., Golgi cisternae, trans-Golgi Network, and post-Golgi vesicles) and endocytic (i.e., early/sorting, recycling, and late endosomes) pathways, though low levels can also be detected at the cell surface (Table 2). While their kinetic properties have not been extensively characterized, limited measurements indicate that endomembrane NHEs are capable of transporting not only Na^+ but also K^+, the main cytoplasmic monovalent cation, in exchange for H^+ (i.e., non-selective alkali cation NHEs). Structurally, members of the SLC9A/NHE and SLC9B/NHA subfamilies share a similar predicted membrane topology with 12 highly conserved transmembrane α-helices at their amino-termini responsible for ion translocation and less-conserved carboxy-termini that are involved in membrane-targeting and regulation of activity (Fig. 1b). By contrast, SLC9C1–2/NHE10–11 are projected to possess 14 transmembrane helices involved in ion exchange, plus an additional 4 transmembrane helices encoding a unique voltage-sensor, followed by a cyclic nucleotide binding domain located in the cytoplasm. However, all of the exchangers are presumed to assemble and function as homodimers. The NHEs/NHAs operate as electroneutral (e.g., $1Na^+:1H^+$) secondary active transporters that can reversibly exchange ions depending on their relative electrochemical concentration gradients. The plasma membrane Na^+-selective NHEs display classical Michaelis-Menten kinetics with respect to the extracellular Na^+ concentration (Fig. 1c). On the other hand, they exhibit positive cooperative binding of intracellular H^+ (i.e.,



Na^+/H^+ Exchangers, Fig. 1 Basic Characteristics of the *SLC9/NHE* Gene Family. (**a**) Genetic diversity and percent amino acids identity of SLC9/NHE isoforms. (**b**) Predicted transmembrane topology of NHE1. (**c**) Kinetic behavior of NHE1

Na^+/H^+ Exchangers, Table 1 Genomic diversity of the human SLC9/NHE gene family

Gene name	Transcript variant	Common name	Genomic locus	Amino acids	Mass (Da)	Comments	NCBI reference sequence
Subfamily A							
SLC9A1		NHE1	1p36.1	815	90,632		NM_003047
SLC9A2		NHE2	2q12.1	812	88,030		NM_003048
SLC9A3	SLC9A3v1	NHE3v1	5p15.3	834	92,724	v1 encodes the longest transcript	NM_004174
	SLC9A3v2	NHE3v2	5p15.3	825	91,774	v2 uses an alternate in-frame acceptor splice site in the mid-coding region, resulting in a shorter protein lacking 9 aa compared to v1	NM_001284351
SLC9A4		NHE4	2q12.1	798	86,886		NM_001011552
SLC9A5		NHE5	16q22.1	896	97,449		NM_004594
SLC9A6	SLC9A6v1	NHE6v1	Xq26.3	701	77,917	v1 encodes the longest transcript	NM_001042537
	SLC9A6v2	NHE6v2	Xq26.3	669	74,162	v2 uses an alternate in-frame splice site in the 5′ coding region, resulting in a shorter protein compared to v1	NM_006359
	SLC9A6v3	NHE6v3	Xq26.3	649	72,260	v3 lacks a portion of the 5′ coding region, and initiates translation at a downstream start codon compared to v1	NM_001177651
	SLC9A6v4	NHE6v4	Xq26.3	617	68,505	v4 differs in the 5’ UTR and 5′ coding region and uses a downstream start codon compared to v1	NM_001330652
SLC9A7	SLC9A7v1	NHE7v1	Xp11.3	726	80,217	v1 encodes the longest transcript	NM_001257291
	SLC9A7v2	NHE7v2	Xp11.3	725	80,131	v2 uses two alternate in-frame splice sites in the 3′ coding region compared to v1, resulting in a shorter protein	NM_032591

(continued)

Na^+/H^+ Exchangers, Table 1 (continued)

Gene name	Transcript variant	Common name	Genomic locus	Amino acids	Mass (Da)	Comments	NCBI reference sequence
SLC9A8	SLC9A8v1	NHE8v1	20q13.13	597	67,275	v1 encodes the longest transcript	NM_001260491
	SLC9A8v2	NHE8v2	20q13.13	581	65,422	v2 uses an alternate splice site in the coding region that maintains the reading frame, but results in a shorter protein	NM_015266
SLC9A9		NHE9	3q23-q24	645	72,565		NM_173653
Subfamily B							
SLC9B1	SLC9B1v1	NHA1v1 / NHEDC1v1	4q24	515	56,054	v1 encodes the longest transcript	NM_139173
	SLC9B1v2	NHA1v2 / NHEDC1v2	4q24	475	52,180	v2 differs in the 3′ coding sequence and UTR, resulting in a distinct C-terminus compared to v1	NM_001100874
SLC9B2	SLC9B2v1	NHA2v1 / NHEDC2v1	4q24	537	57,564	v1 encodes the longest transcript	NM_178833
	SLC9B2v2	NHA2v2 / NHEDC2v2	4q24	480	51,420	v2 differs in the 5' UTR and lacks an in-frame exon in the central coding region compared to v1	NM_001300754
	SLC9B2v3	NHA2v3	4q24	417	44,981	v3 lacks two alternate exons in the central and 3′ coding region, which results in a frameshift and has a distinct C-terminus, compared to v1	NM_001300756
	SLC9B2v4	NHA2v4	4q24	474	50,994	v4 has a distinct C-terminus compared to v1, but identical to v3	NM_001370199
	SLC9B2v5	NHA2v5	4q24	475	50,871	v5 lacks an in-frame exon in the central coding region compared to v1	NM_001370200

(continued)

Na^+/H^+ Exchangers, Table 1 (continued)

Gene name	Transcript variant	Common name	Genomic locus	Amino acids	Mass (Da)	Comments	NCBI reference sequence
Subfamily C							
SLC9C1	SLC9C1v1	NHE10v1 / sperm-NHEv1	3q13	1177	135,206	v1 encodes the longest transcript	NM_183061
	SLC9C1v2	NHE10v2 / sperm-NHEv1	3q13	1129	129,365	v2 lacks an alternate in-frame exon in the 5′ coding region compared to v1	NM_001320531
SLC9C2		NHE11	1q25.1	1124	129,053		NM_178527

Hill coefficient > 1). This feature allows the exchangers to have low activity at resting physiological pH (i.e., ~pH 7.2) but can activate quickly in response to slight increases in intracellular acidity (Fig. 1c).

The following section provides an overview of the tissue and subcellular distributions, functions, and pathophysiological disorders of SLC9 family members (summarized in Table 2). Additional details can be found in the following reviews (Orlowski and Grinstein 2011; Donowitz et al. 2013; Pedersen and Counillon 2019). Due to space constrains, it was not possible to cite much of the primary literature.

SLC9A Subfamily

***NHE1*:** NHE1 is responsible for maintaining cytoplasmic pH near neutral, thereby protecting cells against excessive acidification. NHE1 activity is also coupled functionally with Cl^-/HCO_3^- or anion exchangers to mediate the electroneutral absorption of NaCl, followed by the attendant uptake of water, into cells to restore cell volume following hyperosmotic-induced cell shrinkage. In addition to these housekeeping roles, NHE1 accumulates within certain microdomains of the plasma membrane in some cell types where it has been implicated in more specialized functions. For example, NHE1 accrues at the leading edge of migrating fibroblasts where it associates with the cytoskeletal-associated proteins ezrin, radixin, and moesin. This interaction influences cell shape and facilitates remodelling of the cortical actin cytoskeleton during adhesion and migration. In rat heart, NHE1 localizes at intercalated discs and transverse tubules of both atrial and ventricular myocytes where it is postulated to regulate the pH microenvironment of pH-sensitive proteins at the intercalated discs (e.g., connexin43) and cytoplasmic surface of sarcoplasmic reticulum cisternae (e.g., ryanodine receptors), thereby modulating impulse conduction and excitation-contraction coupling. In mouse hippocampus, NHE1 resides predominantly at presynaptic nerve terminals of both excitatory glutamatergic and inhibitory γ-aminobutyric acid (GABA)-ergic neurons, suggesting an important role in synaptic transmission.

Despite its involvement in numerous physiological processes, ablation of *Nhe1* expression by gene knockout (KO) in mice is not embryonic lethal, but the homozygous mutants suffer from postnatal growth retardation, ataxia, seizures, and significant mortality (~2/3 of pups die by 4 weeks of age). Mutations in *NHE1* have also been documented in humans and are associated with related neurological deficits, including ataxia and epilepsy (Lichtenstein-Knorr Syndrome). The underlying mechanisms are complex and not fully resolved. The neuronal hyperactivity leading to convulsive episodes in constitutive *Nhe1* KO mice was initially attributed, at least in part, to upregulation of voltage-sensitive Na^+ channels in excitatory pyramidal cells of the hippocampus and cortex. However, ensuing analyses using conditional *Nhe1* KO mice indicated that the enhanced network excitability arose predominantly from depressed GABA release from

Na^+/H^+ Exchangers, Table 2 Functional characteristics of the mammalian SLC9/NHE gene family

Gene	Tissue distribution & membrane location	Principal physiological functions	Relations to pathophysiology
SLC9A1/ NHE1	Ubiquitous Plasma membrane/ basolateral in epithelia	Na^+ (Li^+, NH_4^+) /H^+ Cytoplasmic pH regulation Cell volume regulation Fluid secretion Cell shape, adhesion, migration, & proliferation	Ischemia/reperfusion injuries Diabetes-associated vascular hypertrophy Ataxia, seizures, postnatal lethal Lichtenstein-Knorr syndrome Reduced parotid gland secretion
SLC9A2/ NHE2	Multiple tissues (stomach, colon > prostate, testis, kidney > others) Plasma membrane/ apical in epithelia.	Na^+ (Li^+) /H^+ Fluid secretion (Re)absorption of HCO_3^- in renal distal tubules Maintenance of intestine epithelial tight junctions	Gastric achlorhydria Elevated levels of renin in renal cortex Absorptive defects in kidney, colon, and pituitary Reduced parotid gland secretion Low fertility
SLC9A3/ NHE3	Kidney, intestines, other epithelial tissues, brainstem Apical membrane & subapical vesicles of epithelia	Na^+ (Li^+, NH_4^+) /H^+ Na^+ (direct), HCO_3^-, Ca^{2+} and H_2O (indirect) (re)absorption in kidney and intestines Early endosome acidification Regulation of brainstem control of breathing	Diarrhea Metabolic acidosis, hyperkalemia, urinary salt wasting, and hypotension Renal tubular proteinuria Decreased cortical bone mineral density and trabecular bone mass Sudden infant death syndrome?
SLC9A4/ NHE4	Stomach, kidney, Plasma membrane / basolateral in epithelia	Na^+ (NH_4^+) /H^+ Cytoplasmic pH regulation Fluid secretion NH_4^+ absorption by medullary thick ascending limb	Impaired gastric acid secretion Hyperchloremic metabolic acidosis Impaired urinary net acid excretion
SLC9A5/ NHE5	Multiple tissues (spleen > > testis > brain) Plasma membrane & intracellular vesicles	Na^+ (Li^+)/H^+ Cytoplasmic pH regulation Modulation of dendritic spine growth in response to neuronal activity	Elevated BDNF/TrkB signaling, learning, and memory
SLC9A6/ NHE6	Ubiquitous Plasma membrane & recycling endosomes Stereocilia of sensory hair cells	(Na^+, K^+)/H^+ Recycling endosomal and hair cell stereocilia pH regulation Maintenance of apical bile canalicular membranes in polarized hepatocytes	Christianson syndrome Autism spectrum disorder
SLC9A7/ NHE7	Ubiquitous *trans*-Golgi network & associated secretory vesicles	(Na^+, K^+)/H^+ Organellar pH regulation	Intellectual disability Overexpression enhances breast tumor growth and formation
SLC9A8/ NHE8	Ubiquitous Apical membranes in renal and intestinal epithelia Golgi cisternae & *trans*-Golgi network; late endosomes	(Na^+, K^+)/H^+ Renal and intestinal Na^+ absorption, Modulates late endosomal morphology and protein trafficking along the degradative pathway	Impaired mucin synthesis and HCO_3^- secretion in stomach and colon Reduced ocular surface function and degeneration of retinal pigmented epithelial and photoreceptor cells Male infertility
SLC9A9/ NHE9	Ubiquitous Plasma membrane & recycling endosomes Stereocilia of sensory hair cells	(Na^+, K^+)/H^+ Recycling endosomal and hair cell stereocilia pH regulation	Attention-deficit hyperactivity disorder Autism-spectrum disorder

(continued)

Na^+/H^+ Exchangers, Table 2 (continued)

Gene	Tissue distribution & membrane location	Principal physiological functions	Relations to pathophysiology
SLC9B1/ NHA1	Testis, sperm	Unknown (N.B., Drosophila SLC9B1/NHA1 purportedly operates as a H^+-Cl^- cotransporter)	Reduced sperm motility & fertility
SLC9B2/ NHA2	Multiple tissues Plasma membrane & recycling endosomes Synaptic-like microvesicles in β-cells Mitochondria[1]	Na^+ (Li^+)/H^+; Na^+/Li^+ Clathrin-mediated endocytosis Does not regulate endosomal pH Indirectly influences secretagogue-induced, but not basal or depolarization-induced, insulin secretion	Essential hypertension and diabetes? Impaired glucose tolerance and insulin secretion in pancreatic β cells Reduced sperm motility & fertility Promotes cystogenesis in polycystic kidney disease
SLC9C1/ NHE10	Testis, sperm, Plasma membrane of flagella	Required for bicarbonate-dependent stimulation of soluble adenylyl cyclase	Reduced sperm motility & fertility
SLC9C2/ NHE11	Testis, sperm	Unknown	Unknown

Information obtained from References (Orlowski and Grinstein 2011; Donowitz et al. 2013; Khayat et al. 2019; Pedersen and Counillon 2019; Prasad et al. 2019) and citations therein

Na^+/H^+ Exchangers, Table 3 Cation substrate affinities of SLC9/NHE

	SLC9A1 NHE1	SLC9A2 NHE2	SLC9A3 NHE3	SLC9A4 NHE4	SLC9A5 NHE5	SLC9A8 NHE8
Apparent Cation Affinities ($K_{0.5}$)						
Na^+ (mM)	10–16	18–50	4.7–17.0	12	18.6	23–130
Li^+ (mM)	3.4–9.0	2.2–3.5	2.6–7.8		0.32	
K^+ (mM)	19.5	N.A.	N.A.		N.A.	75
Rb^+ (mM)						>200
H^+ (μM)	0.18–0.4	0.13–0.14	0.12–0.35	0.62	0.37	0.32

Values obtained from References (Yu et al. 1993; Peti-Peterdi et al. 2000; Szabó et al. 2000; Chambrey et al. 2001; Nakamura et al. 2005; Xu et al. 2008; Pedersen and Counillon 2019). Range of values for certain substrates reflects analyses of NHEs from different mammalian species (rat, rabbit, and human) and assay conditions

parvalbumin-positive inhibitory interneurons rather than changes in the intrinsic excitatory properties of glutamatergic neurons (Bocker et al. 2019).

On the other hand, NHE1 over-activity contributes to ischemia-reperfusion injury in energy-demanding tissues such as heart and brain. Under hypoxic conditions, cells switch from aerobic to anaerobic metabolism of the limited stores of glucose and glycogen leading to intracellular acidification (due to lactate and H^+ buildup). While activation of NHE1 can rapidly restore intracellular pH, the concomitant increase in intracellular Na^+ cannot be expelled effectively by the Na^+/K^+-ATPase due to depleted ATP levels. Disruption of the transmembrane Na^+ and K^+ gradients triggers a cascade of events leading to a pathological rise in intracellular Ca^{2+} and activation of numerous signaling pathways that exacerbate and accelerate cardiovascular and cerebrovascular injury, a phenomenon known as excitotoxicity. A damaging role for NHE1 in these circumstances is supported by studies showing that specific NHE1 antagonists or genetic ablation alleviate injuries associated with cardiac and neural ischemia both in vitro and in vivo (Karmazyn 2013). Finally, elevated NHE1 activity is implicated in cancer and has been shown to promote tumorigenesis at multiple levels including cell proliferation, migration, invasion, and suppression of apoptosis. Inhibition

of NHE1 attenuates primary tumor growth and metastasis in animal studies, and thus may be an additional target in the design of potent combination chemotherapies (Stock and Pedersen 2017).

Aside from intracellular acidification and cell shrinkage, NHE1 activity can also be regulated by covalent modifications to its C-terminal tail. Phosphorylation by extracellular-regulated kinases 1 and 2 (Erk1/2), calmodulin kinase II (CaMKII), and p38 kinase are stimulatory and allow the exchanger to be active at more alkaline pH. Protein-protein interactions with calmodulin, carbonic anhydrase II, and calcineurin homologous proteins 1, 2, and 3 (CHP1–3) are also known to increase NHE1 activity. Finally, phosphoinositides located in the inner leaflet of the plasma membrane, particularly phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2 or PIP_2), are a key component in NHE1 function since a reduction of PIP_2 levels dramatically decreases NHE1 activity. As a result, NHE1 activity is indirectly dependent on ATP levels because PIP_2 levels are reduced in low-ATP states.

***NHE2*:** NHE2 is highly expressed on the apical membranes of epithelial cells in the gastrointestinal tract and kidneys, among other tissues. NHE2 is thought to contribute to cytoplasmic pH homeostasis as well as luminal Na^+ and indirectly bicarbonate absorption; however, *Nhe2* KO mice display no apparent intestinal or renal absorptive defects. This is likely due to compensatory increases in NHE3 activity, as the expression patterns of these two isoforms overlap considerably. NHE2 plays an important role in gastric parietal cell viability; loss of NHE2 in mice leads to parietal cell degeneration and decreased acid secretion in the stomach. Finally, NHE2 has a key function in repairing epithelial cell damage, as NHE2 KO mice display an impaired ability to repair the epithelial wall and restore tight junctions following damage to the stomach and intestines.

Like NHE1, basal NHE2 activity is dependent on binding CHP1 and is activated by stimuli that elevate protein kinase A (PKA) and protein kinase C (PKC). Interestingly, regulation of NHE2 is sometimes dependent on its location. For example, epidermal growth factor (EGF) increases NHE2 levels in the intestines but does not affect its expression in the kidneys. Similarly, extracellular acidosis stimulates an increase in NHE2 mRNA in the intestines but leads to a decrease in NHE2 mRNA in the kidneys.

***NHE3*:** Similar to NHE2, NHE3 is highly expressed in the apical membrane of intestinal and renal epithelial cells. In addition to its localization at the plasma membrane, NHE3 is present in subapical vesicles that can be trafficked rapidly to and from the cell surface membrane in response to different stimuli. NHE3 plays a major role in renal and intestinal Na^+ absorption, which is critical for water reabsorption and urine concentration. In addition, transport of H^+ ions into the renal lumen is one of the major pathways for bicarbonate reabsorption. *Nhe3* KO mice have diarrhea, increased urine output, decreased blood pressure, and mild acidosis. Thus, despite overlapping expression with NHE2 and NHE8, NHE3 is thought to be the major intestinal and renal NHE.

Loss-of-function mutations in NHE3 have been documented in patients with congenital sodium diarrhea (CSD), a rare condition characterized by severe, uncontrollable diarrhea with high fecal Na^+ loss from birth. Decreased NHE3 activity has also been observed in patients with inflammatory bowel disease (IBD) and may contribute to diarrhea in these individuals as well. In contrast, NHE3 overactivity may be a contributing factor to hypertension and thus could be a potential therapeutic target, although this link has only been documented in animal studies thus far.

NHE3 activity is responsive to both local and systemic stimuli. In the gut, NHE3 activity is adjusted in response to digestion, whereas renal NHE3 activity is regulated in response to total body volume and acid-base homeostasis. Hormonal stimulation of PKA, which phosphorylates the C-terminal tail of NHE3, inhibits its activity and modulates its subcellular location. Activation of PKC also downregulates NHE3 in a similar manner, but this seemingly occurs independently of direct phosphorylation by PKC. Conversely, phosphorylation by protein kinase CK2 or serum- and glucocorticoid-inducible kinase 1 (SGK1) stimulates NHE3 trafficking to the cell surface, therefore increasing its activity. This acute regulation of NHE3 activity also requires the binding of other ancillary proteins, such as CHP1, ezrin, and Na^+/H^+ exchanger regulatory factors 1 and 2

(NHERF1 and NHERF2). In addition to these acute regulatory mechanisms, NHE3 is regulated at the transcriptional and translational levels. Glucocorticoids, thyroxine, aldosterone, butyrate, and chronic metabolic acidosis lead to increased expression of NHE3, whereas chronic exposure to proinflammatory cytokines interferon-gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) lead to a reduction in NHE3 levels.

NHE4: NHE4 is expressed at high levels in the basolateral membranes of stomach parietal and chief cells as well as epithelial cells of the renal thick ascending limb and distal convoluted tubule. NHE4 is also present at lower levels in renal collecting ducts and proximal tubules. In stomach, NHE4 activity is coupled with the Cl^-/HCO_3^- exchanger 2 or anion exchanger 2 (AE2). Together, these two exchangers facilitate the electroneutral absorption of NaCl and, therefore, water absorption. *Nhe4* KO mice display reduced stomach acid secretion, a decreased number of parietal and mature chief cells, and an increased number of necrotic and apoptotic cells. Notably, this phenotype is similar to that of *Ae2* KO mice and suggests that the physiological role of NHE4 is to maintain cell volume and protect against apoptosis during acid secretion. In the kidney, NHE4 is important for ammonia excretion in the urine, where it is thought to catalyze Na^+/NH_4^+ exchange and serves as a principal means of renal acid secretion. *Nhe4* KO mice display reduced NH_4^+ reabsorption, metabolic acidosis, and a distinct inability to increase renal net acid secretion upon oral acid load. In rat and human colon crypt epithelia, NHE4 is activated in response to aldosterone, but not to cAMP or hyperosmolarity. Conversely, in rabbit gastric epithelium, cAMP and hyperosmolarity elevate NHE4 activity. Thus, the regulation of NHE4 is also cell-type dependent. At present, there are no known human diseases related to NHE4 dysfunction.

***NHE5*:** NHE5 transcripts are detected at low levels in most tissues but are highest in spleen $>>$ testis $>$ brain. However, most studies have examined its roles in brain where it localizes to discrete vesicles in neurons of the cerebral cortex, amygdala, hippocampus, and other regions. NHE5 has been shown to regulate pH of the postsynaptic compartment of hippocampal neurons which indirectly controls dendritic spine growth. Following synaptic activity, NHE5 is trafficked from recycling endosomes to the plasma membrane of dendritic spine heads. The increase in NHE5 activity leads to an acidification of the synaptic cleft, which inhibits N-methyl-D-aspartate (NMDA) receptors and attenuates dendritic spine growth. Conversely, NHE5 KO mice, while outwardly normal, display increased synaptic spine density in the brain and enhanced learning and memory that correlates with elevated brain-derived neurotrophic factor (BDNF)/tyrosine receptor kinase B (TrkB) signaling. In addition to its role at the plasma membrane, NHE5 contributes to the endosomal acidification and cell surface trafficking of the TrkA receptor, which in turn promotes neurite outgrowth in rat neuroendocrine PC12 cells treated with nerve growth factor (NGF). While mutations in NHE5 have not been linked to human disease, a study in rats showed an elevated level of NHE5 in C6 glioma cells is responsible for elevated MET and EGF tyrosine kinase receptor signaling and increased tumor growth, proliferation, and invasion.

NHE5, like NHE3, is acutely regulated by trafficking between the plasma membrane and recycling endosomes. Internalization of NHE5 relies on an interaction with β-arrestin2, which depends upon phosphorylation of an acidic serine/threonine di-isoleucine motif in its C-terminal tail by protein kinase CK2. Filamentous actin dynamics are also important for its internalization, as disruption of the actin cytoskeleton with cytochalasin D increased NHE5 abundance at the plasma membrane. On the other hand, trafficking of NHE5 from endosomes to the plasma membrane is enhanced by interactions with secretory carrier membrane protein 2 (SCAMP2) and AMP-activated protein kinase (AMPK), the latter activated upon metabolic stress. Finally, NHE5 transport activity is inhibited by PKA, PKC, and cell shrinkage.

***NHE6*:** NHE6 is widely expressed but is especially abundant throughout the central nervous system. This isoform regulates the pH homeostasis and membrane trafficking of early and recycling endosomes. Mutations in NHE6 are linked to Christianson Syndrome, an X-linked

neurodevelopmental disorder characterized by developmental delay, epileptic seizures, ataxia, hyperkinetic behavior, and degeneration of cortical, hippocampal, and especially cerebellar neurons. It is thought that these neurological deficits are largely due to disrupted endosomal trafficking, as loss of NHE6 in neurons causes over-acidification of early and recycling endosomes and enhanced degradation of their neurotrophic (e.g., TrkB receptor) and ionotropic (e.g., AMPA receptors) cargo in hippocampal neurons. Consequently, these neurons display reduced dendritic arborization, decreased density of mature dendritic spines and impaired neuronal circuit strength and synaptic plasticity. Decreased NHE6 activity has also been linked to Alzheimer's disease. The acidic endosomal pH associated with reduced NHE6 activity correlated with more efficient production and secretion of amyloid beta (Aβ) from endosomes which may contribute to the formation of Aβ plaques.

The mechanisms responsible for regulating NHE6 activity remain largely unknown. However, RACK1 is known to interact with the NHE6 C-terminal tail and appears to have a role in regulating NHE6 trafficking between endosomes and the plasma membrane. Loss of RACK1 leads to a decrease in cell surface levels of NHE6 accompanied by an increase in endosomal pH. Thus, RACK1 activity could play a role in regulating endosomal pH and trafficking by altering NHE6 localization.

***NHE7*:** NHE7 expression is ubiquitous but especially abundant in the brain, skeletal muscle, and various secretory tissues. It localizes mainly to the *trans*-Golgi network (TGN), although lower levels are also detected in the Golgi cisternae as well as post-Golgi vesicles that deliver cargo to the plasma membrane. A gain-of-function mutation in NHE7 has been identified in two unrelated families with X-linked intellectual disability (Khayat et al. 2019). These individuals display moderate to severe cognitive dysfunction, hypotonia, and muscle weakness. This correlated with excessive alkalinization of the TGN and aberrant glycosylation of exported proteins.

The distribution of NHE7 between the TGN and vesicles seems to rely on interactions with SCAMP1, SCAMP2, and SCAMP5. For example, expression of a dominant-negative SCAMP2 results in NHE7 redistribution to recycling endosomes. On the other hand, the factors that regulate NHE7 ion exchange activity are largely unknown. One study, however, has shown that calmodulin has a stimulatory effect on NHE7 activity.

NHE8: NHE8 is expressed in multiple tissues and is found in the *mid*- and *trans*-Golgi cisternae as well as late endosomes/multivesicular bodies. Similar to the other endomembrane NHEs, NHE8 is thought to play a role in regulating the pH of these compartments. NHE8 also seems to be important for endosomal trafficking, as NHE8 knockdown in HeLa cells disrupts protein trafficking and causes late endosomal and lysosomal clustering around the perinuclear region.

In addition to its intracellular localization, NHE8 is found on the apical membrane of epithelial cells in renal proximal tubules and gastrointestinal tract. Like NHE3, NHE8 is involved in Na^+ and bicarbonate reabsorption in the kidney. Notably, NHE3 and NHE8 appear to be developmentally regulated such that NHE8 is the primary brush border NHE in neonates and NHE3 is predominant in adults. Despite this, NHE8 KO mice display no renal deficits due to a compensatory upregulation of NHE3 surface expression and activity. NHE8 KO mice also show no indication of absorptive deficits in the intestines due to compensation by both NHE2 and NHE3. However, NHE8 appears to have a more critical role in mucosal protection of the stomach and intestines, as NHE8 KO mice display decreased mucosal pH and increased susceptibility to ulcers. Consistent with this, decreased NHE8 levels have been documented in the colons of ulcerative colitis patients (Li et al. 2016).

NHE8 activity can be acutely regulated by altering its trafficking to and from the plasma membrane. For example, acid loaded cells display increased NHE8 surface expression despite unchanged total cell protein levels. NHE8 activity is also upregulated at the transcriptional level by somatostatin, but downregulated by glucocorticoids, TNF-α, and EGF signaling.

NHE9: NHE9 is ubiquitously expressed and largely confined to a subpopulation of recycling endosomes that overlap partially with NHE6. Mutations in NHE9 have been linked to attention deficit hyperactivity disorder, autism spectrum disorder, and epilepsy. Autism-associated variants of NHE9 were found to disrupt trafficking of the glutamate aspartate transporter (GLAST) and glutamate uptake in astrocytes, thereby disrupting normal neuronal function. Abnormal elevation of NHE9 activity has also been linked to cancer progression, particularly glioblastomas, and correlated with enhanced EGF receptor signaling that promoted tumor growth, enhanced cell migration, and poor survival outcomes.

SLC9B Subfamily

***NHA1–2*:** NHA1 is expressed exclusively in the testes, where it is localized specifically to sperm flagella. While the properties of NHA1 transport are unknown, loss of NHA1 in mice causes a partial reduction in sperm motility and, consequently, reduced pregnancy rates in females. NHA2 is also found in sperm flagella and its knockdown results in a similar phenotype. Loss of NHA1 leads to a compensatory upregulation of NHA2 and vice versa, indicating these two proteins are functionally redundant in the testes. Double KO of NHA1 and NHA2 causes complete infertility in male mice.

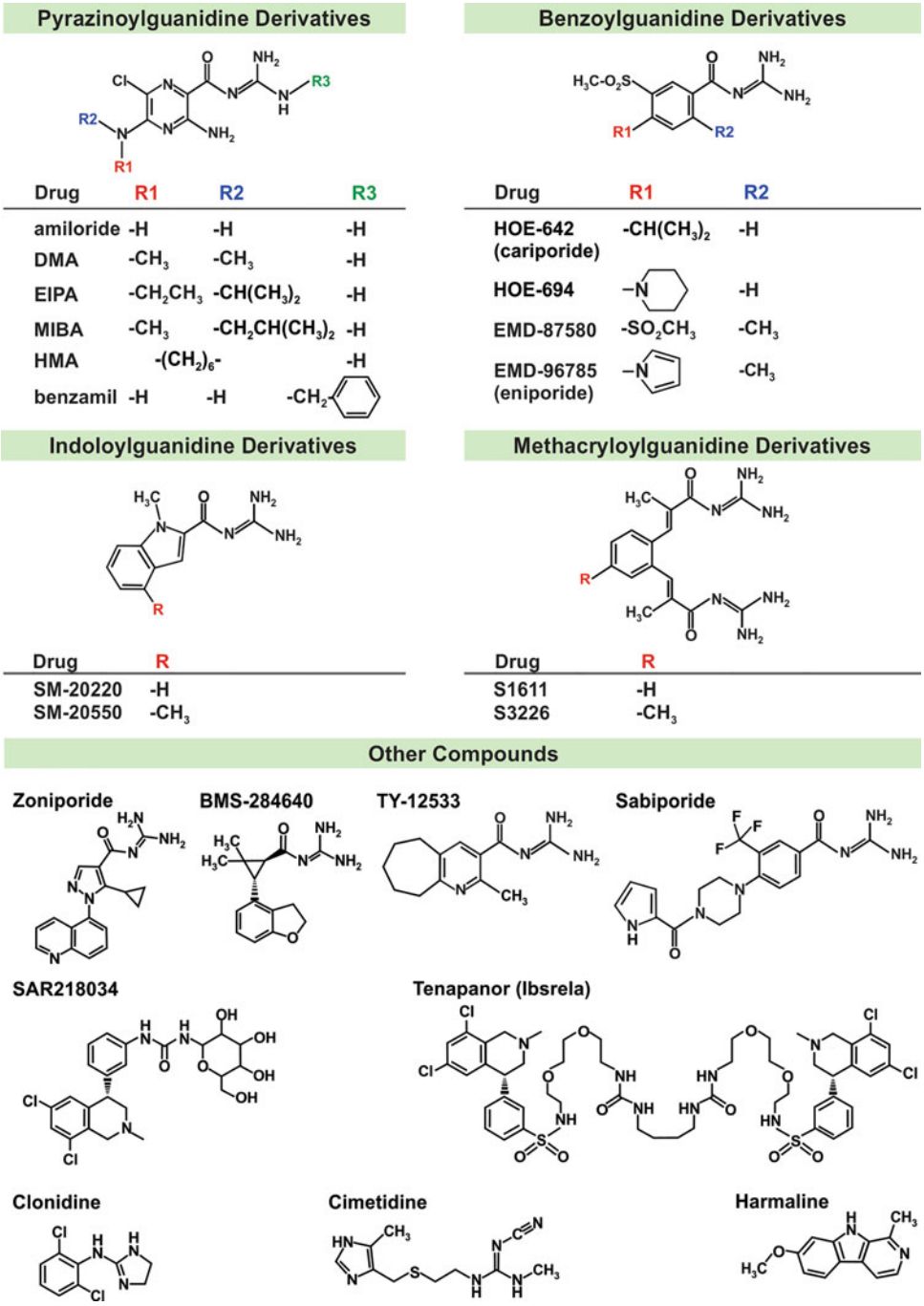
Unlike NHA1, NHA2 is ubiquitously expressed and appears to have specialized localizations and functions depending on the cell type. In β cells of the pancreas, NHA2 is localized to endosomes and has an important role in insulin secretion. NHA2-depleted mice have disrupted clathrin-mediated endocytosis, reduced secretagogue-induced insulin secretion, and abnormal glucose tolerance. Interestingly, however, loss of NHA2 does not affect endosomal pH in β-cells. NHA2 is also found at the plasma membrane of renal distal convoluted tubules where the fine-tuning of Na^+ reabsorption takes place to maintain systemic salt homeostasis. NHA2 has been postulated to be the Na^+/Li^+ countertransporter associated with essential hypertension.

SLC9C Subfamily

NHE10–11: NHE10 was originally referred to as the sperm NHE (sNHE) because it is expressed almost exclusively in the flagella of spermatozoa, a pattern similar to NHA1. Loss of NHE10 renders sperm immotile and causes complete infertility; a phenotype that could be rescued by alkalinizing agents, such as ammonium chloride, or cAMP analogues. Subsequent studies revealed that NHE10 forms a complex with an atypical soluble adenylyl cyclase (sAC/Adcy10) that is directly stimulated by bicarbonate. Activation of sAC activity produces cAMP that then binds to the cyclic nucleotide binding domain of NHE10 and allows it to activate at more depolarized membrane potentials. The resulting sustained NHE10 activity alkalizes the sperm and activates CatSper channels that facilitate Ca^{2+} influx. In turn, the elevated intracellular Ca^{2+} activates signaling pathways that enhance sperm motility. While mutations in *NHE10/SLC9C1* have not been linked to male infertility in humans, the exchanger could be a potential target for contraception. NHE11 is also predominantly expressed in testes, but virtually nothing is known about its function.

Drugs

A wide variety of drugs have been developed to inhibit NHE activity, particularly NHE1 and NHE3, in an effort to treat pathophysiologic conditions where these transporters are over-activated, such as cardiovascular and cerebral ischemic disease and hypertension (Fig. 2 and Table 4). The first drug identified as an NHE inhibitor was the potassium-sparing diuretic, amiloride [3,5-diamino-6-chloro-*N*-(diaminomethylidene)pyrazine-2-carboxamide], which consists of a guanidinium group connected to a pyrazine ring. Amiloride acts as a competitive inhibitor of Na^+ and consequently the potency of this drug is reduced by high Na^+ concentrations. However, amiloride is not a specific NHE inhibitor as it also inhibits epithelial Na^+ channels as well as Na^+/Ca^{2+} exchangers. To create inhibitors with higher potency and specificity for the NHEs,



Na^+/H^+ Exchangers, Fig. 2 Chemical structures of SLC9/NHE inhibitors

additional hydrophobic groups were added to create derivatives such as 5-(*N*-dimethyl)amiloride (DMA), 5-(*N*-ethyl isopropyl)amiloride (EIPA), MIBA, 5-(N-methyl-N-isobutyl)amiloride, 5-(N,N-hexamethylene)amiloride (HMA) and benzamil. Other modifications to amiloride resulted in a

Na^+/H^+ Exchangers, Table 4 Pharmacological properties of SLC9/NHE

	SLC9A1 NHE1	SLC9A2 NHE2	SLC9A3 NHE3	SLC9A4 NHE4	SLC9A5 NHE5	SLC9A8 NHE8
Apparent Drug Affinities (IC_{50} or K_i, μM)						
Amiloride	1–5	1–3	≥100	≈800	21	
DMA	0.023–0.100	0.25–0.70	11–14			
EIPA	0.015–0.020	0.08–0.86	2.4–3.3	2.5–9.0	0.42	
MIBA	0.032					
HMA	0.013		2.4		0.37	
Benzamil	120	320	100			
Cariporide	0.03–3.40	4–62	1–100	547	>30	
HOE694	0.085–0.160	5.0–7.6	640	247	9.1	~5
Eniporide	0.005–0.380	2–17	100–460		>30	
SM-20220	0.005–0.020					
SM-20550	0.010					
Zoniporide	0.059	12	>500			
BMS-284640	0.009	1800	>30		3.36	
TY-12533	0.017					
Sabiporide	0.05	3	>1000			
S1611	4.7	89	0.05			
S3226	3.6	80	0.02			~20
SAR218034	12.4		0.012–0.022		0.97	
Tenapanor	N.A.	N.A.	0.005–0.010			
Cimetidine	26	330	6200		230	
Clonidine	210	42	620		N.A.	
Harmaline	140	330	1000		940	

DMA, 5-(N,N-dimethyl)amiloride; EIPA, 5-(N-ethyl-N-isopropyl)amiloride; MIBA, 5-(N-methyl-N-isobutyl)amiloride and HMA, 5-(N,N-Hexamethylene)amiloride. Range of values for certain drugs reflects analyses of NHEs from different mammalian species (rat, rabbit and human) and assay conditions. N.A., no affinity. Values obtained from References (Counillon et al. 1993; Maidorn et al. 1993; Yu et al. 1993; Schwark et al. 1998; Kuribayashi et al. 1999; Peti-Peterdi et al. 2000; Szabó et al. 2000; Chambrey et al. 2001; Masereel et al. 2003; Touret et al. 2003; Nakamura et al. 2005; Xu et al. 2008; Linz et al. 2012; Spencer et al. 2014; Pedersen and Counillon 2019)

group of benzoylguanidine compounds which contain a phenyl group rather than a pyrazine ring. These include cariporide (HOE-642), HOE-694, eniporide (EMD-96785), EMD-87580 and sabiporide. Similar to the pyrazinoylguanidine derivatives, these drugs are specific NHE inhibitors and do not exert effects on Na^+ channels or Na^+/Ca^{2+} exchangers. Of the NHE isoforms, NHE1 is the most sensitive to amiloride and its derivatives, although other isoforms are also inhibited at higher concentrations (Table 4). NHE3 and NHE4 have markedly reduced affinities for these drugs. Importantly, NHE inhibitors with structures dissimilar to amiloride have also been developed. For example, zoniporide, SM-20220, SM-20550, and BMS-284640 contain bicyclic moieties and are highly selective NHE1 inhibitors. Drugs such as clonidine, cimetidine, and harmaline, which are used for other therapeutic applications, are also weak NHE inhibitors.

Significant interest has been placed on the study of NHE1 inhibition in the hopes of treating cardiac and cerebral ischemia-reperfusion injuries (reviewed in (Avkiran and Marber 2002)). Animal studies have demonstrated that NHE1 inhibition limits tissue damage during ischemia, such as reducing infarct size. However, there has been limited success in clinical trials thus far. A large-

scale phase II clinical trial concluded that eniporide administration during myocardial infarction had no effect on infarct size or clinical outcomes. However, another large-scale trial showed that cariporide had cardioprotective benefits, but only in patients undergoing coronary artery bypass graft surgery when the drug was administered prior to ischemia. Thus, NHE1 inhibitors may be useful for treating ischemia-reperfusion injury in the select cases where it can be administered in advance.

Pharmacological inhibition of NHE1 may also be beneficial for cancer treatment (reviewed in (Stock and Pedersen 2017)). NHE1 is thought to be upregulated in cancer cells, where its ion exchange activity maintains low intracellular H^+ concentrations while creating a highly acidic external tumor microenvironment. Thus, inhibiting NHE1 has the potential to cause intracellular acidosis and trigger apoptosis in cancer cells. NHE1 inhibitors have had considerable success in reducing cancer growth in cell studies; for example, HMA significantly reduced pH and induced apoptosis in human leukemic cells. Animal studies have also demonstrated a reduction in tumor growth, proliferation, and metastasis in response to amiloride treatment. Additional studies evaluating NHE1-specific inhibitors in animal models are needed to further evaluate the efficacy of NHE1 inhibition in cancer therapies.

In addition to NHE1 inhibitors, drugs that specifically inhibit NHE3 have been developed and include S3226, SAR218034, and Tenapanor. Tenapanor inhibits NHE3 activity in the intestines, which reduces the absorption of Na^+ and water from the gut and has received approval in the United States of America to treat patients with constipation-predominant irritable bowel syndrome (Markham 2019). By the same mechanism, Tenapanor may also be useful in treating hypertension. Finally, animal models indicate that S3226 may be beneficial in preventing acute renal failure following ischemia to the kidneys (Hropot et al. 2001), although this has not yet been tested in clinical trials.

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Na^+/K^+-ATPase

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Synonyms

Na $^+$/K $^+$-ATPase: Na $^+$/K $^+$ exchanging ATPase; Sodium pump; Sodium- and potassium-activated adenosine 5′-triphosphatase (EC 7.2.2.13)

Definition

The Na^+/K^+-ATPase is an integral protein of the plasma membrane of animal cells. By utilizing the energy from ATP hydrolysis, it transports 3 Na^+ ions from the cytosol to the extracellular space in exchange for 2 K^+ ions that are taken up from the extracellular medium.

Whereas Na^+ ions are moved against their electrochemical gradient, K^+ ions are taken up against their chemical gradient but following their electrical gradient.

This uneven transport of positive charge – 3 out versus 2 in – is coupled to various elementary functions of the cell: (i) it is the main contributor for the formation and maintenance of the membrane potential of the cell; (ii) it participates in the osmotic regulation of the cell volume; (iii) it provides the basis for neuronal communication in higher organisms; and (iv) it generates the driving force for all Na^+ gradient-coupled secondary active transport systems in all animal cells.

Basic Characteristics

The Subunit Composition of Na^+/K^+-ATPase

The Na^+/K^+-ATPase belongs to the P-type ATPases, a family of more than 50 enzymes that also includes the Ca^{2+}-ATPase of the sarcoplasmic reticulum or the gastric H^+/K^+-ATPase.

P-type ATPases have in common that during ion transport a phospho-intermediate is formed by transfer of the γ-phosphate group of ATP to an aspartic acid found at the beginning of the highly conserved sequence DKTGS/T (Scheiner-Bobis 2002).

The Na^+/K^+-ATPase is an oligomeric protein that consists of α and β subunits. Four different isoforms of the α subunit (α1, α2, α3, and α4) have been identified with relative molecular masses varying between 100 and 113 kDa. They cross the membrane ten times, forming transmembrane domains M1 to M10, and both amino and carboxy termini are localized on the cytosolic side (Scheiner-Bobis 2002). The three β subunit isoforms (β1, β2, and β3) are highly glycosylated proteins of about 60 kDa, with the mass of the protein moiety ranging from 36 to 38 kDa. The β subunit crosses the membrane only once, and the amino terminus is localized on the intracellular side of the membrane. In addition to the α and β subunits, which are absolutely required for catalytic activity, a third cytosolic peptide of 7–11 kDa from the family of FXYD proteins, originally referred to as the γ subunit, appears in some tissues to be involved in regulating the activity of Na^+/K^+-ATPase by influencing its interactions with ATP, ouabain, and Na^+ or K^+ ions. The quaternary structure Na^+/K^+-ATPase with all three subunits has been recently resolved (Morth et al. 2007, 2011).

Na^+/K^+-ATPase Acts as a Sodium-Potassium Pump

Although the ion-transporting process of the sodium pump can be summarized by the equation

$$\mathrm{ATP} + \mathrm{H_2O} + 3\mathrm{Na^+(in)} + 2\mathrm{K^+(out)} \rightarrow \mathrm{ADP} + \mathrm{Pi} + 3\mathrm{Na^+(out)} + 2\mathrm{K^+(in)},$$

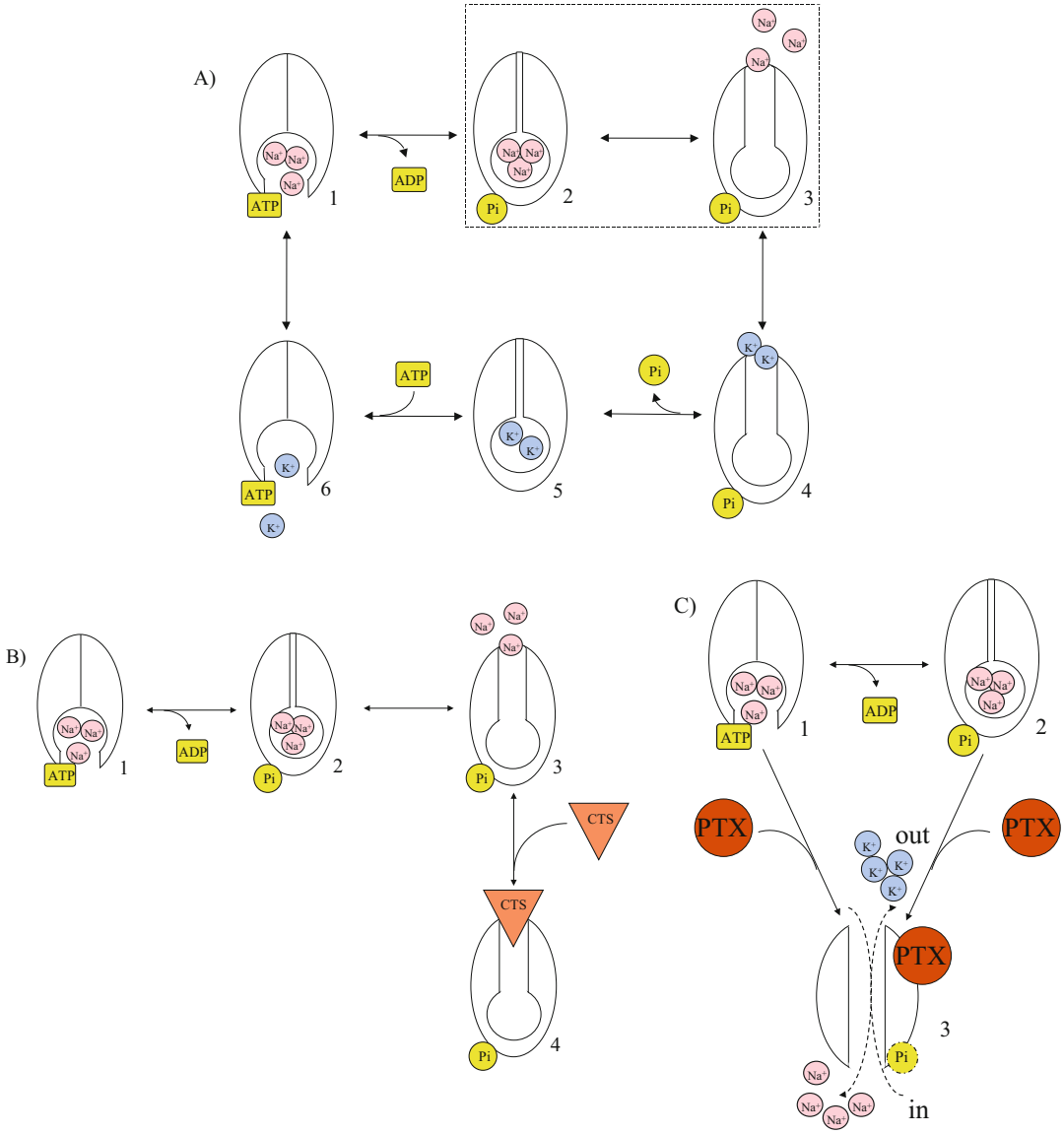
in reality the mechanism of ion translocation is sequential and far more complex than this equation implies. In order to ensure vectorial transport of the Na^+ or K^+ ions and the generation of an ion gradient, the ion-conducting structure of the sodium pump opens at any time of the ion-transporting process only towards one of the two surfaces of the membrane. This is associated with strong conformational changes in the enzyme protein structure, referred to as E_1 and E_2.

Thus, the catalytic cycle of the Na^+/K^+-ATPase can be described by juxtaposition of distinct reaction sequences that are associated with these two different conformational states (Scheiner-Bobis 2002). In the first step of the catalytic cycle, the enzyme in the E_1 conformation binds Na^+ and ATP with very high affinity (K_D values of 0.19–0.26 mM and 0.1–0.2 μM, respectively) (Fig. 1a, Step 1). After autophosphorylation by ATP at the aspartic acid within the sequence DKTGS/T, the enzyme occludes the 3 Na^+ ions (E_1-P($3Na^+$); Fig. 1a, Step 2) and releases them into the extracellular space after attaining the E_2-P $3Na^+$ conformation, characterized by low affinity for Na^+ ($K_{0.5}$ = 14 mM) (Fig. 1a, Step 3). The following E_2-P conformation binds 2 K^+ ions with high affinity (K_D approx. 0.1 mM; Fig. 1a, Step 4). The binding of K^+ to the enzyme induces a spontaneous dephosphorylation of the E_2-P conformation and leads to the occlusion of 2 K^+ ions ($E_2(2K^+)$; Fig. 1a, Step 5). Intracellular ATP accelerates the release of K^+ from the $E_2(2K^+)$ conformation (Fig. 1a, Step 6) and thereby also the return of the $E_2(2K^+)$ conformation to the E_1ATPNa conformation. The affinity of the $E_2(2K^+)$ conformation for ATP, with a $K_{0.5}$ value of about 0.45 mM, is very low.

Drugs

The Na^+/K^+-ATPase Is the Molecular Target for Cardiotonic Steroids (CTS) and Palytoxin

A vast number of toxins that specifically interact with the Na^+/K^+-ATPase and inhibit its catalytic activity have been isolated from various plants and animals (Fig. 2). Most of these substances are steroids, all characterized by the typical sterane backbone that is also found in steroid hormones. The sterane basic structure is made of three six-membered rings (A, B, and C) and a single five-membered ring (D) that form A/B, B/C, and C/D contacts. There are, however, several basic differences in the structures of the



Na^+/K^+-ATPase, Fig. 1 (**a**) The reaction cycle of Na^+/K^+-ATPase**.** In the E1 conformation the enzyme binds Na^+ and ATP with high affinity (Step 1) and becomes phosphorylated at a conserved aspartate residue by the γ-phosphate of ATP. That leads to the occlusion of 3 Na^+ ions within the transmembrane domains of the α-subunit (Step 2) followed by their release to the extracellular space (Step 3). This new conformational state, termed E_2-P, with low affinity for Na^+ binds K^+ with high affinity (Step 4) and occludes this cation after dephosphorylation of the aspartic acid (Step 5). Binding of ATP with low affinity to the $E_2(2\ K^+)$ conformational state leads to K^+ release into the cytosol (Step 6). The dashed box highlights the electrogenic steps of the catalytic cycle. (**b**) Binding of CTS to the sodium pump. CTS bind preferentially after Na + release to the E_2-P conformation of the enzyme and inhibit ATP hydrolysis and ion transport. (**c**) Binding of palytoxin to Na^+/K^+-ATPase. Palytoxin (PTX) binds either to the E1 or to the E1-P conformational state of the enzyme and arrests the natural ion-conducting structure of the α-subunit in a permanently open state (step 3). This rather unique conformation of a pump ion-conducting structure, that normally opens at any time towards one of the two surfaces of the plasma membrane, resembles the situation of ion channels, that, upon stimulation, retain their ion-conducting structure open towards both sides of the membrane. As a consequence of the ion channel–like conformation of the palytoxin/sodium pump complex, Na^+ and K^+cations flow down their concentration gradients (Na + into the cell, K+ out of the cell). The dotted line around the phosphate indicates that phosphorylation is not absolutely required

classical steroid hormones and the sodium pump inhibitors. Whereas in the classical steroid hormones the orientation of the A/B, B/C, and C/D contacts is trans-trans-trans, in steroidal inhibitors of the sodium pump the orientation is cis-trans-cis. In addition, the sodium pump steroidal inhibitors carry either a five-membered and single unsaturated (cardenolides) or six-membered and double unsaturated (bufadienolides) lactone ring at position C-17 (D-ring) (Fig. 2). At C-3 (ring A) both groups can carry one or several sugar molecules (glycosides). The most common sugars that are bound to natural cardiac glycosides are glucose, galactose, mannose, rhamnose, and digitalose. The sugar moiety itself has no activity but its addition to the steroid backbone affects the pharmacodynamics and kinetic profile of the particular glycoside. The type of sugar attached influences the potency of the compounds as it affects their absorption. The same applies for the lactone moiety of the CTS.

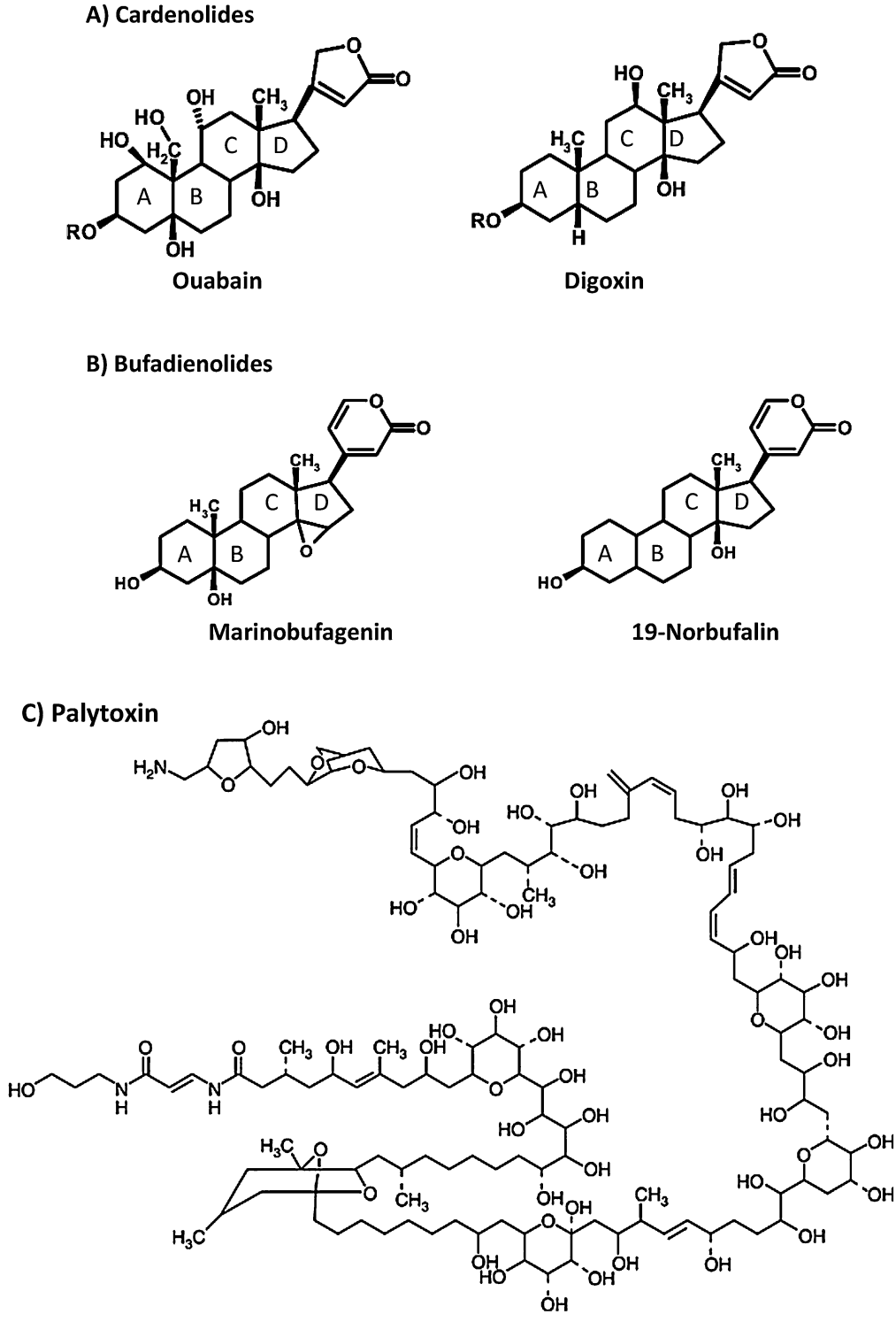
Plant-derived cardenolides or animal-derived bufadienolides are highly specific and strong inhibitors of the sodium pump. As a result of sodium pump inhibition, $[Na^+]i$ on the cytosolic side increases. In the myocardium, where the Na^+/Ca^{2+} exchanger (NCX) is present at high density, the cytosolic elevation of $[Na^+]i$ due to sodium pump inhibition forces the NCX into reverse mode. As a result, instead of extruding Ca^{2+} from the cytosol to the extracellular space, the reverse operational mode of NCX triggers $[Ca^{2+}]i$ elevation and a positive inotropic response in cardiac muscle cells. This mechanism that links sodium pump inhibition to cardiac Ca^{2+} elevation and inotropic response of the heart muscle constitutes the basis for the clinical use of several natural or synthetic cardenolides or bufadienolides in the treatment of chronic heart failure (NYHA II and IV), tachycardia, and dilatative cardiomyopathy. Because of their beneficial effects on the heart, cardenolides or bufadienolides are referred to as cardiotonic (=heart stimulating) steroids (CTS).

The Na^+/K^+-ATPase is the only enzyme known to interact with CTS, which reversibly bind to the extracellular side of the Na^+/K^+-ATPase at the E_2-P conformational state [E_2-P* ouabain] and inhibit ATP hydrolysis and ion transport (Fig. 1b, step 4).

Besides CTS, the highly toxic palytoxin (LD_{50} for rodents is 10–250 ng/kg), produced by corals of the genus *Palythoa*, is also a highly specific inhibitor of Na^+/K^+-ATPase (Fig. 2) (Scheiner-Bobis 2002). Unlike the CTS, however, which stop ATP hydrolysis and ion flow, palytoxin, by binding to either the E1 or the E_1-P conformation (Fig. 1c, steps 1 or 2, respectively), converts the sodium pump into an ion channel with a conductance of approximately 10 pS by arresting its natural ion-conducting structure in a permanently open conformation that allows K^+ to flow down its concentration gradient out of the cell and Na^+ into the cell (Fig. 1c, step 3). Thus, the reason for the high toxicity of palytoxin might be associated with the collapse of the membrane potential, and the ensuing loss of basic cell functions. In addition, depolarization is a key event that affects numerous secondary systems. Thus, the concentration of Ca^{2+} becomes elevated in several organs through the opening of Ca^{2+} channels and leads to the production of inositol trisphosphate, the activation of phospholipase A_2 and metabolism of arachidonic acid, and numerous other physiological responses that all stem from the increased Na^+ influx and the ensuing increase in the concentration of cytosolic Ca^{2+} that accompany the initial K^+ outflow. The strong tumor-promoter activity of palytoxin might be associated with these events (Wattenberg 2006).

Na^+/K^+-ATPase Acts as Signal Transducer for Cardiac Glycosides

The most popular assumption for the action of cardiac glycosides is that an inhibition or partial inhibition may induce inotropy of the heart by raising intracellular cytosolic Ca^{2+}($[Ca^{2+}]_i$) via a coupling of Na^+/K^+-ATPase with the Na^+/Ca^{2+} exchanger (NCX; Fig. 3). This Na^+ lag hypothesis has been recently modified: It is assumed that an inhibition of the sodium pump by cardiac glycosides may lead to a local increase of Na^+ in a tiny reactive space between the plasmalemma and the endoplasmic/sarco-plasmic reticulum (ER/SR) in smooth muscle cells and astrocytes, which has been named *plasmERosome* (Fig. 3) (Blaustein



Na^+/K^+-ATPase, Fig. 2 (continued)

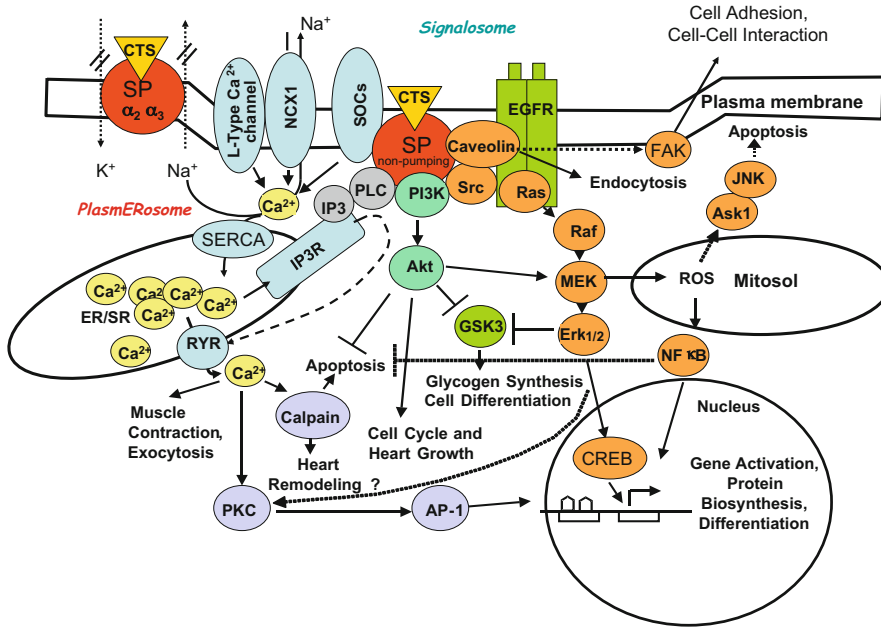
et al. 2000). This may explain why a partial inhibition of the sodium pump isoforms α_2 and α_3 located here result in a small, transient increase in the sub-plasmalemmal Na^+ concentration, which activates NCX1 and leads to a small local rise of Ca^{2+}. This, in turn, stimulates the release of more Ca^{2+} from the ER/SR to the cytoplasm. In arterial smooth muscle cells, this pathway is postulated to lead to arteriolar contraction and might be one of the reasons for essential hypertension.

Nevertheless, numerous recent investigations prove that interaction of CTS with the Na^+/K^+-ATPase located in caveolae at the plasma membrane leads to a series of events that trigger various intracellular signaling cascades. The same results are also obtained when CTS interact with nonpumping sodium pump mutants (Liang et al. 2006), indicating that a local $[Na^+]$ elevation followed by a $[Ca^{2+}]$ rise is not necessarily required for the induction of the signaling process. In this model, named *signalosome*, CTS-induced signaling events might differ depending upon the cell type and the nature of the CTS used. Figure 3 summarizes a series of signaling cascades proposed to explain the results from numerous investigations in various tissues. Thus, binding of CTS leads to a direct interaction of the amino-terminal end of the catalytic α subunit of Na^+/K^+-ATPase with the inositol trisphosphate (IP_3) receptor protein of the ER/SR and/or with phospholipase C (which raises IP3 levels) leads subsequently to a rise of $[Ca^{2+}]_i$. This promotes positive inotropy of the heart, arterial smooth muscle contraction, exocytosis, and leads to remodeling of the heart. Additionally, such effects may be induced by direct interaction of Na^+/K^+-ATPase within the plasma membrane with L-type Ca^{2+} channels. Elevated $[Ca^{2+}]_i$ leads to an activation of protein kinase C (PKC), which activates cardiac genes via transcription factor AP-1. This may lead to cardiac hyperplasia. Besides these possibilities, CTS may activate via the Na^+/K^+-ATPase-Src-caveolin-epidermal growth factor complex the Ras-Raf-MEK-ERK1/2 pathway. CTS-induced activation of the Ras-Raf-MEK-ERK1/2 pathway may also activate the formation of reactive oxygen species (ROS) in mitochondria that stimulate nuclear factor κB (NFκB). In kidney tubule and heart cells, activation of genes by this route leads to differentiation of the tissue. In epithelial cells this same signaling cascade involving Src-Ras-Raf-MEK-ERK1/2 stimulates transcription factors CREB and ATF-1 and promotes expression of tight junction proteins such as claudins and formation of blood-tissue barriers. In tumor cells, cardiac glycosides activate apoptosis via Ask1 and JNK, leading thereby to cell death, whereas in heart and kidney cells stimulation of ROS formation leads to the activation of NFκB and inhibition of apoptosis. The amino terminal end of the α subunit of Na^+/K^+-ATPase also interacts with phosphatidylinositol 3-kinase (PI3K). CTS binding to Na^+/K^+-ATPase may thus activate protein kinase B (Akt), which induces hypertrophy of the tissue and blocks apoptosis simultaneously. Akt also inactivates glycogen synthase kinase (GSK-3 α/β), which stimulates glycogen synthesis and is a master switch in regulating cell fate, specificity, and tumorigenesis. Binding of CTS to the Na^+/K^+-ATPase-Src-caveolin-epidermal growth factor complex stimulates the focal adhesion kinase (FAK), which alters cell adhesion and cell–cell interactions but also induces the endocytosis of Na^+/K^+-ATPase. This may then lead to the degradation of CTS. It is unclear whether the sodium pump is able to be recycled.

Endogenous Cardiac Glycosides Act as Hormones

Cardenolides and bufadienolides are generally considered to be of plant origin. Bufadienolides,

Na^+/K^+-ATPase, Fig. 2 Specific inhibitors of Na^+/K^+-ATPase: (**a**) Cardenolides are characterized by a five-membered lactone at position C17 of the steroid moiety. (**b**) Bufadienolides carry a six-membered lactone at C17. (**c**) Palytoxin ($C_{129}H_{223}N_3O_{54}$) produced by corals of the genus *Palythoa*. (R in ouabain is rhamnose; R in digoxin: 3 digitoxoses)



Na+/K+-ATPase, Fig. 3 Mechanisms of action of cardiac glycosides via the sodium pump in the plasmERosome and in the signalosome. The Na^+ lag hypothesis assumes the existence of a sub-plasmalemmal space called the plasmERosome. A cardioactive steroid (CST)–dependent transient local rise of $[Na^+]_i$ in this compartment caused by stimulation of store-operated channels (SOC) or by CTS-dependent inhibition of α_2 and α_3 sodium pump subunits may subsequently lead to a local rise of $[Ca^{2+}]_i$. This signal is amplified by filling up the sarco/endoplasmic reticulum (SR/ER) via the sarcoplasmic Ca^{2+}-ATPase (SERCA) to a higher extent with Ca^{2+}. Any agonist-stimulated Ca^{2+} release leads to an amplified Ca^{2+} release via the inositol tris-phosphate receptor (IP_3R) that stimulates the opening of the ryanodine Ca^{2+} channel (RYR), thus resulting in a higher degree of vasoconstriction. In a noninhibited Na^+/K^+-ATPase, Ca^{2+}in the PlasmERosome will be extruded by the Na^+/Ca^{2+}exchanger (NCX1) and the plasmalemmal Ca^{2+}-ATPase. When Na^+/K^+-ATPase acts as a signalosome in caveolae, endogenous and exogenous CTS induce various signal transducing pathways, even with a nonpumping enzyme. The abbreviations used are: AP-1 (activator protein 1), Ask-1 (apoptosis signal-regulating kinase 1), CREB (cAMP response element–binding protein), ERK 1/2 (extracellular signal–regulated kinases 1 and 2), FAK (focal adhesion kinase), GSK3 (glycogen synthase kinase 3), IP_3 (inositol (1,4,5) trisphosphate) IP_3R (IP_3 receptor), JNK (c-Jun N-terminal kinase), MEK (mitogen activated ERK-activating kinase, a MAP kinase), MKK (MAP kinase kinase), NCX1 (Na^+/Ca^{2+}exchanger), NF-κB (nuclear factor κB), PI3K (phosphatidylinositol 3-kinase), PKC (protein kinase C), PLC (phospholipase C), Raf (Raf, a MAP kinase kinase kinase), Ras (a MAP kinase kinase), ROS (reactive oxygen species), SOC (store-operated Ca^{2+} channel), Src (sarcoma kinase), SP (sodium pump)

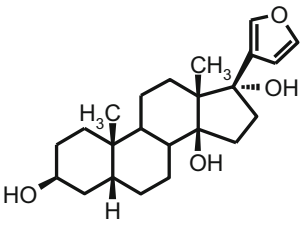
however, have also been found in the skin of *Bufo sp*. and have been considered for a long time to be the only CTS synthesized in animals. Numerous, more recent investigations have shown, however, that CTS are not only produced in various animals but also that they act as hormones that can trigger specific cell responses at nanomolar concentrations (Wattenberg 2006).

Several cardiac glycosides have meanwhile been isolated and identified in mammals (Schoner and Scheiner-Bobis 2007a, b, 2008). The cardenolide ouabain was isolated from human blood and bovine adrenal glands and hypothalamus. Digoxin was isolated from urine of humans not being treated with this drug. The bufadienolides marinobufagenin, telocinobufagin, and 19-norbufalin were isolated from human fluids and tissues. There is a high probability that the zona fasciculata cells of the adrenal cortex, and possibly also the hypothalamus, are able to synthesize cardenolides. An adrenal tumor secreting ouabain and producing hypertension has been described. Na^+-dependent release of ouabain, angiotensin II, endothelin, and

catecholamines from cells of the midbrain may stimulate the release of endogenous ouabain from adrenal glands. An increase of sympathetic tone in stress and physical exercise leads to a rapid rise of endogenous ouabain and to a rapid fall after discontinuation. A constant and small rise of ouabain plasma concentrations (in the nanomolar concentration range, either from endogenous or exogenous sources) leads to arterial hypertension and remodeling of the heart and the arterial wall. Fifty percent of Caucasians with uncomplicated arterial essential hypertension and low plasma renin activity have been shown to exhibit elevated concentrations of endogenous ouabain. Possibly, endogenous digoxin, whose biosynthesis has also been demonstrated to take place in the adrenal gland, counteracts the secretion of endogenous ouabain from midbrain cells and lowers the sympathetic tone. This suppressive effect of digoxin could represent a very important mechanism for the therapeutic action of exogenously applied digoxin as well. There is good evidence that the secretion of the bufadienolide marinobufagenin from adrenal glands is activated via angiotensin II and catecholamines from the adrenal glands when the Na^+ concentration increases in midbrain and hypothalamus. Marinobufagenin and telocinobufagin are also elevated in essential hypertension. Contrary to the case with other cardenolides, marinobufagenin shows a natriuretic action on kidney tubular cells containing mainly the α_1 isoform of Na^+/K^+-ATPase (Fedorova et al. 2001).

Rostafuroxin, A Cardenolide Derivative with Antihypertensive Action

If 50% of Europeans with essential hypertension are affected by this disease because of an elevated secretion of endogenous ouabain, then there might be a chance to block its interaction at the cardiac glycoside binding site of Na^+/K^+-ATPase and thus lower blood pressure. Initial studies involving the cardenolide analogue rostafuroxin (PST 2238; Fig. 4) demonstrated that very low concentrations of this compound can overcome ouabain-induced hypertension in experimental animals (Ferrari et al. 2006). These first promising results from animal experiments could not be confirmed in five double-blind, crossover phase-II studies involving 410 hypertonic (140–169 mmHg) patients; a blood pressure–reducing effect was not found at any of the tested concentrations of the drug (Staessen et al. 2011). This rather disappointing outcome should not, however, preclude any further investigations in the same direction.

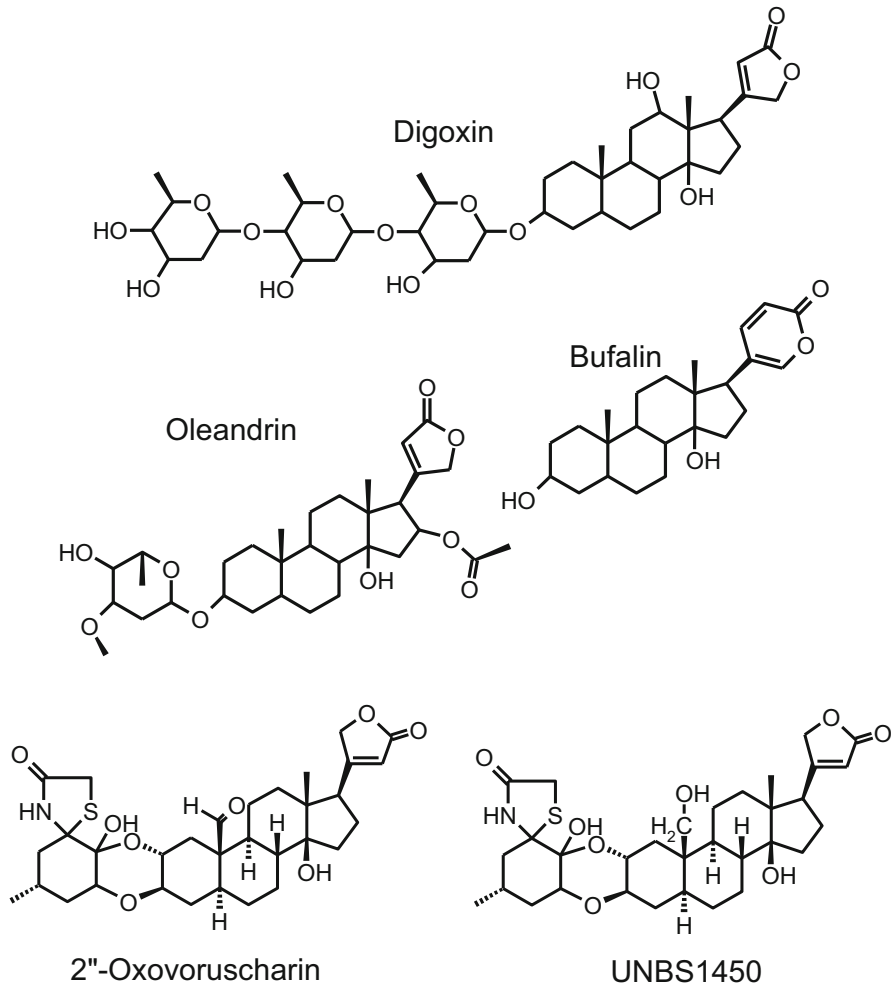


Ouabain antagonist
Rostafuroxin (PST 2238)

Na^+/K^+-ATPase, Fig. 4 Ouabain antagonist

Natural and Synthetic Cardenolides and Bufadienolides as Anti-Cancer Agents

There is increasing evidence that proliferation of cancer cells is affected by cardiac glycosides and that the extent of this effect depends on the nature of the steroid derivative and on the cancer cell line being used for in vitro testing (Prassas and Diamandis 2008; Menger et al. 2013; Slingerland et al. 2013). Thus, digitalis or ouabain have been shown to induce apoptosis in human breast cancer or neuroblastoma cells, respectively, whereas bufalin and the class of the more hydrophobic cardenolides like digoxin, oleandrin, and the 2″–oxovoruscharin and its derivative UNSB1450 (Fig. 5) have been shown in vitro and in vivo to have anti-cancer activity primarily in leukemia, lymphoma, melanoma, and ovarial and prostate cancer cells (Prassas and Diamandis 2008; Menger et al. 2013; Slingerland et al. 2013). Thus far, three cardiac glycosides have entered phase I clinical trials to determine their potency as anti-cancer agents. Two of them, Anvirzel™



Na^+/K^+-ATPase, Fig. 5 Cardiac glycosides and their derivatives with anti-cancer action

(extract from *Nerium oleander* containing the CTS oleandrin and oleandigenin) and PBI-02504 (more concentrated extract from *Nerium oleander*) had positive effects on a rather small percentage of treated patients. Thus, 7 out of 45 patients treated with PBI-02504 achieved stable disease conditions for more than 4 months (Hong et al. 2014).

The activity of the compound UNSB1450 has been tested in 57 human cancer models and it was always more potent than usual reference compounds such as irinotecan, mitoxantrone, oxaliplatin, paclitaxel, or temozolomide (Slingerland et al. 2013). UNSB1450 binds with high affinity to all α subunit isoforms of Na^+/K^+-ATPase. It affects the actin cytoskeleton and can therefore be considered not only antiproliferative but also anti-migratory (Slingerland et al. 2013). With the above background, UNSB1450 entered phase I clinical trials in Belgium. Unfortunately, however, the phase I study was interrupted in 2011 for financial reasons before reaching conclusions about the maximum tolerated dose.

Although at the current stage none of the CTS and derivatives tested have been certified as an anti-cancer remedy, the various phase I clinical studies are encouraging and should lead to continued investigation in this direction.

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N-Acetyl-5-Methoxytryptamine

► Melatonin

NAD(P)H Oxidase

► NADPH Oxidase

NAD(P)H:Oxygen Oxidoreductase

► NADPH Oxidase

NADPH Oxidase

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Synonyms

NAD(P)H oxidase; NAD(P)H:oxygen oxidoreductase

Definition

NADPH (nicotinamide adenine dinucleotide phosphate) oxidase (EC 1.6.3.1) is an enzyme that produces superoxide anions by transferring electrons from reduced NADPH (the electron donor) to oxygen (O_2). In the reaction, each NADPH molecule donates two electrons, which reduce two oxygen molecules, respectively, yielding two superoxide anions. NADPH oxidase is not a single protein but a multi-subunit protein complex (Babior 2004). While the term NADPH oxidase generally refers to such a protein complex, sometimes it is also ambiguously used to describe the catalytic subunit of the enzyme (aka the NOX subunit). The NADPH oxidase family contains different isoforms of the catalytic subunit, which may differ in the enzyme composition, subcellular localization, expression patterns, and/or biochemical properties. Some isoforms of NADPH oxidase produce hydrogen peroxide instead of superoxide.

Basic Characteristics

Prototype NADPH Oxidase

The prototype of NADPH oxidase was originally discovered in professional phagocytes (i.e., white blood cells or leukocytes that can ingest invading microorganisms), including neutrophils, eosinophils, monocytes, and macrophages. The basic composition of NADPH oxidase in phagocytes includes two membrane-bound subunits gp91phox (currently classified as NOX2) and p22phox; three cytosolic subunits p67phox, p47phox, and p40phox; as well as a small GTPase Rac1 or Rac2. Within the NOX2 protein, there is one molecule of FAD (flavin adenine dinucleotide) bound to the cytosolic C-terminus, as well as two heme groups located in the membrane-associated portion of the protein. FAD and likely the heme groups comprise the electron transfer mechanism that is essential for carrying out the redox reaction. In its inactivated state, the cytosolic subunits and Rac locate in the cytosol. Upon activation, these subunits translocate to the membrane and bind with NOX2 and p22phox. It is thought that activation of NADPH oxidase is caused by the migration of the cytosolic components to the cell membrane so that the complete oxidase can be assembled (Babior 2004).

Isoforms

In mammalian cells, the NADPH oxidase family contains seven isoforms of the NOX subunit, namely, NOX1 to NOX5 and DUOX1 and DUOX 2. It is thought that a specific NOX subunit determines the final enzymatic complex composition, modes of activation, and the products of the enzymatic reaction (Bedard and Krause 2007; Altenhofer et al. 2015).

NOX1: NOX1 is also known as NOH-1, MOX1, or GP91-2. NOX1 is predominantly expressed in colon epithelial cells and vascular smooth muscle cells. Activation of NOX1 requires the membrane-bound stabilizing subunit p22phox, two cytosolic subunits NOXO1 (in lieu of p47phox) and NOXA1 (in lieu of p67phox), and the small G protein Rac. NOX1 generates superoxide (Bedard and Krause 2007; Altenhofer et al. 2015).

NOX2: NOX2 is also known as gp91phox or CYBB. NOX2 is the prototype of NADPH oxidase. NOX2 is highly expressed in phagocytic leukocytes such as neutrophils and macrophages. NOX2 is also widely expressed in other types of cells at varying levels. Optimal activation of NOX2 requires the membrane-bound stabilizing subunit p22phox; three cytosolic subunits p67phox, p47phox, and p40phox; as well as

Rac. NOX2 generates superoxide (Bedard and Krause 2007; Altenhofer et al. 2015).

NOX3: NOX3 is also known as GP91-3. NOX3 is mainly expressed in the inner ear. NOX3 is also found to be expressed in various fetus organs (including the kidney, liver, lung, and spleen). Activation of NOX3 requires membrane-bound p22phox, cytosolic NOXO1, and probably NOXA1, but not Rac. NOX3 generates superoxide (Bedard and Krause 2007; Altenhofer et al. 2015).

NOX4: NOX4 is also known as RENOX, KOX-1, or KOX. NOX4 appears to be ubiquitously expressed in the body, as well as in various tumor cells. The activity of NOX4 requires membrane-bound p22phox, but does not require any cytosolic subunits. The activity of NOX4 appears to be largely influenced by its level of expression. The intracellular localization of NOX4 appears to be variable. In addition to the conventional plasma membrane compartment, NOX4 (probably a splice variant of NOX4 called NOX4D) has also been detected in the nucleus, mitochondria, and endoplasmic reticulum. NOX4 primarily generates hydrogen peroxide (Bedard and Krause 2007; Altenhofer et al. 2015).

NOX5: Like NOX3, NOX5 is also mainly expressed in fetus tissues. NOX5 activation is driven by intracellular calcium, which binds to the EF hand domains located in the N-terminal region of NOX5. The calcium sensitivity of NOX5 can be enhanced by calmodulin. The activity of NOX5 requires neither any cytosolic subunits nor the membrane-bound stabilizing subunit. NOX5 is also expressed in the testis and lymphoid tissues. NOX5 generates superoxide (Bedard and Krause 2007; Altenhofer et al. 2015).

DUOX1 and DUOX2: DUOX1 and DUOX2 are also, respectively, known as Thox1, LNOX1, or NOXEF1, and Thox2, LNOX2, or NOXEF2. DUOX1 and DUOX2 are mainly expressed in the thyroid gland. The membrane-bound stabilizing subunits for DUOX1 and DUOX2 are DUOXA1 and DUOXA2, respectively, instead of p22phox. Activation of DUOX1 and DUOX2 does not require cytosolic subunits, but is stimulated by calcium. Both DUOX1 and DUOX2 generate hydrogen peroxide (Bedard and Krause 2007; Altenhofer et al. 2015).

Biological Functions

The basic function of NADPH oxidase is to produce superoxide and hydrogen peroxide, two members of a group of oxygen-containing substances called reactive oxygen species (ROS). In the body, superoxide or hydrogen peroxide can be further converted to other ROS molecules, such as hypochlorous acid, singlet oxygen, hydroxyl radical, and peroxynitrite, via spontaneous or enzyme-catalyzed processes. In activated phagocytes, NOX2 functions to kill invading microorganisms and parasites by generating a large quantity of ROS. In human beings, a deficiency in NADPH oxidase function in phagocytes causes chronic granulomatous disease, which is characterized by severe infections that are extremely difficult to treat. About 60% of cases of chronic granulomatous disease are caused by mutations of the NOX2 gene; other cases can be caused by genetic mutations of p22phox, p67phox, or p47phox. Other confirmed functions of individual NADPH oxidase isoforms include the following:

1. In the inner ear, NOX3 plays a key role in the generation of otoconia, small calcium carbonate crystals found in the vestibular apparatus, which are utilized to perceive linear acceleration and gravity. Mice deficient in NOX3 are unable to form otoconia and therefore suffer from a disturbed equilibrium exhibiting a so-called “head-tilt” phenotype.
2. DUOX1/2 expressed in the thyroid is involved in the biosynthesis of thyroid hormones, which requires hydrogen peroxide (Babior 2004; Bedard and Krause 2007).

In addition to these specialized functions of NADPH oxidase in specific organs, evidence suggests that NADPH oxidases may have other more generalized functions in a spectrum of cells. Theoretically, all NADPH oxidases may change the redox balance inside cells by producing ROS, thereby influencing functions of certain proteins that are redox-sensitive. Many of these redox-sensitive proteins are involved in intracellular signal transduction. By modulating such cell signaling pathways, it is argued that NADPH oxidases may regulate a variety of biologic or pathophysiologic processes, including cell proliferation,

differentiation, survival, apoptosis, tumorigenesis, angiogenesis, fibrosis, inflammation, and tissue regeneration/wound healing. Under different conditions, a role of NADPH oxidases can be either beneficial or detrimental. On the other hand, evidence also suggests that aberrant expression or activation of NADPH oxidases may increase oxidative stress. Hence it is thought that NADPH oxidases may be involved in the pathogenesis of various oxidative stress-related diseases, such as cardiovascular disease, kidney disease, liver disease, lung disease, neurodegenerative disease, cancer, inflammatory disease, and aging. More specifically, current experimental data have pointed to a role of NOX1 in atherosclerosis, diabetic retinopathy, and liver fibrosis and a role of NOX4 in stroke, diabetic nephropathy, and osteoporosis (Bedard and Krause 2007; Altenhofer et al. 2015).

Drugs

Based on the rationale that oxidative stress may participate in the pathogenesis of different diseases, and the fact that NADPH oxidases are the only known enzymes solely dedicated to generate ROS, NADPH oxidase inhibitors are believed to be a promising therapeutic option for diseases associated with oxidative stress. Developing isoform-specific NADPH oxidase inhibitors seems to be a bottleneck in research; so far all of the reported NADPH oxidase inhibitors are not strictly isoform selective. Therapeutic efficacies and potential side effects of these novel NADPH oxidase inhibitors still remain to be established by long-term animal studies and clinical trials (Altenhofer et al. 2015; Drummond et al. 2011).

GKT136901 and GKT137831: These compounds are pyrazolopyridine derivatives. They inhibit NOX1, NOX4, and NOX5, with IC_{50} (concentrations with 50% inhibition of activity) values in a sub-μM range. They are approximately tenfold less effective against NOX2 oxidase. They show minimal off-target effects against more than 100 protein targets tested, including endothelial nitric oxide synthase (eNOS) and xanthine oxidase. These compounds have a high oral bioavailability and favorable pharmacokinetic and safety profiles in vivo. In animal studies, GKT136901 or GKT137831 showed beneficial effects against atherosclerosis, ischemic retinopathy, hepatic fibrosis, tumor angiogenesis, diabetic nephropathy, and osteoporosis (Altenhofer et al. 2015; Drummond et al. 2011).

ML171: ML171 (also known as 2-acetylphenothiazine) is a specific NOX1 oxidase inhibitor with an IC_{50} value of <300 nM for NOX1 and is >tenfold less effective for NOX2/3/4 and xanthine oxidase. ML171 appears to target the NOX1 catalytic subunit but not its cytosolic regulators (NOXO1, NOXA1, or Rac). ML171 shows some inhibitory effects toward serotonin and adrenergic receptors with low potencies. This compound is predicted to have favorable pharmacokinetics and safety profiles for in vivo applications (Altenhofer et al. 2015; Drummond et al. 2011).

VAS2870 and VAS3947: VAS2870 and VAS3947 are triazolopyrimidines, which inhibit NOX2 with IC_{50} values being 0.77 and 2.0 μM, respectively. VAS3947 also inhibits NOX1 and NOX4 with similar potencies (IC_{50} ~10 μM). VAS2870 is also reported to have inhibitory effects on NOX4 and NOX5 (IC_{50} values unknown), but has no inhibition on xanthine oxidase or eNOS. The mechanism(s) of NADPH oxidase inhibition by these compounds are not clear. In animal studies, VAS2870 shows beneficial effects on experimental stroke (reduced brain infarct volume and improved neurological outcome) (Altenhofer et al. 2015; Drummond et al. 2011).

S17834: S17834 is a polyphenol reported to have NADPH oxidase-inhibiting properties. It does not inhibit xanthine oxidase or eNOS. NOX isoform selectivity of S17834 is unknown. An off-target effect of S17834 is the activation of adenosine monophosphate-activated protein kinase (AMPK), which may mask the benefits produced by NADPH oxidase inhibition. In chronic animal studies, in vivo administration of this compound suppressed the development of atherosclerosis (Altenhofer et al. 2015; Drummond et al. 2011).

DPI, Apocynin, AEBSF, and Plumbagin: DPI (diphenylene iodonium), apocynin, AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride], and

plumbagin are historically used as pharmacological tools to inhibit NADPH oxidase in the laboratory. These inhibitors lack NOX isoform selectivity and are associated with significant off-target effects and/or low potencies. Hence these compounds are not suitable for further drug development (Altenhofer et al. 2015; Drummond et al. 2011).

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NCX

▶ Na^+/Ca^{2+} Exchangers

NCX1

▶ Na^+/Ca^{2+} Exchangers

NCX2

▶ Na^+/Ca^{2+} Exchangers

NCX3

▶ Na^+/Ca^{2+} Exchangers

Neprilysin Inhibitors

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Neprilysin and the Natriuretic Peptides

Neprilysin (NEP), also known as neutral endopeptidase, CD10, enkephalinase, common acute lymphoblastic leukemia antigen, and endopeptidase 24.11, is a well-known enzyme that was described in the early 1970s and fully characterized at the turn of the century (Bayes-Genis et al. 2016a). NEP is ubiquitous, mainly is expressed in the kidneys, but also present in the lungs, endothelial cells, vascular smooth-muscle cells, cardiac myocytes, fibroblasts, neutrophils, adipocytes, testes, and brain, with the highest concentrations in the proximal tubules of nephrons. NEP is also promiscuous in function, with >50 putative peptide substrates with varying levels of in vitro and/or in vivo evidence of functional relevance (Erdös and Skidgel 1989).

In the cardiovascular system, NEP cleaves numerous vasoactive peptides, some of them with mainly vasodilating effects including natriuretic peptides, adrenomedullin, and bradykinin, and other with mainly vasoconstrictor effects: angiotensin I and II, and endothelin-1 among others. Nevertheless, the relative affinity of neprilysin is variable among substrates, with highest affinity for atrial natriuretic peptide (ANP), C-type natriuretic peptide (CNP), and angiotensin I and II, and lowest affinity for brain natriuretic peptide (BNP), endothelin-1, and bradykinin (Mangiafico et al. 2013). The natriuretic peptides ANP and BNP are of predominantly cardiac origin, whereas CNP is largely sourced from endothelial cells throughout the systemic vasculature. Bioactive NPs act via G protein-coupled transmembrane receptors activating cyclic guanosine monophosphate (cGMP) as a

second messenger (Potter 2011). ANP and BNP act via the NPR-A receptor to exert natriuretic, diuretic, hemoconcentrating, and vasodilating effects in association with suppression of the renin-angiotensin-aldosterone and sympathetic nervous systems, and trophic effects that oppose cardiac hypertrophy and fibrosis. CNP, operating via the NPR-B receptor, is not natriuretic but is central to vasomotion, opposes vascular cell hyperplasia, and has a unique role in long bone development. All three NPs are cleared via the NPR-C receptor in concert with proteolysis (Potter 2011; Charles et al. 1996).

Neprilysin is responsible for the initial proteolytic cleavage of ANP and CNP; it also plays a role in processing BNP but does not cleave the amino-terminal prohormone fragments (Lafferty et al. 1989; Kenny et al. 1993; Stephenson and Kenny 1987; Yandle et al. 1989). Much of the impact of inhibiting neprilysin in preclinical and clinical settings has been presumed to be due to enhancement of NP bioactivity. The ranking of avidity of the enzyme for the NPs is CNP > ANP > BNP. In health, proteolytic cleavage and removal by the "clearance" NP receptor NPR-C play equal roles in metabolism of the NPs, but in high-NP states such as HF, it seems likely that neprilysin plays an increasingly important role (Potter 2011).

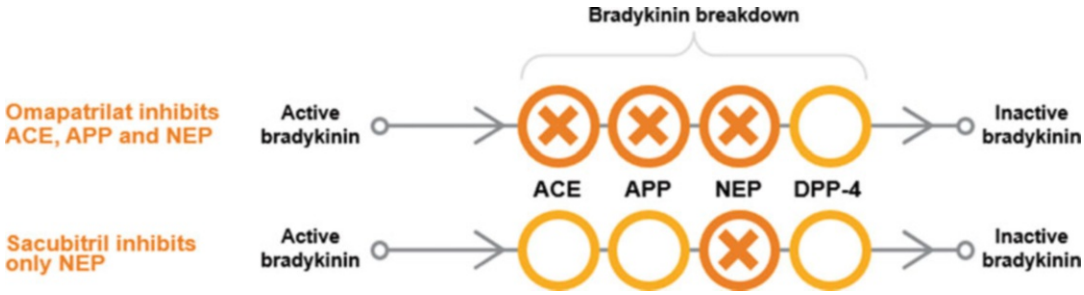
Neprilysin Inhibitors

For decades, neprilysin has been an important biotarget, in which academia and industry have actively researched in conjunction for inhibitors (NEPI) useful in clinical practice. More recently, neprilysin has also been proposed as a putative biomarker given the detection of circulating soluble neprilysin (sNEP) (Bayes-Genis et al. 2015a, b). The present review will focus mainly in the road to development of NEPIs, and less on sNEP. At present, data on sNEP are scarce, and there are several open issues regarding pre-analytical and analytical methods that deserve further insight before sNEP may be considered a valid and robust biomarker in precision medicine and prognostication.

The development of multiple neprilysin inhibitors has enabled clarification of the role of the enzyme in regulating levels of NPs and other substrates. While during the 1980s and 1990s, it seemed that all efforts to treat HF were only focused on the modulation of the renin-angiotensin-aldosterone system (RAAS) and the sympathetic nervous system, in parallel, several therapeutic failures happened in an attempt to find a formulation (fine tuning) to inhibit neprilysin. Such failures were both caused by the way to inhibit neprilysin (NEPI monotherapy), as well as due to inappropriate route of administration and dosage.

Thiorphan was the first synthetic NEPI and was followed by many other agents, suitable for both oral and parenteral administration. In healthy humans and during hypertension and HF, NEPIs alone induced elevations in endogenous plasma concentrations of ANP and BNP in association with increased circulating and urine cGMP and at least brief beneficial hemodynamic responses. Nevertheless, the hypotensive effects of NEPI alone were modest and transient, and in severe HF NEPI had no significant effect (Cavero et al. 1990). Other NEPIs had similar fate. Candoxatril was shown to have favorable hemodynamic effects compared with placebo in patients with chronic HF (Lang et al. 1991), and ecadotril led to numerically more deaths, as well as no evidence of clinical efficacy compared with placebo in patients with HF (Cleland and Swedberg 1998). Consequently, the development of both candoxatril and ecadotril for HF was discontinued.

Evidence of concurrent activation of the RAAS, together with augmentation of NP bioactivity by NEPI monotherapy, inspired development and testing of agents that combine NEPI and ACE-inhibiting activity: the so-called vasopeptidase inhibitors. Several vasopeptidase inhibitors have been developed, including omapatrilat, fasidotril, sampatrilat, and mixanpril (Nathisuwan and Talbert 2002). In the IMPRESS trial of 573 patients with NYHA class II–III HF, omapatrilat decreased rates of a composite endpoint (death, hospitalization, or discontinuation of treatment for worsening HF) to 6% compared to 10 % on lisinopril ($p = 0.035$) (Rouleau et al. 2000). However, in the phase III trial Omapatrilat



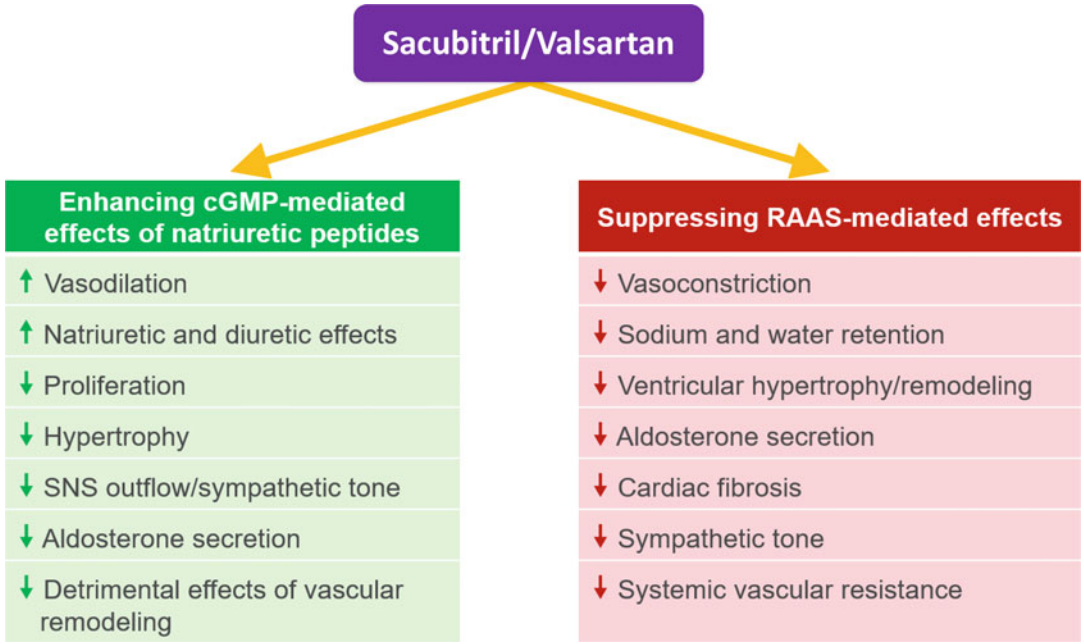
Neprilysin Inhibitors, Fig. 1 Impact on bradykinin breakdown by omapatrilat and sacubitril/valsartan. Sacubitril/valsartan does not excessively increase bradykinin. (ACE = angiotensin-converting enzyme; APP = aminopeptidase P; AT_1 = angiotensin II type 1; DPP-4 = dipeptidyl peptidase-4; NEP = neprilysin)

Versus Enalapril Randomized Trial of Utility in Reducing Events (OVERTURE) of 4400 patients, omapatrilat was not superior to enalapril with respect to the primary endpoint of death from any cause or admission for HF (Packer et al. 2002). These indications of benefit from vasopeptidase inhibition were overshadowed by an excess of severe angioedema, which led to cessation of vasopeptidase development. The increased incidence of angioedema (24 patients [0.8%] vs. 14 patients [0.5%] for omapatrilat and enalapril, respectively) is believed to have been caused by excess bradykinin produced by the combined vasopeptidase inhibitor effects of inhibiting not only ACE and neprilysin but also aminopeptidase P (Fig. 1). In the OVERTURE Trial, omapatrilat was given once daily (to reflect its use in patients with hypertension), even though its pharmacologic advantages over enalapril in patients with HF were not maintained throughout the 24-h dosing interval (Rouleau et al. 2000). These issues could be avoided by the combination of NEPI with an ARB blocker into one compound, instead of ACEI, and to prescribe them twice daily (Fig. 2).

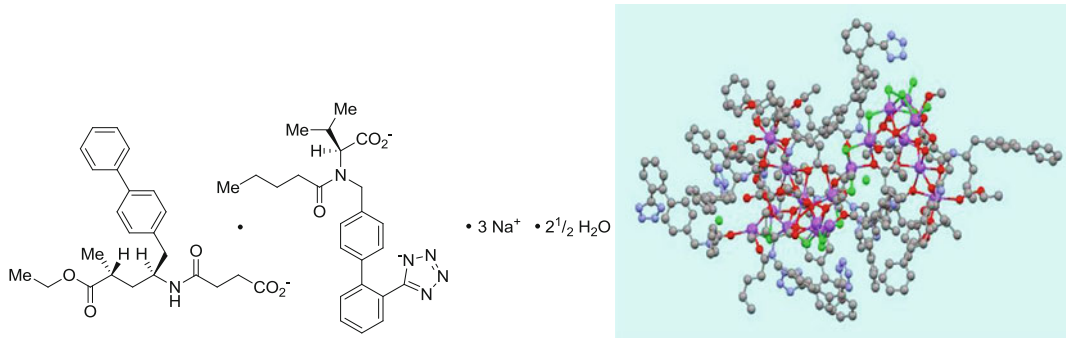
ARNIs in HFrEF and HFpEF

The combination of NEPI and ARB, also known as angiotensin receptor neprilysin inhibitor (ARNI) (Fig. 3), has been evaluated in large clinical trials with both reduced and preserved ejection fraction. Table 1 summarizes the impact of NEPI, ACEI, vasopeptidase inhibitors, and ARNI on vasoactive peptides and physiological effects (Bayes-Genis et al. 2016b).

The PARADIGM-HF trial was the largest (n = 8442) and most geographically diverse (1043 clinical centers) trial in HF performed to date (McMurray et al. 2014). This was an intention-to-treat study evaluating the clinical benefit of the ARNI sacubitril/valsartan against enalapril in patients with LVEF<40% (protocol subsequently amended to LVEF <35%). Patients were required to have BNP levels of ≥150 pg/mL or ≥100 pg/mL with hospitalization secondary to HF in the preceding 12 months (or NT-proBNP ≥600 pg/m or ≥400 pg/mL), in addition to taking stable beta-blockers and ACEI/ARB doses that were equivalent to enalapril 10 mg daily in the 4 weeks prior to screening. Subjects were randomized in a double-blinded manner to receive sacubitril/valsartan 200 mg twice daily (n = 4187) or enalapril 10 mg twice daily (n = 4212) in combination with conventional systolic HF therapy including beta-blockers (93% of patients), diuretics (80%), MRAs (56%), and digoxin (30%), in addition to implantable cardioverter defibrillators (ICD) (15%) or cardiac resynchronization therapy (CRT) (7%). The trial was early terminated due to overwhelming reductions in death from cardiovascular causes and the primary endpoint (CV death or first hospitalization for worsening HF). After a mean treatment duration of 27 months, treatment with sacubitril/valsartan was associated with significant reductions in the primary endpoint, as



Neprilysin Inhibitors, Fig. 2 Simultaneous inhibition of NEP and suppression of the RAAS with sacubitril/valsartan has complementary effects. (cGMP = cyclic guanosine monophosphate; NEP = neprilysin; NP = natriuretic peptide; RAAS = renin-angiotensin-aldosterone system; SNS = sympathetic nervous system)



Neprilysin Inhibitors, Fig. 3 Sacubitril/valsartan 2D scheme and 3D structure. Sacubitril/valsartan is a crystalline salt complex comprising sacubitril and valsartan in their anionic forms, sodium cations and water molecules

there were 914 events in the sacubitril/valsartan group and 1117 in the enalapril group (21.8 vs. 26.5%, respectively, HR 0.80; $p < 0.001$). The incidence of both components of the composite endpoint were significantly reduced with sacubitril/valsartan as CV death occurred in 13.3 vs. 16.5% of enalapril patients (HR 0.80, $p < 0.001$), and first hospitalization from worsened HF occurring in 12.8 and 15.6% of patients, respectively (HR 0.79, $p < 0.001$). All-cause mortality rates were significantly reduced (17.0 vs. 19.8%, HR 0.84, $p < 0.001$) and the average reduction in the KCCQ clinical score reflects significantly fewer symptoms or physical limitations secondary to HF in the sacubitril/valsartan group. There was no difference in the incidence of new-onset AF or worsened renal function.

Neprilysin Inhibitors, Table 1 Angiotensin receptor neprilysin inhibitors (ARNI) overcome the challenges associated with previous approaches to neprilysin inhibition

	NEPi	ACEI	NEPi + ACEI[a]	ARNI[b]
Effects on peptide levels				
Angiotensin-II	↑	↓	↓	↔↓
Renin	↓	↔	↔↑	↑
Aldosterone	↓	↔	↓	↓
NPs or cGMP	↑	↔	↑	↑
Endothelin-1	↑	↔	↑	↓
Big-Endothelin-1			↑	↑
Bradykinin	↑	↑	↑↑	↑
Physiological effects				
Blood pressure	↔	↓	↓	↓
Sodium excretion	↑	↑	↑	↑↑
CV hypertrophy	↔↓	↓	↓↓	↓↓
CV fibrosis	↓	↓	↓↓	↓↓

ACEI = angiotensin converting enzyme inhibitor; ARNI = angiotensin receptor neprilysin inhibitor; cGMP = cyclic guanosine monophosphate; CV = cardiovascular; NEPi = neprilysin inhibitor; NP = natriuretic peptide
[a]Only data for omapatrilat considered
[b]Only data for sacubitril/valsartan considered

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Due to the unethical nature of withholding an ACE inhibitor or ARB in patients with HFrEF, sacubitril/valsartan could not be compared directly with placebo (McMurray et al. 2015). Therefore, the investigators made indirect comparisons, using putative placebos. They quantified the effect of sacubitril/valsartan on a number of key cardiovascular outcomes and on all-cause mortality. Compared in this way, sacubitril/valsartan was shown to have striking effects on all outcomes examined, with the relative risk reduction for cardiovascular mortality ~32–34% (for cardiovascular morbidity and mortality, it was 39–43%). There were even larger effects on heart failure hospitalization, with relative risk reductions of 46–49% (McMurray et al. 2015).

Additional analysis from PARADIGM-HF indicates that fewer sacubitril/valsartan-treated patients required intensification of medical treatment for heart failure (520 vs. 604; HR 0.84; $p = 0.003$) or an emergency department visit for worsening heart failure (HR 0.66; $p = 0.001$) (Packer et al. 2015). The patients in the sacubitril/valsartan group had 23% fewer hospitalizations for worsening heart failure (851 vs. 1079; $p < 0.001$) and were less likely to require intensive care (768 vs. 879; 18% rate reduction, $p = 0.005$), to receive intravenous positive inotropic agents (31% risk reduction, $p < 0.001$), and to have implantation of a heart failure device or cardiac transplantation (22% risk reduction, $p = 0.07$). These advantages were apparent even though the enalapril group had a meaningfully higher mortality rate throughout the trial, leading to the preferential exclusion of high-risk enalapril-treated patients with progressing symptoms, and the enalapril group had greater intensification of background therapy, which would have been expected to ameliorate deleterious changes in clinical status. Importantly, sacubitril/valsartan

also significantly reduced sudden cardiac death relative enalapril (HR 0.80, $p = 0.008$) (Desai et al. 2015).

The age-strata analysis showed that the rate of death and heart failure hospitalization increased with age, yet this gradient was not as pronounced in PARADIGM-HF as in prior trials. The benefit of sacubitril/valsartan over enalapril was similar across age categories and intolerance of sacubitril/valsartan leading to treatment withdrawal was uncommon, even in elderly individuals. Of note, a larger number of patients with a broader range of ages were included in PARADIGM-HF than in any previous trial in HFrEF ($\geq$75 years ($n = 1563$); $\geq$80 years ($n = 587$)) (Jhund et al. 2015).

The biomarker sub-study revealed that, as expected, neprilysin inhibition with sacubitril/valsartan increased levels of both urinary cyclic GMP and plasma BNP (Packer et al. 2015). In contrast, in comparison with enalapril, patients receiving sacubitril/valsartan had consistently lower levels of NTproBNP (reflecting reduced cardiac wall stress) and troponin (reflecting reduced cardiac injury) throughout the trial. The contrasting effects of sacubitril/valsartan on the two types of natriuretic peptides represent an important finding, because the levels of the two peptides characteristically parallel each other during the course of heart failure (Packer et al. 2015). However, because BNP (but not NT-proBNP) is a substrate for neprilysin, levels of BNP reflect the action of the drug, whereas levels of NTproBNP will reflect the cardioprotective effect of the drug.

Strengths of PARADIGM-HF include its randomized, prospective, active-treatment controlled design, and clinically relevant endpoints. The trial was adequately powered to detect differences in hospitalization and mortality, two endpoints that clinicians focus therapy on preventing in HF patients. Despite a higher incidence of hypotension in the sacubitril/valsartan arm, discontinuation of therapy was significantly less common overall in the sacubitril/valsartan group (17.8 vs. 19.8%, $p = 0.02$). Additionally, fewer patients taking sacubitril/valsartan required a discontinuation of therapy due to renal failure (0.7 vs. 1.4%, $p = 0.002$) or adverse events (10.7 vs. 12.3%, $p = 0.03$). Angioedema occurred in 19 patients receiving sacubitril/valsartan and 10 patients on enalapril, not reaching statistical significance. None of the reported angioedema included airways compromise.

The next frontier was HFpEF. Approximately, 50% of HF patients have HFpEF. Therapies for HFpEF are directed toward symptom management and cardiovascular risk factors due to the lack of clinical trials that demonstrate therapeutic benefits with agents commonly utilized in HFrEF. The safety and efficacy of sacubitril/valsartan among HFpEF patients showed promising results in a phase 2 trial (Solomon et al. 2012). The PARAMOUNT trial was a randomized, double-blind, parallel-group, active controlled trial (Solomon et al. 2012). Patients were eligible if they were aged $\geq$40 years with an LVEF of at least 45% and a documented history of HF with associated signs or symptoms (dyspnea on exertion, orthopnea, paroxysmal dyspnea, and peripheral edema). Additional requirements included a NT-proBNP level greater than 400 pg/mL. Patients were randomized to sacubitril/valsartan 50 mg twice daily or valsartan 40 mg twice daily. Both arms were titrated to their final doses of 200 mg twice daily or 160 mg twice daily, respectively, over 2–4 weeks. The primary endpoint was a change from baseline in NT-proBNP at 12 weeks. Baseline characteristics were similar between groups. Most patients were elderly, female, overweight, and classified as NYHA class II. The change in NT-proBNP was seen at week 4 in the sacubitril/valsartan group compared to the valsartan group, although not reaching significance ($p = 0.063$). However, at 12 weeks, NT-proBNP was significantly reduced in the sacubitril/valsartan group compared to valsartan ($p = 0.005$). The findings from PARAMOUNT suggested that sacubitril/valsartan could have favorable effects in patients with HFpEF.

PARAGON-HF was designed to test the hypothesis that sacubitril/valsartan would improve outcomes in HFpEF (Solomon et al. 2019). A total of 4,822 patients were randomly assigned to sacubitril/valsartan or valsartan. The comparator was valsartan because most HFpEF

patients already take a renin-angiotensin system inhibitor. Patients were required to have signs and symptoms of HF, LVEF of 45% or greater, evidence of natriuretic peptide elevation, and structural heart disease. The median follow-up was 34 months. The primary endpoint was a composite of total (first and recurrent) heart failure hospitalizations and cardiovascular death. The rate ratio for the primary endpoint was 0.87 (95% confidence interval [CI] 0.75–1.01; $p = 0.059$). This reduction was just short of statistical significance and was driven by a decline in heart failure hospitalization with no effect on cardiovascular death or all-cause mortality.

Several sensitivity analyses, including assessment of investigator-reported outcomes, favored sacubitril/valsartan. Several secondary endpoints, such as quality of life, change in New York Heart Association (NYHA) class, and percentage of patients with worsening renal function, favored sacubitril/valsartan. Sacubitril/valsartan was associated with more hypotension, but less hyperkalemia and less renal dysfunction compared with valsartan, findings similar to those seen in the PARADIGM-HF trial.

Importantly, there was heterogeneity in the population with respect to treatment response. In particular, there was greater benefit in patients with ejection fraction below the median of 57%, with a 22% reduction (rate ratio 0.78; 95% CI 0.64–0.95) and in women, with a 28% reduction (rate ratio 0.73; 95% CI 0.59–0.90) in the primary endpoint (Solomon et al. 2019).

In conclusion, neprilysin is back to the center stage after an eventful career spanning almost half century. The impact of neprilysin blockade and natriuretic peptide rise observed with the new ARNIs has revolutionized the way we treat heart failure and impacted our knowledge on neprilysin-regulated neurohormones pathophysiology. Current findings indicate that the benefit of sacubitril/valsartan observed in PARADIGM-HF in HFrEF could extend to HF patients with ejection fraction below the normal range, including those designated HFmrEF, as suggested by a sub-analysis of PARAGON-HF, but not in truly HFpEF patients.

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Neurodegeneration

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Synonyms

Neuronal cell death; Neuronal cell deterioration

Definition

Neurodegeneration refers to the processes whereby neuronal cells progressively deteriorate,

loose their structure and/or function, and eventually die.

Since the body's ability to replace lost neurons (i.e., such as via neurogenesis) is quite limited when compared to many non-neuronal cells, degenerative processes affecting neurons can be particularly devastating.

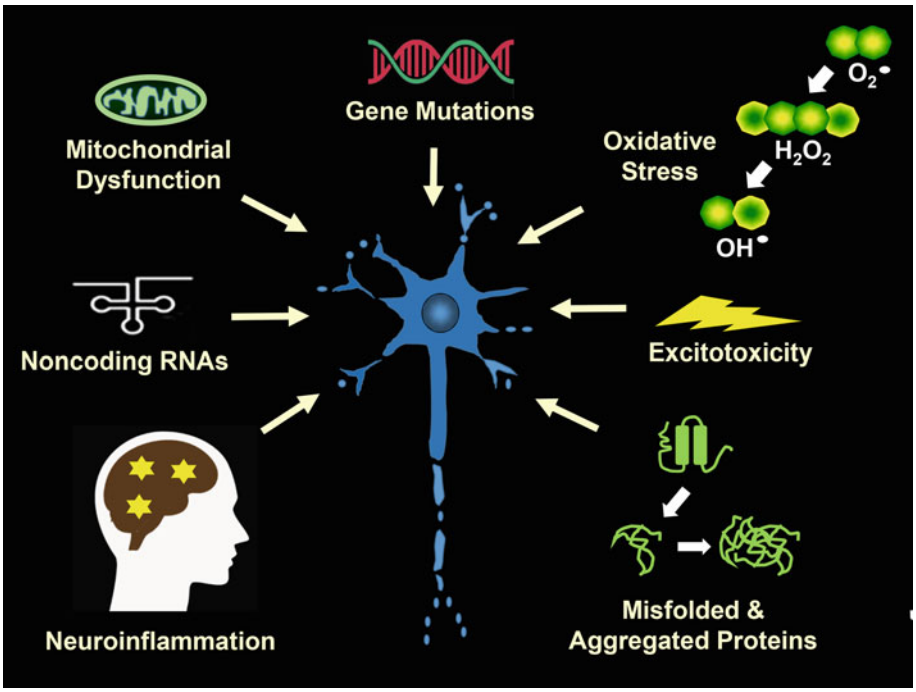
Basic Mechanisms

The basic mechanisms underlying the neuronal cell degeneration and death observed in the neurologic disorders as diverse as Alzheimer's disease and stroke have not been fully elucidated. However, a number of distinct biological factors and processes clearly contribute to neurodegeneration including gene mutations and noncoding RNAs, increased oxidative stress and free radical damage, impaired mitochondrial function, excitotoxicity, immunologic and inflammatory mechanisms, misfolded and aggregated proteins, impaired trophic factor support, and altered cell signaling (see illustration in Fig. 1). In the process of neuronal degeneration, cells eventually die as a result of apoptosis, necrosis, or a combination of these processes (Martin 2001).

Table 1 provides an overview of the major differences between necrosis and apoptosis.

Necrosis

Necrosis occurs as a pathological response to cell injury most commonly resulting from trauma, ischemia, hypoxia, neurotoxins, or infection. Necrosis typically occurs when a cell is too severely damaged for the orderly energy-dependent process of apoptosis (see below) to occur. Following one or more of the insults listed above, neuronal degeneration or death occurs in groups of contiguous cells in a localized region, and the initiation of inflammatory processes can be clearly observed in tissue sections (Schwartz and Osborne 1995). The processes of necrosis are summarized in Table 1 on the left and illustrated at the top of Fig. 2.



Neurodegeneration, Fig. 1 Illustration of some of the major biological processes and factors that can lead to neurodegeneration

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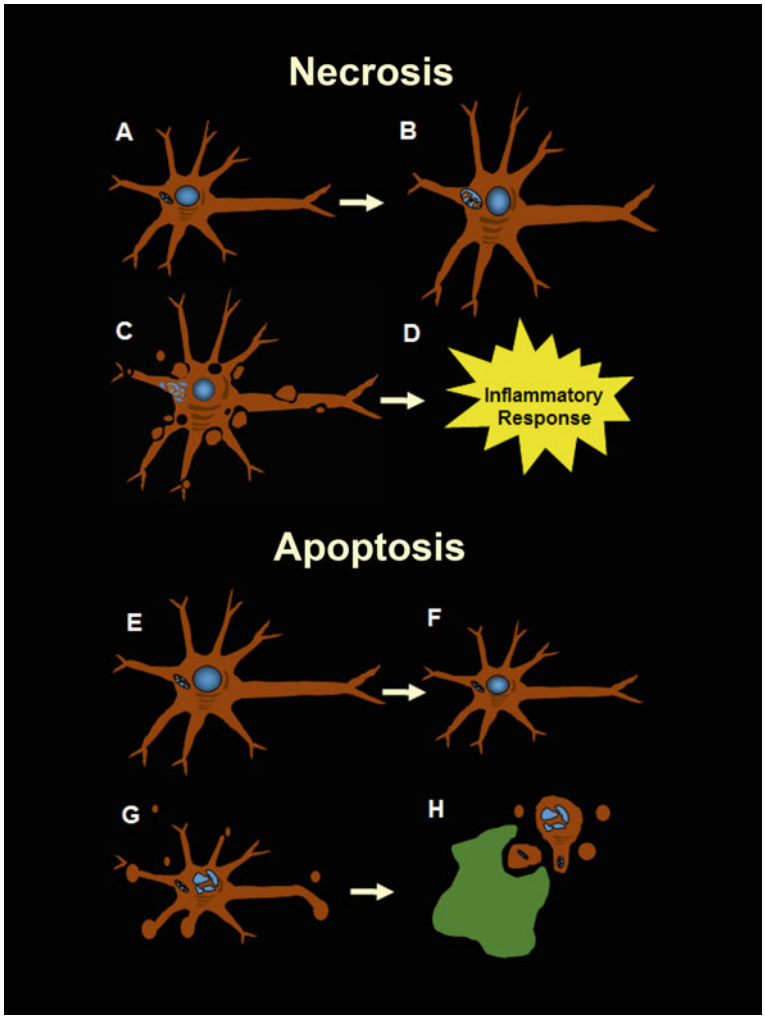
Neurodegeneration, Table 1 Comparison of necrosis and apoptosis

Necrosis	Apoptosis
Cellular swelling	Cellular shrinkage
	Nuclear and cellular pyknosis
Little or no chromatin condensation	Chromatin condensation
Rupture of organelles and plasma membrane	Organelles and plasma membrane not usually ruptured
Release of cytoplasmic contents and inflammation	Release of cytoplasmic contents and inflammation not usually present
Random DNA degradation	DNA fragmentation
Caspases not involved	Activation of caspases
	Cytoplasmic blebbing
	Formation of apoptotic bodies which are engulfed and cleared by phagocytes

Apoptosis

The term apoptosis is derived from an ancient Greek word meaning "dropping off" like leaves falling from trees in the autumn season. In modern cell biology, the term is used to describe the orderly form of programmed cell death that occurs in multicellular organisms. Neuronal apoptosis is triggered by a number of factors including lipid peroxidation (and membrane damage) induced by reactive oxygen species, genetic mutation, or DNA damage (or degradation) resulting from radiation or other destructive agents. A loss of trophic factor support, as well as some of the same factors that induce necrosis (see above), can also initiate apoptotic processes. Cell death by apoptosis also occurs extensively during the development of the mammalian nervous system and is required for the formation of appropriate connections between neurons and their targets. The processes involved in apoptosis generally differ from necrosis (see Table 1) in several important details, most notably, that neuronal death with apoptosis usually involves individual cells that are phagocytized before they can release their cytoplasmic contents and induce an inflammatory response in adjacent tissues. In addition, in the process of apoptosis, phagocytes are able to recognize dying or degenerating cells by their expression of death-related cell surface epitopes. Furthermore, mitochondria are preserved until the late stages of apoptosis, whereas they typically swell and disintegrate early in necrosis. The major cellular changes observed in neuronal apoptosis are summarized in Table 1 on the right and illustrated at the bottom of Fig. 2.

As noted above, neuronal apoptosis serves a number of important roles in normal brain development and is a key mechanism by which defective or damaged neurons are removed from the brain. However, in a number of brain disorders including Alzheimer's disease (AD), dementia with Lewy bodies (DLB), and Parkinson's disease (PD), inappropriate apoptosis may occur leading to accelerated neuronal loss and progressive disease symptoms. Conversely, in neural tumor cells, such as neuroblastoma and medulloblastoma cells, apoptotic pathways may be disabled, and the cells become resistant to chemotherapeutic drugs that kill cancer cells by inducing apoptosis. Neuroblastoma and medulloblastoma are important pediatric solid tumors that arise in the sympathetic neuron lineage and cerebellum, respectively. Apoptosis may be accelerated or retarded by a variety of hormones, metabolic by-products, electrolytes, and other endogenous substances. As examples, altered serum levels of thyroid hormone or ammonia, altered plasma or extracellular levels of excitatory amino acids such as glutamate and aspartate, imbalances of calcium and other electrolytes, and lactic acidosis are all known to initiate or modify apoptotic processes. Apoptosis is also influenced by synaptic communication in both the central and peripheral nervous systems. For example, in transsynaptic degeneration, neurons deteriorate and often undergo apoptosis if they fail to be innervated (from the afferent side) due to the loss of presynaptic neurons. This process has been observed in the lateral genicular body after optic nerve lesions and in the inferior olivary nucleus after destruction of the central tegmental tract. Efferent, motor neurons degenerate if they fail to match with target muscle fibers or their muscle targets are lost, such as after amputation of a limb.



Neurodegeneration, Fig. 2 *Top*: Illustration of the major cellular changes observed in neuronal necrosis. A normal neuronal cell (**a**) when exposed to an insult (e.g., trauma, ischemia, hypoxia, neurotoxins, infection, etc.) initially swells (**b**), mitochondria and other organelles swell and rupture, the plasma membrane lyses (**c**), and spillage of the cellular contents into the extracellular space follows. A general inflammatory response (**d**) is then triggered and macrophages attack and phagocytize the cellular debris. *Bottom*: Illustration of the major cellular changes observed in neuronal apoptosis. A normal neuronal cell (**e**) when exposed to specific triggers (e.g., lipid peroxidation, genetic mutation, DNA damage, excitotoxic injury, etc.) initially shrinks (**f**), chromatin becomes pyknotic and condenses and then migrates to the nuclear membrane, DNA fragmentation and degradation occurs, and several organelles (e.g., Golgi apparatus, endoplasmic reticulum) disappear. Afterward, blebbing of the plasma membrane occurs (**g**), and the cell then fragments into small apoptotic bodies that are subsequently phagocytosed and digested (**h**) without triggering inflammation

Regulation of Apoptosis

Apoptosis is regulated by a complex molecular cascade that controls the activation of a family of cysteine proteases known as caspase proteins (caspases-1–14). Caspases are responsible for breaking down vital structural and functional proteins, leading to the characteristic cytomorphology associated with apoptosis. While multiple molecular pathways (e.g., mitochondrial, death receptor, endoplasmic reticulum pathways) have been identified that lead to the activation of caspases, the mitochondrial

(intrinsic) pathway is most associated with neuronal apoptosis. The mitochondrial pathway regulates caspase activity by controlling mitochondrial release of cytochrome c by pro- and anti-apoptotic members of the Bcl-2 family of proteins. These proteins interact through dimerization in the mitochondrial membrane. The ratio of pro-apoptotic (e.g., Bax, Bad) to anti-apoptotic (e.g., Bcl-2, Bcl-XL) protein levels is a key determinant in regulating cytochrome c release and subsequent caspase activation. For example, high Bax/Bcl-2 and Bax/Bcl-XL ratios promote cytochrome c release, while low Bax/Bcl-2 and Bax/Bcl-XL ratios inhibit cytochrome c release. Downstream of mitochondria, cytochrome c forms a complex with caspase-9 and Apaf-1 to form the apoptosome. This complex cleaves procaspase-3 to form activated caspase-3 which can initiate cellular disassembly. While caspase-3 is known as a downstream effector caspase, its activity can still be inhibited by members of the inhibitor-of-apoptosis (IAP) protein family (e.g., XIAP); XIAP is itself potentially inhibited by Smac/Diablo, a mitochondrial protein (Michel et al. 1999).

As discussed above, apoptotic activity can be triggered by a broad array of stimuli including oxidative stress (e.g., ischemia, hypoxia), pro-inflammatory cytokines, excitotoxicity, neurotrophin withdrawal, mitochondrial dysfunction, and abnormal intracellular calcium concentrations. A number of these stimuli can alter Bcl-2 family protein expression via potent regulatory genes such as p53 and par-4 in order to promote cytochrome c release and induce caspase-3 activation. Increased neuronal apoptosis has been demonstrated in classic neurodegenerative disorders including AD. Interestingly, these disorders are also characterized by alterations in apoptotic regulatory proteins including several Bcl-2 family proteins and caspases. For example, Bcl-2 and caspase-3 are increased in the cortex of postmortem AD brains. These increases are thought to be due to a compensatory upregulation in response to the neurodegenerative process. In contrast, lower Bcl-2 levels have been reported in the frontal cortex of patients with autism, a classic neurodevelopmental disorder. Accumulating data suggest important roles for apoptotic pathways in the pathophysiology of a spectrum of neuropathological disorders (Michel et al. 1999).

Apoptosis-Necrosis Cell Death Continuum

There is now a substantial body of evidence of an apoptosis-necrosis cell death continuum. In this continuum, neuronal death can result from varying contributions of coexisting apoptotic and necrotic mechanisms. Moreover, the long-standing paradigm in the field of cell biology that specifically divided cell death processes into those that are regulated (apoptosis) and those that are accidental and unregulated (necrosis) has been overturned by the discovery that tumor necrosis factor (TNF) can induce regulated cell death with apoptotic or necrotic features depending on the specific context. Recently discovered forms of regulated (non-apoptotic) cell death include necroptosis, ferroptosis, parthanatos, cyclophilin D-(CypD)-dependent necrosis, neutrophil extracellular trap (NET)-associated cell death (termed NETosis), pyroptosis, and autophagic cell death (autophagy) (Fricker et al. 2018). The most studied (non-apoptotic) forms of cell death with specific relevance to neurodegeneration to date include necroptosis and autophagy. These processes are discussed briefly below. The other forms of cell death noted above have been reviewed elsewhere (Brinkmann et al. 2013: Green et al. 2014; Galluzzi et al. 2014; Lupfer et al. 2015; Fricker et al. 2018).

Necroptosis

Necroptosis is a programmed form of necrosis that may have evolved as an inflammatory defense mechanism against pathogens and to eliminate infected cells (Mocarski et al. 2015). Necroptosis is dependent on the kinase activity of receptor-interacting kinase 1 (RIP1), kinase activity of RIP3, and expression of the mixed lineage kinase domain-like (MLKL) (Galluzzi et al. 2014). The process is initiated by the binding of the TNF family of cytokines such as TNFα, Fas/CD95, and TRAIL (TNF-related

apoptosis-inducing ligand) to membrane receptors to activate intracellular RIP family kinases. Necroptosis is gaining attention as a mediator of neuroinflammation and neurodegeneration, and it has been linked to a variety of inflammatory conditions such as multiple sclerosis, amyotrophic lateral sclerosis (ALS), stroke, ischemia-reperfusion injury, myocardial infarction, arteriosclerosis, and Crohn's disease. It is currently unknown, however, whether necroptosis is a driving force in these diseases or a secondary consequence of some other pathologic process (Fricker et al. 2018).

Autophagy

Autophagy is a catabolic (lysosome-dependent) process used by cells to eliminate misfolded proteins, protein aggregates, and damaged organelles. The process is also used to recycle important cellular constituents such as amino acids, lipids, nutrients, and metabolites. Autophagy is a normal, orderly biological process that is accelerated by a variety of cellular stressors, such as nutrient starvation, DNA damage, growth factor withdrawal, organelle damage, and the accumulation of abnormal proteins. Because neurons are postmitotic, they are dependent on high basal levels of autophagy compared to non-neuronal cells since misfolded proteins and damaged organelles cannot be diluted through cell division. Moreover, the axons and dendrites of neurons have long and extended cytoplasms that present additional challenges for preventing dysfunctional organelles and cellular waste from accumulating over time. While autophagic processes in young neurons are efficient, aged neurons are vulnerable to slowdowns in the proteolytic clearance of autolysosomal substrates. Such slowdowns can result in the accumulation of ubiquitinated protein aggregates which lead to impairments in neuronal function and degeneration. The importance of autophagy in the pathophysiology and treatment of age-related neurodegenerative illnesses such as AD and PD is becoming increasingly recognized (Son et al. 2012; Nixon 2013; Fujikake et al. 2018).

Important Mediators of Neurodegeneration

Oxidative Stress

A variety of metabolic pathways generate highly reactive by-products known as free radicals including hydrogen peroxide, superoxide anions, and hydroxyl radicals. These substances can be used by various cells as part of the immune response to serve useful functions such as to combat infectious organisms and neoplastic cells and to execute cells programmed for death during the normal course of development. In abnormal circumstances such as associated with traumatic and ischemic injury or neurodegenerative diseases such as AD and PD, free radicals may be excessively produced, aberrantly controlled, or inadequately scavenged. In such cases, free radicals cause injury as a result of membrane lipid peroxidation, DNA damage, iron accumulation, and protein nitrosylation. Excess free radicals are normally scavenged and inactivated by several endogenous substances such as vitamin E (-α-tocopherol), which can quench lipid peroxidation, superoxide dismutase which scavenges superoxide radicals, and glutathione peroxidase which removes hydrogen peroxide and lipid peroxides. Therefore, alterations or deficits in any of these endogenous substances can contribute to and/or initiate neurodegeneration.

Excitotoxic Amino Acids

Excitotoxic amino acids play a deleterious role in a number of neurologic diseases and are known to contribute to neurodegeneration. These substances are released in response to a wide variety of insults to the CNS and include glutamate, aspartate, and several oxidation products of cysteine and homocysteine. For example, in stroke, excitatory amino acids are released in the penumbra of ischemic lesions and further released when perfusion is restored and thus are believed to contribute significantly to reperfusion injury. These compounds are also released following traumatic brain injury, during prolonged seizures, and are thought to contribute to the neurotoxicity associated with the amyloid plaques observed in

AD. Overactivation of N-methyl-D-aspartate (NMDA) receptors (a subtype of glutamate receptor) by glutamate leads to alterations in a number of signal systems and ion channels activating apoptosis.

Mitochondrial Dysfunction, Energy Failure, and Ion Dysregulation

Mitochondria play critical roles in cellular respiration, metabolism, energy production, as well as intracellular signaling, free radical production, and apoptosis. Accordingly, they play important roles in both neuronal survival and death. Since neurons have high-energy requirements, they are especially vulnerable to injury and death from dysfunctional mitochondria (Golpich et al. 2017). Neuronal degeneration as a consequence of energy failure within mitochondria can be precipitated by ischemia, as well as several acquired and genetic disorders of metabolism. For example, mitochondrial energy disruption and neurodegeneration occur in Wernicke's encephalopathy, an acquired metabolic disorder resulting from ethanol abuse and/or thiamine deficiency. Similar neuropathology can be observed in Leigh's syndrome, an inherited neurometabolic disorder in which point mutations in mitochondrial DNA are evident. Friedrich's ataxia, the most common inherited ataxia, is an autosomal recessive disease in which protein aggregates appear to disrupt mitochondrial iron metabolism, leading to abnormal free radical formation and altered energy metabolism. In the cases highlighted above, specific irreversible processes lead to a decrease in high-energy phosphates (e.g., ATP, creatine phosphate), possibly leading further to elevated acyl-CoA levels that inhibit multiple metabolic processes. Local electrolyte (ion) imbalances and/or ion channel dysfunction are also thought to contribute significantly to neurodegenerative processes. Ion changes are commonly among the early events in apoptosis, and in fact, alterations in calcium homeostasis are among the best-documented factors in neurodegeneration. Direct evidence of the importance of ion channels in neurodegeneration comes from genetic disorders that affect specific ion channels (i.e., channelopathies). Channelopathies may underlie certain forms of migraine, episodic ataxias, and epilepsy. Indirect evidence that ion dysregulation plays an important role in some forms of neurodegeneration comes from preclinical studies (i.e., stroke models in animals) in which calcium and sodium channel blockers reduce infarct size.

Neuroinflammation

There is extensive evidence that inflammatory processes contribute to the pathology of multiple neurodegenerative illnesses. The term "neuroinflammation" is used to broadly represent the inflammatory processes occurring in the CNS involving both the innate and adaptive immune systems (Gelders et al. 2018). Microglia are the principal innate immune cells in the brain, although astrocytes, macrophages, natural killer cells, mast cells, oligodendrocytes, and neurons all contribute to innate immune responses. Microglia play a major role in homeostatic processes in the brain including synaptic pruning; clearing of apoptotic cells, misfolded proteins, and waste products; and providing trophic support for neurons. They represent the first line of defense in the brain and react to pathogens and tissue damage by releasing oxygen free radicals and secreting a variety of substances known to stimulate local inflammation such as inflammatory cytokines, complement and coagulation proteins, as well as binding proteins. As an example, inflammatory factors found in degenerating sites in Alzheimer's disease brains include activated microglia, the cytokines interleukin IL-1 and IL-6, an early component of the complement cascade, Clq, as well as acute-phase reactants such as C-reactive protein. A significant role of the adaptive immune system in neurodegenerative disorders is exemplified by alterations in subsets of T- and B-cells and increases in autoantibody levels in the blood, cerebrospinal fluid, and brain tissues. The positive (anti-inflammatory) or negative (pro-inflammatory) role of these subsets of T- and B-cells appears to be dependent on the levels of specific cytokines and chemokines. The aging process (a major contributor to many neurodegenerative illnesses) is associated with negative effects on both innate and adaptive immune responses. In the setting of unhealthy

aging, microglia display prolonged activation to insults where pro-inflammatory cytokines (e.g., IL-6, TNF, IL-1b) are elevated, and the microglia also exhibit reduced motility, impaired phagocytosis, and impairments in their ability to provide trophic support to neurons (Stephenson et al. 2018).

Neurotrophin Support and Altered Cell Signaling

A continuous supply of a variety of polypeptide molecules known as neurotrophic factors (or neurotrophins) is essential to the nervous systems of all vertebrates throughout development as well as in adult life (Huang and Reichardt 2001). These important molecules interact with specific receptors and initiate a variety of cellular signaling systems. During the period of target innervation, limiting amounts of neurotrophic factors regulate neuronal numbers by allowing survival of only some of the innervating neurons, the remaining being eliminated by apoptosis. Increasing evidence indicates that several neurotrophic factors also influence the proliferation, survival, and differentiation of precursors of a number of neuronal lineages. In the adult, neurons continue to be dependent on trophic factor support, which may be provided by the target or by the neurons themselves. Altered trophic factor support and cell signaling as a result of excess free radicals or peroxynitrites has been implicated in the neurodegenerative processes associated with several neurologic diseases. Furthermore, the ability of neurotrophins to promote survival of peripheral and central neurons during development and after neuronal damage has stimulated the interest in these molecules as potential therapeutic agents for the treatment of nerve injuries and neurodegenerative diseases. Examples of therapeutically relevant neurotrophins (from a neurodegenerative disease standpoint) are nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) (see further discussion below).

Misfolded and Aggregated Proteins

Mammalian cells typically contain thousands of different proteins that must fold into specific three-dimensional structures to acquire functional activity. The biologically active conformation (i.e., the native state) of these proteins, while thermodynamically favorable, is often only marginally stable under physiological conditions. Metastable proteins can adopt a multitude of conformational states including misfolded conformations that are prone to forming toxic aggregates, including soluble oligomers and fibrillar amyloid deposits, which are linked with neurodegeneration in AD and PD, and many other pathologies. To maintain homeostasis, all cells contain an extensive network of molecular chaperones to regulate protein synthesis, folding, and degradation. In the context of neurodegenerative disease, these molecular chaperones can become dysfunctional (Hartl 2017).

Gene Mutations, Noncoding RNAs, and Exosomes

While most of the common neurodegenerative disorders are sporadic with unknown etiology, it is now widely believed that the susceptibility to one or more of these illnesses probably involves a combination of genetic and environmental risk factors. Many of these illnesses (e.g., AD, PD, amyotrophic lateral sclerosis – ALS) have familial forms that have been particularly useful for genetic analyses where specific mutations have revealed the importance of protein and enzyme abnormalities to the illness pathology. Examples include the amyloid precursor protein and presenilin genes in AD, α-synuclein and Parkin in PD, and superoxide dismutase in ALS. Next-generation sequencing has resulted in a rapidly evolving literature over the last several years for both the identification of candidate genes and the development of diagnostic markers for neurodegenerative diseases. Gene-expression studies have implicated biological pathways such as inflammation, protein homeostasis, and RNA splicing as critical to the development and progression of multiple neurodegenerative disorders. Another rapidly evolving area of (genetics-related) research focuses on the "epigenetic" contributions of noncoding RNAs (ncRNAs) to the pathophysiology of

neurodegenerative diseases. Noncoding RNAs (ncRNAs) are functional RNA molecules that are transcribed from DNA but not translated into proteins. Typically, ncRNAs function to regulate gene expression at the transcriptional and posttranscriptional level. They play a major role in heterochromatin formation, histone modification, DNA methylation targeting, and gene silencing. ncRNAs are now known to play critical roles in brain evolution, development, homeostasis, stress responses, and plasticity (Salta and De Strooper (2017)). Among the ncRNAs, the most widely studied group with relevance to neurodegeneration are the microRNAs (miRNAs) which are now known to play dynamic roles in neurodevelopment, neuroplasticity, and stress responses (Sharma and Lu 2018). Recent research has demonstrated that miRNAs are often transported in body fluids within exosomes, small cell-derived vesicles that function in intercellular communication processes. Once released into the extracellular fluid, exosomes fuse with other cells and transfer their cargo to the acceptor cell. Exosomes can thus transport miRNAs that can have positive or negative effects on neural plasticity. Exosomes are also involved in the spread of "toxic" proteins in neurodegenerative disorders which are mutated or "misfolded" proteins and serve as template for the formation of oligomers.

Pharmacological Intervention

There are multiple mechanisms known to underlie neurodegeneration that at least theoretically could be targeted for pharmaceutical intervention. Currently however, there is no clinically available therapeutic agent that can reliably protect the brain from progressive neurodegenerative processes for sustained periods. Due to the extensive amount of preclinical research that has been conducted in recent years, there is a basis for optimism, however. It appears likely that some of these approaches may result in clinically effective therapeutic modalities in the near future. A short (representative) overview of some of the investigational approaches to combat neurodegeneration is provided below.

Inhibitors of Oxidative Stress

There are both epidemiologic evidence and prospective data from in vitro studies and animal experiments that support the premise that lipophilic antioxidants such as vitamin E, coenzyme Q10 (CoQ10), vitamin A, carotenoids, docosahexaenoic (DHA), and eicosapentaenoic acids (EPA) may have a therapeutic role in neurodegenerative illnesses. Several of these compounds have been evaluated in prospective clinical trials, however, to date; the data have provided negative, conflicting, or equivocal results with some studies showing slightly positive effects and others showing little or no effect. A number of issues require further attention in this area such as the identification of optimal doses of the various antioxidant compounds as well as the evaluation of selected combinations of these agents. These issues are important since specific compounds are known to scavenge or inactivate specific oxidative agents, and thus a single compound would not intuitively be expected to combat free radicals originating from several sources.

Modulators of Glutamate Transmission

The prototypic glutamate NMDA antagonist dizocilpine (MK-801) has been shown to reduce the detrimental effects of excess glutamate (as well as other insults to neurons) in vitro as well as in a variety of animal models, thus supporting this general pharmacological approach in human neurodegenerative illnesses. Unfortunately, dizocilpine is too toxic for use in humans. Several glutamate antagonists have been (or are in the process of being) evaluated both preclinically and clinically as neuroprotective agents. Memantine (another NMDA antagonist) is available adjunctively (to be administered with acetylcholinesterase inhibitors) for the treatment of moderate to severe AD. Riluzole (a drug which inhibits glutamate release) is available clinically for the treatment of ALS and is somewhat effective in slowing progression. Riluzole has also been shown to reduce infarct size in stroke and

brain injury after trauma in animal models, and accordingly human studies are anticipated in the near future. Currently there is considerable interest in developing compounds that modulate metabotropic glutamate receptors (mGluRs). A variety of mGluRs agonists and antagonists as well as positive and negative allosteric modulators are in varied stages of development and testing (Ribeiro et al. 2017). Other agents that modulate glutamate receptors such as AMPA antagonists and compounds that interact allosterically at the polyamine and glycine receptor sites are also being evaluated.

Targeting Mitochondrial Dysfunction

It is clear that mitochondrial dysfunction is present in multiple neurodegenerative diseases; however, it is not always clear if the dysfunction is a causal factor in the illness or a result of some other pathologic process. The types of mitochondrial dysfunction that have been characterized in neurodegenerative illnesses include oxidative damage, altered mitochondrial dynamics and quality control, impaired ATP synthesis, NAD+ depletion, disrupted calcium homeostasis, and the production of abnormal protein or peptide aggregates. Thus, mitochondria are thought to contain multiple targets that are potentially druggable. In animal models, mitochondria-targeted antioxidants such as MitoQ and mitochondria-targeted peptides such as elamipretide have been found effective as have treatments designed to enhance mitochondrial biogenesis by increasing the activity of transcription factors such as PGC1α and NRF2 or of AMPK. In addition, replenishing NAD+ pools with molecules such as NMN67 or improving mitochondrial dynamics (e.g., mitochondrial division inhibitor-1, mdivi-1) has also shown benefit in animal models. Unfortunately, the approaches evaluated to date (e.g., NRF2, MitoQ) showed no effect in clinical trials (Murphy and Hartley 2018).

Neuroinflammation

Currently, reducing neuroinflammation in neurodegenerative diseases is one of the most active areas of focus in drug discovery research. Inflammatory processes associated with neurodegenerative disease suggest a number of therapeutic targets, including inhibitors of complement activation or cytokines, free radical scavengers, and inhibitors of microglial activation. In retrospective studies, the use of nonsteroidal anti-inflammatory drugs (NSAIDs) has been associated with a reduced incidence or slowed progression of AD, indicating a potential for therapeutic use of this class of agent. However, to date, no NSAIDs have been found to be effective in prospective clinical trials in neurodegenerative diseases. Other areas of current focus include the modulation of eicosanoid signaling, agonists of peroxisome proliferator-activated receptor-γ (PPARγ) (a nuclear hormone receptor that acts as a transcription factor in the regulation of inflammatory gene expression), and human plasma-derived products such as immune globulins. Other compounds in various stages of testing include minocycline, an antibiotic that crosses the blood-brain barrier and exhibits anti-inflammatory and neuroprotective effects, and cannabinoid receptor ligands (e.g., CB2 agonists) that have anti-inflammatory activity. Novel epigenetic approaches (e.g., histone deacetylase 2 (HDAC2) inhibitors) for modulating the activation states of microglia and astrocytes are also currently under investigation (Fu et al. 2019).

Inhibitors of Apoptosis, Necroptosis, and Growth Factor-Like Molecules

As indicated earlier, apoptosis is inhibited by certain proteins, such as Bcl-2 and Bcl-x. In contrast, Bax and the tumor suppressor protein, p53, have been shown to enhance the onset of apoptosis. Accordingly, drugs which have the ability to enhance the expression of Bcl-2 and Bcl-x or to inhibit the expression of Bax and p53 could theoretically have the potential to reduce neurodegeneration. Drugs that inhibit apoptosis-inducing enzymes including caspases may also have a role. The expression of Bcl-2 and other proteins in this family is also modulated by trophic factors such as NGF and basic fibroblast growth factor (FGF), endogenous neurotrophins

which have been shown to block cell death and preserve the phenotype of various cells in the nervous system. Unfortunately, NGF, FGF, as well as most other peptide molecules fail to adequately penetrate the brain from peripheral administration and are thus considerably limited from a therapeutic standpoint. Recent interest has thus focused on low molecular weight growth factor-like molecules, molecules that increase the release of growth factors in the brain (or increase their receptors), or various downstream signaling molecules. There is also increasing evidence that inhibition of necroptosis using inhibitors of RIP1, RIP3, or MLKL can confer neuroprotective effect in neurodegenerative disorders (Zhang et al. 2017).

Autophagy Inducers

Defective autophagy has been reported in multiple neurodegenerative diseases; thus the induction of autophagy for enhancing the clearance of misfolded proteins is considered a potential treatment strategy. The therapeutic potential of this therapeutic strategy has been demonstrated in a variety in vitro systems and in vivo transgenic models of neurodegenerative diseases. Examples include mTOR inhibitors like rapamycin or its analogs that stimulate autophagy as well as mTOR-independent autophagy inducers such as lithium, carbamazepine, rilmenidine, and trehalose (Panda et al. 2019).

Other Investigational Approaches

A myriad of additional pharmaceutical approaches to neurodegenerative diseases at varying stages of development includes new calcium channel antagonists, compounds that stimulate the expression of antioxidant enzymes such as superoxide dismutase, small molecules that can serve as chemical chaperones to stabilize unfolded monomer conformations and/or to destabilize misfolded oligomers, as well as metal chelators that can sequester metal ions to block protein aggregation. Finally, it may turn out that the most effective strategy to combating devastating neurodegenerative diseases will be a combination therapy (or multi-target) approach such that taken to treat cancer, hypertension, infectious disease, etc. (Trippier et al. 2013).

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Neurokinin A Receptor

► Neurokinin/Tachykinin Receptors

Neurokinin B Receptor

► Neurokinin/Tachykinin Receptors

Neurokinin/Tachykinin Receptors

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Synonyms

Neurokinin A receptor; Neurokinin B receptor; Neurokinin-1 receptor; Neurokinin-2 receptor; Neurokinin-3 receptor; Substance P receptor; Tachykinin 1 receptor; Tachykinin 2 receptor; Tachykinin 3 receptor

Definition

Metabotropic tachykinin or neurokinin receptors (NK-1R, NK-2R, and NK-3R encoded, respectively, by tachykinin (*TACR*)*1*, *TACR2*, and *TACR3* genes) belong to the G-protein-coupled receptor family. After the binding of peptides belonging to the tachykinin family of peptides (substance P, hemokinin-1, neurokinin A, neurokinin B, eledoisin, ranakinin, neuropeptide K, kassinin), tachykinin receptors mediate many physiological and pathophysiological actions.

Basic Mechanisms

Neurokinin receptors (NK-1R, NK-2R, and NK-3R) belong to the G-protein-coupled receptor family (also known as 7TM receptors, serpentine receptors, or seven-transmembrane domain receptors). The binding of tachykinins to these

receptors promotes a change in the Gα subunit coupled to the NK receptor. Seven-transmembrane-helix receptors show the same structural unit (Graefe and Mohiuddin 2020): three extracellular and three intracellular loops flanked by seven intermembrane domains, an amino-terminal extracellular domain which is responsible for the receptor specificity, and a carboxy-terminal cytoplasmic domain involved in the desensitization of the receptor (in this mechanism, the phosphorylation of both threonine and serine residues of the C-terminus plays an important role). Once tachykinins bind to the NK receptor, the binding to protein Gα is mediated by the third cytoplasmic loop of the receptor; subsequently, a change in the Gα subunit occurs allowing the exchange of GTP for GDP, and hence the signaling cascade is induced. Five subtypes of this subunit (Gαs, Gαi, $G\alpha q_{11}$, $G\alpha_{12/13}$, Gαo), associated with several signaling pathways, have been described. Tachykinins (substance P (SP), hemokinin-1, neurokinin A (NKA), neurokinin B (NKB)), after binding to NK receptors (the conserved carboxy-terminal domain of tachykinins, Phe-X-Gly-Leu-Met-NH_2, interacts with them), control several signaling pathways by activating the second messenger adenylate cyclase which promotes the synthesis of cyclic adenosine monophosphate cytoplasmic and the activation of protein kinase A (these mechanisms are mediated by the Gαs subunit); by stimulating the release of [^{3}H]-arachidonic acid which inhibits various types of adenylate cyclase and by means of the mitogen-activated protein kinases (MAPKs) which increases the phosphorylation of extracellular signal-regulated kinases (mediated by the Gαi subunit); by stimulating the synthesis of inositol (1,4,5) trisphosphate (which promotes the release of Ca^{2+} from the endoplasmic reticulum) and diacylglycerol (which activates protein kinase C) (mediated by the $G\alpha q_{11}$ subunit); by regulating changes in cytoskeletal rearrangement (depending on the activation of the Rho/Rock signaling pathway that modulates the myosin regulatory light chain protein) when cells are preparing to migrate (mediated by the $G\alpha_{12/13}$ subunit); or by activating the Wnt-β- catenin signaling pathway (mediated by the Gαo subunit) (Muñoz and Coveñas 2018).

SP, via the NK-1R, regulates many cell signaling pathways controlling cell function (e.g., promotes the synthesis of DNA and the activation of phospholipases A_2/C), exerts an antiapoptotic action (activating the basal kinase activity of Akt (protein kinase B) via phosphatidylinositol-3 kinase), favors the synthesis of pro-inflammatory cytokines (e.g., interleukins 1, 6, and 12, tumor necrosis factor (TNFα)), and activates pro-inflammatory transcription factors (e.g., nuclear factor kappa B (NF-κB); an NF-κB binding site has been located in the promoter of the *TACR1* gene, a promoter which also contains binding sites for other transcription factors (e.g., AP1, Sp1, Oct2) that control inflammatory genes) (Muñoz and Coveñas 2019). SP, via the NK-1R, is involved in cell proliferation (activating the MAPK cascade (including p38MAPK and ERK1/2) that induces the activation of the AP-1 transcription factor, a heterodimer of c-jun and c-fos), cell migration (promotes changes in cellular shape (e.g. blebbing) in which the Rho/Rock system is involved), angiogenesis (the NK-1R expressed in endothelial cells mediates its proliferation), glycogen breakdown (to obtain glucose for cell metabolism) and inflammation (Muñoz and Coveñas 2019). SP, via the NK-1R, also induces c-myc mRNA/protein synthesis (involved in the progression through S to the G2/M phase of the cell cycle) and the formation of the epidermal growth factor receptor complex which contains the adapter proteins Grb2 and SHC. SP regulates the expression of the hypoxia-inducible factor and increases the expression of vascular endothelial growth factor C and, by enhancing the activation of several signaling pathways (Akt, JNK, ERK1/2), increases the expression of degradative matrix metalloproteinases. SP regulates the NK-1R/Hairy and Enhancer of Split 1, a transcriptional inhibitor of the Notch signaling pathway, induces the mammalian target of rapamycin signaling axis, and activates p70 S6 kinase and the eukaryotic initiation factor 4E-binding protein 1.

The activation of the NK-2R (coupled to the $G\alpha q_{11}$ protein) activates the RhoA-Rho kinase

pathway and the release of adenosine triphosphate which is mediated by protein kinase C, Ca^{++}/calmodulin signals, and multidrug resistance protein transporters. The ablation of the *TACR2* promotes gastric emptying disturbances and upregulates the expression of vasoactive intestinal polypeptide and nitric oxide synthase; NF-κB and Creb signalings are involved in these changes. An association between the age-related decline of rostral cholinergic basal forebrain volume and a NK-3R single-nucleotide polymorphism has also been reported as it is known that puberty onset time is associated with the single-nucleotide polymorphism A63P located in the *TAC3* gene.

Tachykinin receptors are widely distributed by the peripheral/central nervous systems; they are also found in immune (e.g., macrophages, dendritic cells, monocytes, lymphocytes) and endothelial cells, smooth muscle, skin, thyroid gland, spleen, thymus, lymph nodes, lung, genitourinary/gastrointestinal tracts, and cancer cells (in which the NK-1R is overexpressed) (Muñoz and Coveñas 2018). Thus, the NK-1R is involved in immunoregulatory and vasodilatory (endothelial cells express the receptor) mechanisms mediated by SP. The distribution of the NK-2R is less widespread than that found for the NK-3R (this is mainly expressed in brain limbic areas). Tachykinins (mainly SP and NKA) are widely distributed by the peripheral and central nervous systems, are present in body fluids (breast milk, blood, cerebrospinal fluid), and are located in non-nerve cells (endothelial cells, fibroblasts, smooth muscle, platelets, Leydig cells, immune cells (eosinophils, macrophages, monocytes, lymphocytes)) and cancer cells (Muñoz and Coveñas 2019). The distribution for both SP and NKA is more widespread than that observed for NKB. The *TAC1* gene (in chromosome 7) encodes SP (it can be processed originating SP biological active fragments (SP_{1-4}, SP_{1-7}), neuropeptide K and NKA, and the *TAC3* gene, NKB. SP carboxy- and amino-terminal sequences are cleaved by proteolytic enzymes: carboxy-terminal fragments (e.g., SP_{6-11}) show a high affinity for the NK-3R, whereas amino-terminal fragments (e.g., SP_{1-4}, SP_{1-7}) bind to NK-1R and NK-2R (Duarte et al. 2016). SP is hydrolyzed by angiotensin-converting hormone (plasma) and p-endopeptidase (extracellular fluid) and has a lower half-life in tissues (seconds/minutes) than in plasma (hours). Tachykinins bind to all three NK receptors (a lack of specificity due to the conformational flexibility of the tachykinergic peptides occurs) showing different degrees of affinity for them: SP shows the highest affinity for the NK-1R, and for this reason it has also been named SP receptor; hemokinin-1 (present in non-neural tissues) also shows a high affinity for the NK-1R; NKA has a high affinity for the NK-2R and NKB for the NK-3R (Muñoz and Coveñas 2019). Chemical modifications can change affinity; thus the substitution to NKB with MePhe and Asp improves receptor binding and provides a NK-3R selective analog. The NK-1R has different active conformations, each of which shows a different affinity for distinct antagonists or agonists. This receptor has seven hydrophobic alpha-helical transmembrane domains. The intracellular C-terminus and the extracellular N-terminus of the NK-1R, respectively, contain potential phosphorylation and glycosylation sites: NK-1R signaling is regulated by phosphorylation/glycosylation mechanisms. SP, via the NK-1R, promotes the phosphorylation, desensitization (a low concentration of SP favors the return of the NK-1R to the cell membrane; a high concentration induces the ubiquitination/degradation of the receptor), and internalization (clathrin-dependent mechanism) of the NK-1R. Two isoforms, with different signaling capabilities (e.g., in cancer cells), of the NK-1R are known: the full-length and the truncated (the oncogenic isoform of the NK-1R that mediates tumor cell malignancy) forms (Muñoz and Coveñas 2018). In cancer cells, the full-length form is downregulated, whereas the truncated form is upregulated. The expression of the NK-1R was increased when SP activated the NF-κB. SP binds to the extracellular loops of the NK-1R, but non-peptide NK-1R antagonists bind in a pocket relatively deep between the III–VI transmembrane segments of the receptor. Gln165 (IV), His197 (V), His265 (VI), and Tyr287 (VII) residues play an important role in the binding of non-peptide NK-1R antagonists to the NK-1R, whereas other residues (Ser169,

Glu193, Lys194, Phe264, Phe267, Pro271, Tyr272) also contribute to this binding. Moreover, it is known that transmembrane domains 5 and 6 are involved in NK-3R ligand binding and trafficking, whereas the third intracellular loop in NK-3R signaling. The activation of the NK-3R affects gene expression (genes involved in synaptic plasticity, cell growth, and cell signaling) and chromatin structure (relaxation) by means of histone acetylation.

To date, the SP/NK-1R system is the most tachykinergic studied system, and the involvement of the tachykinin/NK receptor system in many physiological and pathophysiological mechanisms is known. The broad physiological actions exerted by tachykinins are explained by the binding capacity of tachykinins to all three NK receptors. Table 1 shows some examples of this involvement, not only for SP but also for NKA and NKB. The data shown in Table 1 suggest that NK receptor antagonists could be potentially used for the treatment of many human pathologies.

Drugs

Peptides are mainly released under a pathological situation or after a strong cell activation, and it seems that NK receptor antagonists exert a therapeutic effect acting on deranged tachykinergic systems in which a high amount of peptides is released. Although tachykinins, via NK receptors, are involved in the molecular bases of many physiological/patophysiological mechanisms, only five NK receptor antagonists (all of which are non-peptide NK-1R antagonists) have currently been approved for use in humans for the treatment of acute/delayed chemotherapy-induced nausea/vomiting and for postoperative nausea/vomiting: aprepitant (Emend, L-754,030, MK-869; oral administration; Cinvanti, aprepitant injectable solution), fosaprepitant dimeglumine (Ivemend, a water-soluble prodrug of aprepitant which is converted to aprepitant by ubiquitous phosphatases; intravenous administration), rolapitant (Varubi, oral administration), netupitant (oral administration) and fosnetupitant (a prodrug form of netupitant which is converted by phosphatases to its active form; intravenous administration) (Fig. 1). In preclinical studies, the effective action of NK receptor antagonists has been demonstrated, but in general, the action has unfortunately been ineffective in clinical trials. This can be explained by the low dose of the drug administered, the inappropriate selection of patients/end points, and/or the lack of knowledge on the molecular interaction between NK receptors and their antagonists. It is pivotal to know in depth the structure-function relationship of tachykinins-NK receptors for a rational design of new drugs. To date, no NK-2R or NK-3R antagonist (Figs. 2, 3) has been approved to be administered in humans; although they all were safe, this was mostly due to a lack of efficacy. NK-2R or NK-3R antagonists have been tested in clinical trials for the treatment of depression, diarrhea, schizophrenia, gastrointestinal disorders, asthma, panic disorder, and major depressive disorder. NK-1R antagonists exert (in a dose-dependent manner: the greater the concentration, the greater the therapeutic effect; the greater the number of NK-1Rs, the greater the concentration of the NK-1R antagonist) antiemetic, anxiolytic, analgesic, anti-inflammatory, anticonvulsant, antipruritic, apoptotic, antitumor, antidepressant, antiangiogenic, antimetastatic, and antiviral effects. The therapeutic effect exerted by NK receptor antagonists depended on the number of NK receptors expressed and on the degree of affinity of NK receptor antagonists for NK receptors.

In many pathologies, the SP/NK receptor system is upregulated (Table 1). In inflammatory processes the NK-1R and SP are upregulated; in chronic stress, acute pancreatitis, asthma, and HIV-positive patients, the NK-1R expression was also upregulated; in the latter patients, the SP level was also increased in blood, and it is known that SP, via the NK-1R, increases HIV replication in immune cells. In the skin, SP induces the degranulation of mast cells increasing capillary permeability and edema formation. In mastocytosis (abnormal growth/accumulation of mast cells), an increase in the plasma level of SP and in the number of NK-1R-positive cells has been observed. Moreover, changes in the levels of both the NK-1R and SP have been observed in

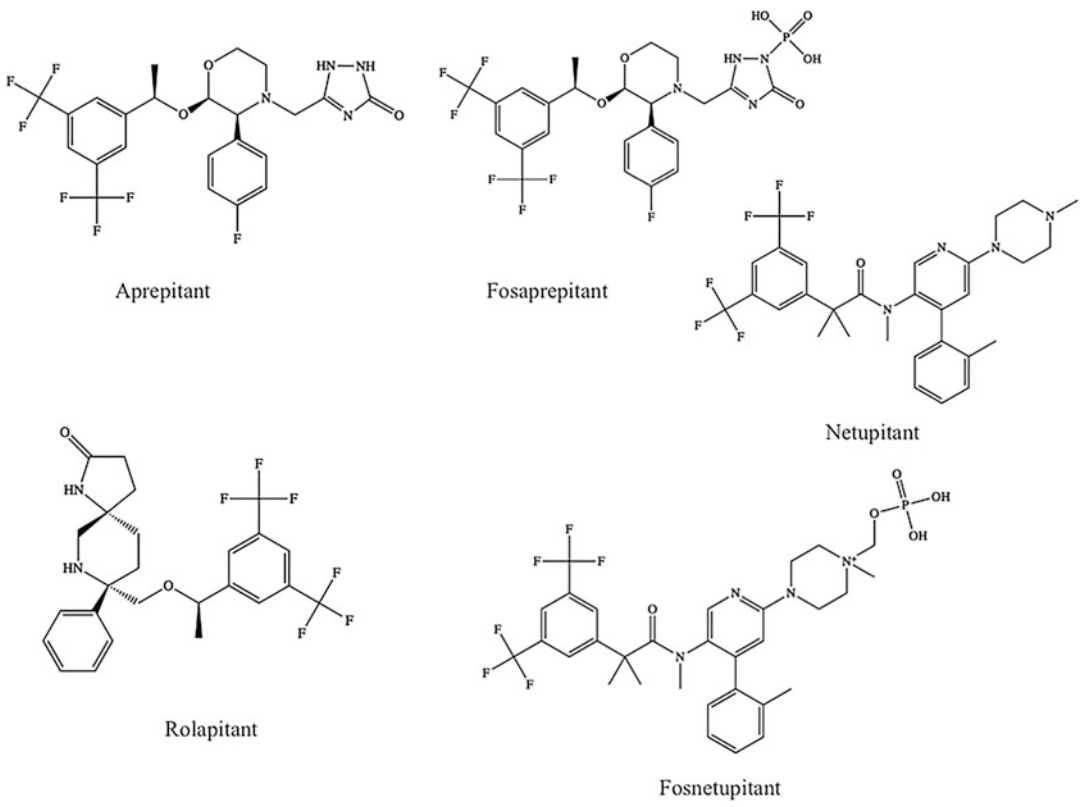
Neurokinin/Tachykinin Receptors, Table 1 SP, NKA, and NKB: physiological and pathophysiological implications

	SP	NKA	NKB
Physiology	Hematopoiesis Locomotion Melanogenesis Memory Permeability of the blood-brain barrier Recruitment of leukocytes and mast cells Release of acetylcholine, histamine, glutamate, dopamine, prolactin, and adrenocorticotropin and growth hormones Respiratory and cardiovascular mechanisms Secretion of chloride, mucus, bile, water, and electrolytes Sperm and gastrointestinal tract motility Synthesis of inflammatory cytokines	Gastrointestinal motility and secretion Hematopoiesis Immune system regulation Memory Release of kisspeptin, gonadotropin-releasing hormone, serotonin, growth hormone, growth hormone-releasing hormone, acetylcholine, adenosine triphosphate, and luteinizing hormone Respiratory and cardiovascular mechanisms	Activation of hypothalamic kisspeptin neurons Angiogenesis Binding to copper ions Cardiovascular, respiratory, and reproductive mechanisms Control of dopaminergic neurons Filopodia growth and sprouting Learning Memory Regulation of heat-defense pathways Release of 5-hydroxytryptamine, testosterone, follicle-stimulating hormone, peptide YY, luteinizing hormone, vasopressin and gonadotropin-releasing hormone Thermoregulation
Pathophysiology	Aggressive behavior Alcoholism Anxiety Arthritis Asthma Bacterial infection (sepsis, meningitis) Bipolar disorder Cancer Chronic bronchitis Crohn's disease Depression Dermatitis Diabetes Drug reinforcement and reward Eczema Epilepsy Fibromyalgia	Allergic rhinitis Anxiety Asthma Bladder dysfunction Cancer Defensive behavior Depression Epilepsy Gastric ulcer Genitourinary diseases Inflammation Irritable bowel syndrome with diarrhea Pain Psoriasis Schizophrenia Spasmogenic activity Stress Taste	Alcoholism Cancer C-reactive protein (an immune mediator) pathogenesis Fear memory consolidation Influences the behavioral responses to cocaine Irritable bowel syndrome Menopausal hot flushes Nonobstructive azoospermia Polycystic ovarian syndrome Regulation of the puberty onset Reproductive function Schizophrenia Stress Uterine leiomyomata

(continued)

Neurokinin/Tachykinin Receptors, Table 1 (continued)

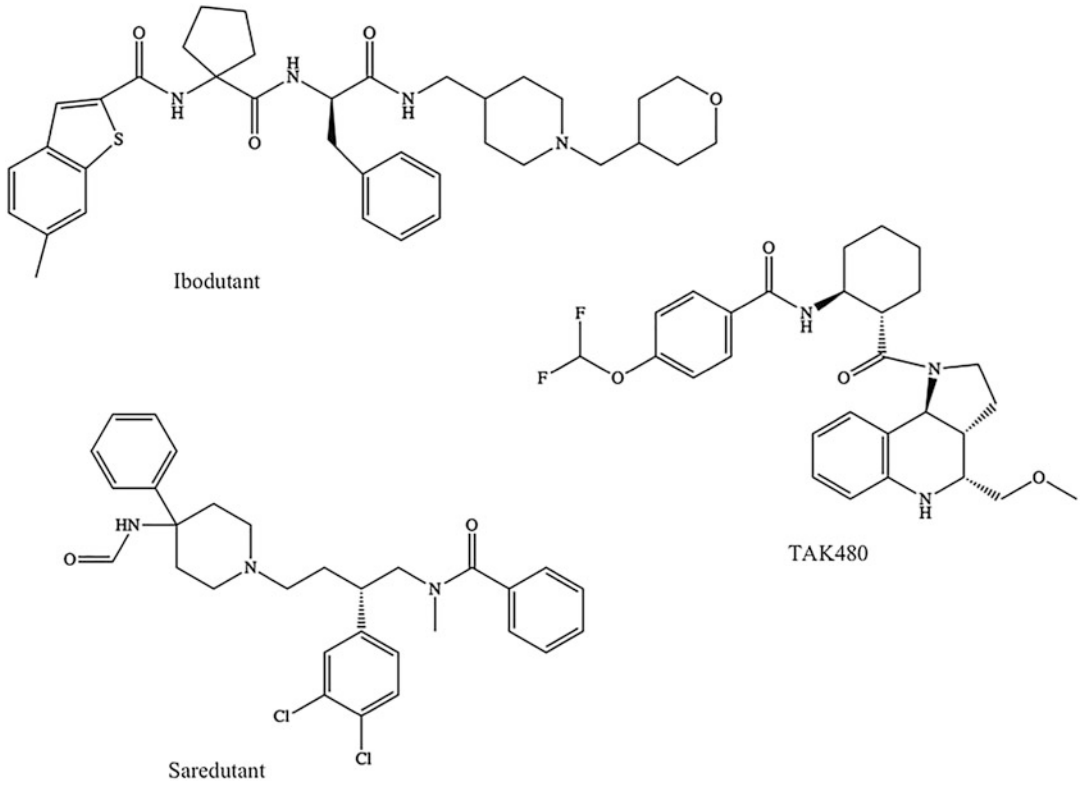
	SP	NKA	NKB
	Heart failure Hepatitis Hirschsprung's disease Inflammation Migrane Mycosis fungoides Myocardial infarction Myocarditis Nausea and vomiting Neurodegeneration Obesity Pain Pancreatitis Pruritus Psoriasis Schizophrenia Seizure Smoking (inhaled cigarette smoke favors the release of SP and promotes the generation of ROS and neurogenic inflammatory mechanisms) Suicide Thrombus formation Ulcerative colitis Urinary incontinence Virus infection and replication (HIV, respiratory syncytial virus, encephalomyocarditis virus, herpes virus, measles virus)		



Neurokinin/Tachykinin Receptors, Fig. 1 Chemical structures of non-peptide NK-1R antagonists: aprepitant, fosaprepitant, fosnetupitant, netupitant, and rolapitant

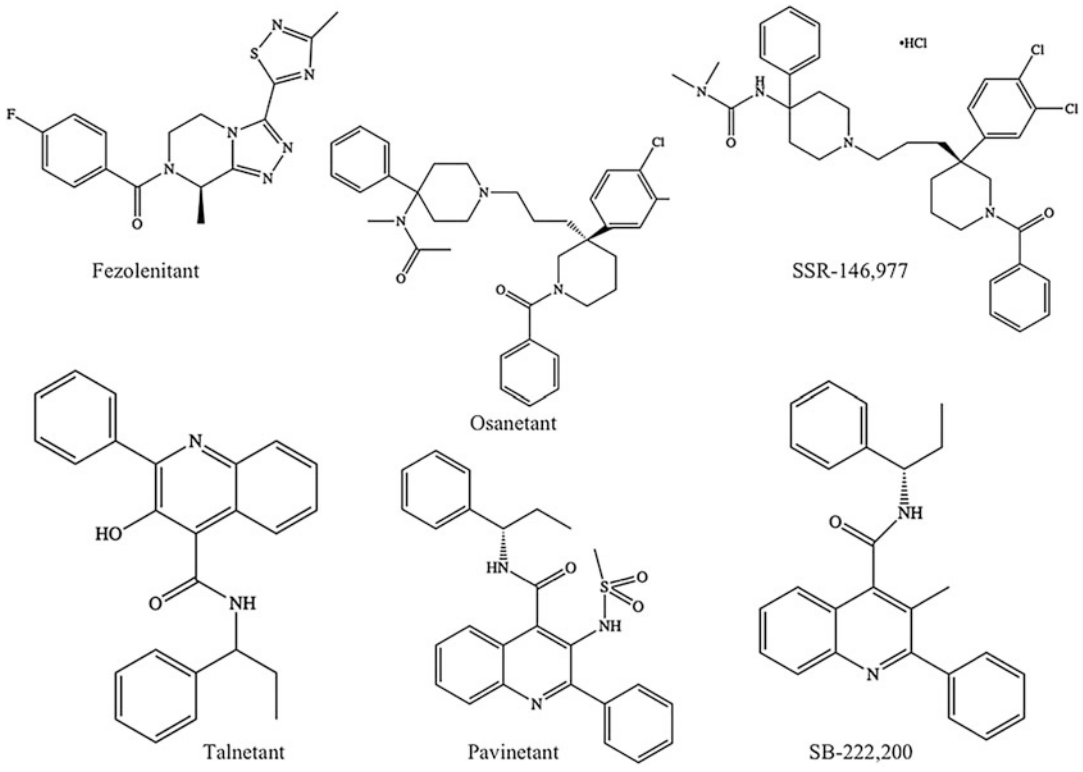
bipolar disorder and schizophrenia. In the latter disease, fibromyalgia, major depression (antidepressant drugs decrease SP synthesis), and in people attempting suicide, the level of SP was increased. In Crohn's disease and ulcerative colitis, SP (in the inflamed mucosa) and the NK-1R (in arterioles and lymphatic tissue) were upregulated. SP is involved in rheumatoid arthritis: the joint with severe arthritis is densely innervated by SP-containing fibers, and an increase in the level of SP in the synovial fluid/serum has been reported; synovial endothelial cells express the NK-1R. In pseudomembranous colitis and asthma, an overexpression of the NK-1R has been observed, whereas a high level of SP has been found in the nasal secretion of coughing subjects, and it is known that SP is involved in the proliferation, migration, invasion, and metastasis of cancer cells in which the NK-1R is overexpressed. In acute cystitis, the SP/NK-1R system is upregulated, and when bacteria infect the urinary bladder and promote pain and dysuria, the SP/NK-1R system which mediates pain is activated. The upregulation of the SP/NK-1R system in the abovementioned pathologies suggests that the NK-1R is a therapeutic target and that the use of NK-1R antagonists is a potential therapeutic strategy for the treatment of these pathologies.

NK-1R antagonists are divided into non-peptide (compounds with different chemical compositions) (Fig. 1) and peptide (also known as SP analogue antagonists; in the SP molecule, L-amino acids are replaced by D-amino acids) antagonists. The latter are not brain-penetrant, can exert a toxic effect, show poor potency, and are degraded rapidly by peptidases (its use is limited in clinical practice), whereas non-peptide NK-1R antagonists show, in general, opposite



Neurokinin/Tachykinin Receptors, Fig. 2 Chemical structures of NK-2R antagonists: ibodutant (MEN-15,596), TAK-480 and saredutant (SR-48,968)

characteristics. The latter antagonists are safe and well tolerated, even at high dose (1,140 mg/day for 45 days), and serious side effects have not been reported; instead, they were mild or moderate: headaches, somnolence, fatigue, irritability, tinnitus, dizziness, and pharyngitis. NK-1R antagonists are moderate inhibitors of the CYP3A4 metabolic pathway and, for certain drugs, a dose reduction is required. These antagonists reduce the intensity of the itch (patients with pruritus showed an increase in the serum level of SP; the release of this peptide from C fibers promotes a neurogenic inflammation). Proximal airways and larynx are innervated by C vagal afferent fibers containing SP which enhance cough via the NK-1R, and, in fact, antagonists of this receptor are antitussive drugs. Doxorubicin (an anthracycline antineoplastic drug) increases fibrosis in the cardiac tissue and apoptosis in cardiomyocytes, promoting a cardiomyopathy. NK-1R antagonists attenuate cardiac fibrosis by preventing cardiomyocyte apoptosis, decrease cardiotoxicity, and increase cancer cell sensitivity to doxorubicin. NK-1R antagonists decrease edema formation and blood-brain barrier dysfunction, reduce liver inflammation, and promote the synthesis of cytokines (e.g., IL-6, IL-10) that exert a hepatoprotective effect. In seizure, the level of SP is augmented, and NK-1R antagonists increase the anticonvulsant efficacy of sodium channel blockers. NK-1R antagonists improve obesity, decrease alcohol intake, prevent the induction of the vomiting pathways mediated by the nucleus tractus solitarius and area postrema, and, in patients with irritable bowel syndrome, reduce anxiety and pain. In cancer, the SP/NK-1R system is involved in cancer progression, migration, invasion, and metastasis: the receptor is overexpressed in tumor cells and SP promotes the proliferation of these cells. A higher expression of the NK-1R is



Neurokinin/Tachykinin Receptors, Fig. 3 Chemical structures of NK-3R antagonists: fezolinetant, osanetant (SR-142,801), pavinetant (AZD-4,901), SB-222,200, SSR-146,977, and talnetant (SB-223,412)

related to a higher invasion/metastatic potential, larger tumor size, and poorer survival. This means that the NK-1R can be used as a biomarker for cancer leading to earlier diagnosis and treatment. Like the NK-1R, in cancer patients, an increase in the plasma level of SP has been reported, and this observation can also be used as a predictive factor for tumor development and/or a high risk of developing cancer. The NK-1R is involved in the viability of tumor cells, and hence this receptor is a new target for the treatment of tumors with NK-1R antagonists (e.g., aprepitant) (Fig. 1). Aprepitant is a broad-spectrum anticancer drug (e.g., retinoblastoma, osteosarcoma, glioma, neuroblastoma, carcinoma). NK-1R antagonist and fosaprepitant also exert an antitumor action (Fig. 1). Aprepitant is mainly metabolized in human liver by cytochrome P450, family 3, subfamily A (CYP3A4); it shows half-life ranges from 9 to 13 h and binds to plasma proteins. Aprepitant promotes apoptosis in cancer cells by increasing mitochondrial reactive oxygen species (a Ca^{2+} flux from the endoplasmic reticulum into mitochondria occurs); activates the caspase-3-dependent apoptotic pathway, via suppression of antiapoptotic target genes of NF-κB; increases apoptotic markers (propidium iodide/annexin-V) and the level of pro-apoptotic proteins (cleaved caspase-3 and 9, Bam, Bim, PARP) and mRNA expression of pro-apoptotic targets (p21, p73, Bax, Bid, Bad); decreases antiapoptotic proteins (Bcl-xL, Bcl-2); downregulates the expression of lymphoid enhancer-binding factor 1, cyclin B1, cyclin D1 CDK4, and CDC25A; blocks c-myc expression (the molecule by which p73 regulates NF-κB activity); alters cell cycle and DNA replication rates; and induces a cell increase in the G_0/G_1 phase and a decrease of cells in the S phase. Aprepitant also decreases the cardiotoxicity induced by doxorubicin and increases tumor cell sensitivity to this drug.

Table 1 shows the involvement of the NKA/NK-2R system in physiological and pathophysiological mechanisms. In uterine leiomyomata, the upregulation of the TACR2 mRNA has been described. The inhibition of the NK-2R is a therapeutic strategy for the treatment of irritable bowel syndrome (Szymaszkiewicz et al. 2019), and, in the acute inflammation of the intestine, NK-2R antagonists promote recovery from colitis, prevent inflammation, and counteract changes in enteric glial gene expression. Ibodutant (MEN-15,596) (Fig. 2), a NK-2R antagonist, improves stool pattern, abdominal pain, and overall symptoms in patients suffering from irritable bowel syndrome with diarrhea. In an experimental model of visceral hypersensitivity, the same antagonist blocked abdominal nociception. Ibodutant reduced NK-2R internalization in colon smooth muscle cells (this could exert a beneficial effect in gut diseases showing hypermotility, since NK-2R mediates spasmogenic activity on enteric smooth muscle cells) and also blocked bronchoconstriction. The NK-2R plays an important role in the neuro-immuno cross talk causing airway inflammation, including severe asthma. In this disease, the NK-2R is upregulated, and this upregulation is attenuated by bone marrow-derived mesenchymal stem cells which induce the synthesis of anti-inflammatory substances. The blockade of the NK-2R (located on the vagal endings) inhibited respiratory changes (e.g., respiratory rate increase) induced by NKA. In allergic rhinitis, mast cells overexpress the NK-2R; by targeting these receptors, the allergic airway inflammation occurring in this disease can be prevented. NK-2R also mediates the activation of dendritic cells which induce the type 1 immune response, and hence NK-2R antagonists could be used for the treatment of chronic inflammation produced by excessive type 1-dominant immunity. In the bladder, NK-2R antagonists attenuated increase bladder compliance, intravesical pressure, and bladder-afferent firing, whereas in recurrent major depressive disorder, monocytes upregulated the expression of the NK-2R and NKA favored TNFα release and the activation of NF-κB (the latter was blocked by NK-2R antagonists). In psoriasis, NK-2R-immunoreactive nerves and non-neuronal inflammatory cells (expressing NKA and NK-2R) were increased compared to healthy controls, and the number of cells expressing the NK-2R was related to pruritus intensity. The NK-2R antagonist, saredutant (SR-48,968) (Fig. 2), blocked gastric motility, and another NK-2R antagonist (TAK-480) (Fig. 2) accelerated defecation, blocked smooth muscle contraction, and improved visceral hypersensitivity. Saredutant counteracted stress-related conditions by normalizing the activity of the hypothalamic-pituitary-adrenal axis and by increasing the hippocampal expression of the brain-derived neurotrophic factor. Saredutant also exerts antidepressant and anxiolytic (without impairing cognition) effects and inhibits the release of growth hormone/growth hormone-releasing hormone and the contraction of vein smooth muscle cells mediated by NKA.

Table 1 shows the involvement of the NKB/NK-3R system in physiological and pathophysiological mechanisms. In uterine leiomyomata, the expression of NKB and NK-3R is upregulated. NKB has been involved in reproductive mechanisms and in gynecological disorders since favors the release of follicle-stimulating and luteinizing hormones. The NK-3R antagonist, ESN-364, blocks the hypothalamic-pituitary-gonadal axis and regulates gonadotropin secretion (e.g., luteinizing hormone). NK-3R antagonists (e.g., talnetant (SB-223,412)) inhibited the release of testosterone, luteinizing hormone, and follicle-stimulating hormone, and another NK-3R antagonist, pavinetant (AZD-4,901) (Fig. 3), decreased the release of luteinizing hormone in patients with polycystic ovary syndrome (characterized by a high release of this hormone). Using NK-3R antagonists, the rodent litter size was decreased. NKB signaling is increased in menopausal women in whom NK-3R antagonists (pavinetant) have been tested against menopausal hot flushes with promising results (effective and safe) (Anderson et al. 2019). In schizophrenia, NK-3R antagonists (e.g., talnetant, osanetant) (Fig. 3) improved positive symptoms. The NKB/NK-3R has also been involved in fear consolidation, since when a genetic silencing of the *TAC2*-expressing neurons occurred, an impaired

fear consolidation appeared, whereas osanetant (SR-142,801, a NK-3R antagonist) inhibited fear memory consolidation. An increase in NKB level was observed before social isolation stress symptoms appeared. Osanetant exerts an anxiogenic effect, and, after a stressing stimulus, the NK-3R is internalized and translocated into the nucleus. In the nucleus of smooth muscle cells, the presence of NKB has also been observed. SB-222,200 (Fig. 3), a selective NK-3R antagonist, respectively, counteracted the decrease and increase of surface and cytoplasmic NK-3R densities induced by a dopamine D1/D2 receptor agonist (apomorphine), but not the increase in nuclear NK-3R density induced by the same agonist. SB-222,200 also blocked the development of oral squamous cell carcinoma in cases of bone destruction, although NK-3R agonist analogues showed antitumor/antimigration effects and NKB antiangiogenic actions.

In humans, NK receptor antagonists are safe and well tolerated; they are available as potential therapeutic agents, but unfortunately this potential is currently minimized. The antitumor and antipruritic action of NK-1R antagonists (in combination with radiotherapy/cytostatic drugs or alone) must be studied in depth (e.g., an adequate dose of NK-1R antagonists for the treatment of a specific pathology must be established) as much pivotal data demonstrate that the SP/NK-1R system is involved in both pathologies and hence they are currently the most promising future lines of research. The potential therapeutic dose of the NK-1R antagonist administered must be established according to a determined pathology (cancer, pruritus) by its pharmacokinetic characteristics (e.g., elimination/excretion, absorption, distribution by different tissues and plasma). A lower dose will not reach the necessary threshold to promote a therapeutic effect, and this might be related to the number of NK receptors that must be blocked by the NK receptor antagonist. In this sense, it has been suggested that to obtain a therapeutic antitumor action, aprepitant (>20 mg/kg/day) must be administered daily and for a long period of time depending on the response to treatment. NK receptors are promising targets in many diseases, and NK receptor antagonists could be used for numerous therapeutic interventions.

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Neurokinin-1 Receptor

► Neurokinin/Tachykinin Receptors

Neurokinin-2 Receptor

► Neurokinin/Tachykinin Receptors

Neurokinin-3 Receptor

► Neurokinin/Tachykinin Receptors

Neuronal Cell Death

► Neurodegeneration

Neuronal Cell Deterioration

▶ Neurodegeneration

Neuropathology

▶ Ca^{2+}-Binding Proteins

Neuropeptide Y

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Synonyms

NPY

Definition

Neuropeptide Y (NPY) is a 36 amino acid polypeptide with tyrosine residues at both ends of the molecule. It is characterized structurally by a "PP-fold" consisting of an extended polyproline helix and an α-helix connected by a β-turn. Based on structural and evolutionary criteria, NPY is closely related to **peptide YY** (PYY) and **pancreatic polypeptide** (PP).

Basic Characteristics

NPY is primarily (but not exclusively) synthesized and released by neurons, which in the peripheral nervous system are predominantly sympathetic neurons. In most cases, NPY acts as a co-transmitter that is preferentially released upon high-frequency nerve stimulation. NPY can be metabolized by the enzyme ***dipeptidylpeptidase 4*** (also known as ***CD26***) (Elmansi et al. 2019) to generate the biologically active fragment $\boldsymbol{NPY_{3–36}}$.

NPY has been highly conserved during evolution and has many effects in the central and peripheral nervous system. In the central and peripheral nervous system, NPY auto- and heteroreceptors (mostly Y_2 subtype) mediate prejunctional inhibition of **neurotransmitter release**. Central effects include a potent stimulation of food intake, anxiolytic effects (Reichmann and Holzer 2016), anti-seizure activity, and various forms of neuroendocrine modulation (Kalra and Crowley 1992). Based on these effects, it has been proposed to play a role in addiction (Thorsell and Mathé 2017), **learning** and **memory** (Gøtzsche and Woldbye 2016), **anxiety** and post-traumatic stress disorder (Reichmann and Holzer 2016), and obesity and **appetite control** (Loh et al. 2015), the latter probably reflecting a central and peripheral effect.

In the peripheral nervous system, NPY was primarily identified as a potent vasoconstrictor that has direct effects on blood vessels and also potentiates **vasoconstriction** by other agents (mostly via Y_1 receptors) (Franco-Cereceda and Liska 1998). Despite reductions of renal blood flow, NPY enhances diuresis and natriuresis in the kidney (Bischoff and Michel 1998). NPY can inhibit pancreatic insulin release and inhibit **lipolysis** in adipocytes (Loh et al. 2015). It also can regulate gut motility and gastrointestinal and renal epithelial secretion. In some cell types, e.g., in vascular smooth muscle cells, NPY appears to enhance cell growth. Peripheral effects of NPY have been proposed to be relevant in cardiovascular diseases including **atherosclerosis**, congestive **heart failure** and arrhythmia (Tan et al. 2018), inflammatory bowel disease (El-Salhy and Hausken 2016), **osteoporosis** (Horsnell and Baldock 2016), and some forms of **cancer**, particularly those of childhood (Tilan and Kitlinska 2016).

NPY, PYY, and PP act upon the same family of receptors, which are classified together as **NPY receptors**. Based on IUPHAR recommendations, the NPY receptors are designated by a capital Y, and the various receptors within the family are designated by subscript numbers (Michel et al. 1998). Five mammalian subtypes of NPY receptors have been cloned and are designated Y_1, Y_2,

Y_4, Y_5, and y_6. The Y_4 receptor preferentially binds PP, whereas NPY (and PYY) are much less potent at the Y_4 receptor than at the other subtypes; hence, this PP-preferring subtype will not be discussed here. The y_6 receptor is found in mice and rabbits, represents a nonfunctional pseudo-gene in humans and primates, and is absent from the rat genome (Khor et al. 2016).

Sequence comparisons show that receptors Y_1, Y_4, and y_6 are more closely related to each other than to the receptors Y_2 and Y_5 (Michel et al. 1998). This is apparent not only from sequence identity but also from other features, such as cysteines believed to form disulfide bonds and the size of the third cytoplasmic loop, which is large in Y_5. The receptors Y_2 and Y_5 are equally distantly related to one another as to the $Y_1/Y_4/y_6$ group. In fact the $Y_1/Y_4/y_6$ group, the Y_2, and the Y_5 receptor are more distantly related to one another than any other G-protein-coupled receptors that bind the same endogenous ligand, despite the fact that Y_1, Y_2, and Y_5 each bind two distinct endogenous ligands, namely, NPY and PYY. However, based on pharmacological recognition profiles, the receptors Y_1 and Y_5 are more similar to one another than to the receptor Y_4 (see below).

- Y_1 receptors have been cloned from humans, rats, mice, and from non-mammals such as *Xenopus laevis*. The genomic organization of the Y_1 subtype gene has been determined in humans and mice, and the human gene has been located on chromosome 4q(31.3–32). Three splice variants in the 5′ region of the human Y_1 receptor yield multiple promoters with tissue-specific expression patterns (Cerda-Reverter and Larhammar 2000). Two splice variants of the murine Y_1 receptor have been described. While both variants bind NPY, the form with a shortened seventh transmembrane-spanning region and a lacking C-terminal tail does not appear to couple to signal transduction as efficiently as the full-length form. Messenger RNA for the Y_1 receptor has been detected in a variety of human, rat, and murine tissues including the brain, heart, kidney, and gastrointestinal tract.
- Y_2 receptors were originally cloned from human SMS-KAN cells. Later cloning studies in rats demonstrated that the cloned Y_2 receptor is also the molecular correlate of a previously proposed PYY-preferring receptor in the gastrointestinal tract. Messenger RNA for the Y_2 receptor has been detected in various parts of the CNS, whereas only low levels of Y_2 mRNA were found in human peripheral tissues.
- Y_5 receptors were cloned from humans and rats. Interestingly, the corresponding gene resides on human chromosome 4q in the same location as the human Y_1 receptor gene, but in opposite orientation (Cerda-Reverter and Larhammar 2000). Messenger RNA for Y_5 receptors was detected by Northern blotting and in situ hybridization in several rat brain areas, including those believed to be important for the regulation of food intake, as well as in testis.

All known NPY receptors belong to the large superfamily of G-protein-coupled, heptahelical receptors. They appear to use similar signal transduction pathways, and no clear and consistent alignment of a specific receptor type with a distinct transduction pathway has been identified. In almost every cell type studied (with the possible exception of some prejunctional receptors), NPY receptors act via pertussis toxin-sensitive G-proteins, i.e., members of the G_i and G_o family. The typical signaling responses of NPY receptors are similar to those of other G_i/G_o-coupled receptors. Thus, inhibition of adenylyl cyclase is found in almost every tissue and cell type investigated and also with all cloned NPY receptor subtypes upon heterologous expression. Additional signaling responses that are restricted to certain cell types include inhibition of Ca^{2+} channels, e.g., in neurons, and activation and inhibition of K^+ channels, e.g., in cardiomyocytes and vascular smooth muscle cells. Based on experiments with Ca^{2+} entry blockers, it has been postulated that NPY stimulates Ca^{2+} channels in the vasculature. In some cell types, members of the NPY family can mobilize Ca^{2+} from intracellular stores. While this appears to involve inositol phosphates in some

cells, inositol phosphate-independent Ca^{2+} mobilization has been postulated in other cell types. A sensitivity of certain responses to NPY to the cyclooxygenase inhibitor, indomethacin, indicates possible activation of a phospholipase A_2 by NPY receptors, but this has yet to be demonstrated definitively. Activation of a phospholipase D or of a tyrosine kinase, which can occur with some G_i/G_o-coupled receptors, has also not clearly been demonstrated. Thus, in general, Y receptors demonstrate a preferential coupling to pertussis toxin-sensitive G-proteins, i.e., the G_i and G_o family, which is followed by the responses typically under the control of these G-proteins (Michel et al. 1998).

Drugs

NPY receptors and NPY-induced responses were originally classified based on agonist orders of potency, but the advent of several subtype-selective antagonists has at least partly superseded the use of agonists for classification purposes. In some cases, however, particularly in vivo, agonists may still be required for receptor characterization. This is based on the use of NPY, PYY, [Pro^{34}]-substituted analogues (which may or may not contain an additional [Leu^{31}] substitution), and on C-terminal fragments of NPY and PYY (including the endogenous $NPY_{3–36}$ and $PYY_{3–36}$).

Y_1 receptors are characterized by an agonist order of potency of NPY $\geq$ PYY $\geq$ [Pro^{34}]-substituted analogue >PP > C-terminal fragment. Some C-terminal fragments that contain exchanged residues may act as partial or full agonists at Y_1 receptors (Hofmann et al. 2018) and in some cell lines even as antagonists; whether such partial antagonism also occurs with intact tissues or in vivo remains to be determined. Y_1-selective antagonists include BIBO 3304 ((R)-N-[[4-(aminocarbonylaminomethyl)-penyl]methyl]-N2-(diphenylacetyl)-argininamide trifluoroacetate, K_i or K_B 0.2–1 nM) and BIBP 3226 ((R)-N^2-diphenylacetyl)-N-[(4-hydroxyphenyl)methyl]-arginine amide, K_i or K_B 1–10 nM), with BIBP 3435 ((S)-N^2-(diphenylacetyl)-N-[(4-hydroxyphenyl)methyl]-argininamide) being a much less active stereoisomer which can be used as an inactive control for the latter one. Other Y_1 receptor antagonists include SR 120819A ((R,R)-1-(2-[2–2-naphthylsulphamoyl-3-phenyl-propionamido]-3-[4[N-(4-[dimethylaminomethyl]-*cis*-cyclohexylmethyl)amidinophenyl]propionyl)-pyrrolidine) or the polypeptide GR 231118 (also known as 1229U91 or GW1229, homodimeric Ile-Glu-Pro-Dpr-Tyr-Arg-Leu-Arg-Tyr-$CONH_2$), but the latter has also been reported to be an agonist at Y_4 receptors in some cases.

The Y_1 receptor has been crystallized with the two high-affinity antagonists UR-MK299 ((R)-N(α)-diphenylacetyl-N(ω)-[2-([2,3-(3)H]propionylamino)ethyl]aminocarbonyl-(4-hydroxybenzyl) arginin-amide) and BMS-193885 (dimethyl 4-[3-[3-[4-(3-methoxyphenyl)piperidin-1-yl]propylcarbamoylamino]phenyl]-2,6-dimethyl-1,4-dihyd ropyridine-3,5-dicarboxylate;(2S)-2-hydroxypr opanoic acid), and the structure has been solved with high resolution. By NMR analysis, mutagenesis and homology modeling full-length NPY was docked into the structure providing a first vision on ligand interaction on a molecular level (Yang et al. 2018).

Y_2 receptors are characterized by an order of potency of NPY ≈ PYY $\geq$ C-terminal fragment >> [Pro^{34}]-substituted analogue > PP. BIIE 0246 ((S)-N^2-[[1-[2-[4-[(R,S)-5,11-dihydro-6(6 h)-oxodibenz[b,e]azepin-11-yl]-1-piperazinyl]-2-oxoethyl] cyclopentyl]acetyl]-N-[2-[1,2-di-hydro-3,5-(4H)-dioxo-1,2-diphenyl-3H-1,2,4-triazol-4-yl]ethyl]-argininamide) is a Y_2-selective antagonist with an affinity of 3 nM. More recently, JNJ-31020028 (*N*-[4-[4-[2-(diethylamino)-2-oxo-1-phenylethyl] piperazin-1-yl]-3-fluorophenyl]-2-pyridin-3-ylbenzamide) was discovered that has been set up as tool to further understand Y_2 receptors in vivo (Aydin et al. 2011).

Structural characterization of the receptor by NMR and homology modeling clearly demonstrated the relevance of the C-terminus for the binding mode of NPY (Kaiser et al. 2015). Furthermore, Y_2 receptor-selective peptides with high metabolic stability are currently studied in

clinical trials to treat obesity (Østergaard et al. 2018).

Y_5 receptors are characterized by an order of potency of NPY $\geq$ PYY $\approx$ [Pro^{34}]-substituted analogue $\approx$ $NPY_{2–36} \approx PYY_{3–36} >> NPY_{13–36}$; rat PP had very low potency at the rat and human Y_5 receptor, while human and bovine PP had affinities similar to those of NPY and PYY. Y_5-selective antagonists include CGP 71683A (*trans*-naphthalene-1-sulfonic acid 4-[4-amino-quinazolin-2-ylamino) methyl]-cyclohexylmethylamide hydrochloride) with an affinity of 1 nM. Furthermore, Y_5 receptor-selective agonists are based on aminoisobutyric acid at position 32 (Cabrele et al. 2001).

As additional tools, transgenic mice overexpressing NPY and knockout mice lacking NPY, Y_1 receptors or Y_5 receptors have been published.

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Neuroprotection

▶ Ghrelin, Physiological Roles and Clinical Relevance of

Neuroprotection-Neurorescue

▶ Monoamine Oxidases and Their Inhibitors

Neurosteroids

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Definition

Produced *de novo* in the central nervous system, neurosteroids are steroids that affect neuronal function, without having any hormonal activity (Baulieu and Robel 1990). They exert their action either through genomic pathways, by interacting with a nuclear receptor leading to regulation of transcription, or through non-genomic pathways, that include interaction with plasma membrane or cytoplasmic receptors (Schverer et al. 2018).

The common point of neurosteroids is that they can modulate neuroplasticity, thus leading to behavioral consequences that will be developed below.

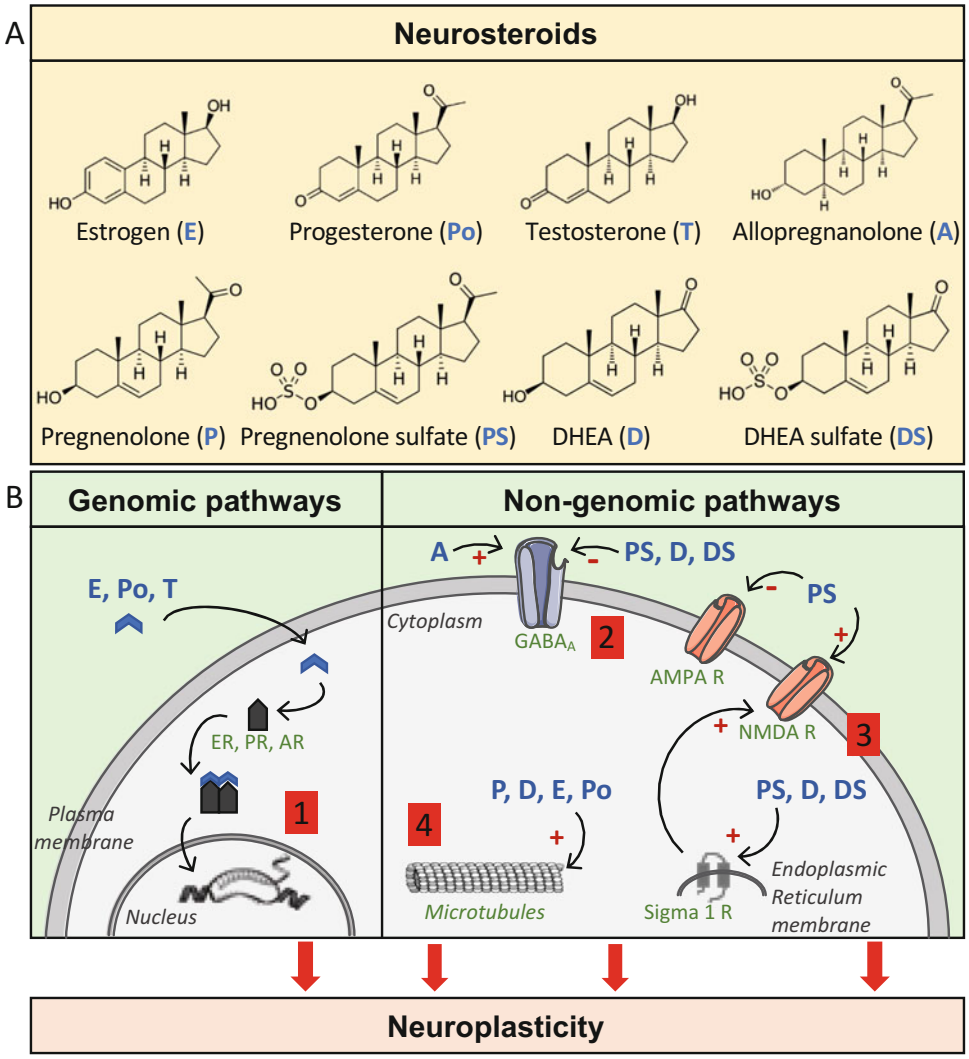
Basic Characteristics

Biosynthesis

Early studies have reported that some steroids were present in the brain even when the steroidogenic glands adrenals and gonads were ablated. Unlike peripheral steroids, neurosteroids are devoid of any hormonal activity, meaning that they are not secreted and transported via systemic circulation. Rather, they are produced in the central nervous system in both neurons and glial cells, thanks to the same enzymatic system found in peripheral steroidogenic cells. The biosynthesis of neurosteroids is thus similar to that of steroids, starting in the mitochondria by the conversion of cholesterol into pregnenolone. This is the result of an enzymatic complex composed of the mitochondrial enzyme cholesterol side-chain cleavage, ferredoxin and ferredoxin reductase (Do Rego et al. 2009). Pregnenolone is then transformed into metabolites in the smooth endoplasmic reticulum and the cytoplasm through pathways that have been well characterized. The resulting metabolites are progestrogens, estrogens, androgens, mineralocorticoids, and glucocorticoids (Do Rego et al. 2009). The most common metabolites of pregnenolone characterized as neurosteroids are progesterone, estradiol, testosterone, pregnenolone sulfate, dehydroepiandrosterone, dehydroepiandrosterone sulfate, and allopregnanolone (Fig. 1a). They all can affect brain activity, either through genomic or through non-genomic pathways.

Regulation of Neuroplasticity Through Genomic Pathways

As detailed below, neurosteroids are molecules that can modulate brain activity, acting on neuroplasticity processes. Neuroplasticity allow the brain and the organism to adapt to changing environment, via learning and memory processes. It involves the remodeling of brain structure and function in response to internal and external stimuli, occurring in specific brain areas, throughout the lifespan in both healthy and pathological conditions. Functional plasticity can be observed when repetitive electrical activity induces a long-term potentiation or depression of synaptic efficacy in brain regions. Structural plasticity refers to the complexity of the neuronal arborization (i.e., number and length of ramifications), and the dynamic of dendritic spines, which are small protrusions that serve as postsynaptic biochemical compartments receiving excitatory inputs. Neuroplasticity is a phenomenon regulated by a



Neurosteroids, Fig. 1 (**a**) Chemical structure of neurosteroids and their abbreviations. (**b**) Mechanism of action of neurosteroids. 1. In genomic pathways, the neurosteroids estrogen, progesterone, and testosterone enter in the cytoplasm of the cells, bind to their specific receptor that undergo dimerization and enter in the nucleus. The complex activates the transcription of genes involved in neuroplasticity. 2. Allopregnanolone is a positive allosteric modulator of the GABAA receptor, whereas pregnenolone sulfate, dehydroepiandrosterone, and dehydroepiandrosterone sulfate inhibits it. 3. Pregnenolone sulfate inhibits the AMPA receptor and potentiates the activity of the NMDA receptor. Pregnenolone sulfate, dehydroepiandrosterone, and dehydroepiandrosterone sulfate activate the sigma 1 receptor that in turn potentiates the activity of the NMDA receptor. 4. Pregnenolone, dehydroepiandrosterone, estrogen, and progesterone promote the activity of the microtubules. All these actions influence neuroplasticity. (Abbreviations. *ER* estrogen receptor, *PR* progesterone receptor, *AR* androgen receptor, *GABA$_A$* stands for the GABA$_A$ receptor, *AMPA R* AMPA receptor, *NMDA R* NMDA receptor, *Sigma 1 R* sigma 1 receptor)

plethora of actors, including a large variety of proteins involved in intracellular signaling pathways, and both the excitatory and inhibitory neurotransmissions, respectively, mediated by the neurotransmitters glutamate and γ-aminobutyric acid (GABA).

The genomic pathways of neurosteroids involve nuclear receptors (Fig. 1b). They are

well characterized for estrogen, progesterone, and testosterone that specifically bind to estrogen, progesterone, and androgen receptors, respectively. These receptors are anchored in the cytoplasm by chaperone proteins, and the binding of the ligand releases this anchoring, allowing dimerization of the receptor (Sever and Glass 2013). This leads to the unmasking of the nuclear localization sequence, followed by the migration of the complex in the nucleus, the association with transcriptional coactivators, and the recognition of specific DNA sequences (Sever and Glass 2013). The consequence of the genomic pathways of neurosteroids is the activation of gene transcription, leading to a series of effects, such as a trophic effect on neurons and glia, the modulation of neurotransmission, and the synthesis of proteins that promote neuroplasticity (Arnal et al. 2017) (Fig. 1b).

Regulation of Neuroplasticity Through Non-genomic Pathways

Some neurosteroids have no nuclear receptor identified. Instead, they exert their activity through non-genomic pathways, by modulating plasma membrane or cytoplasmic targets (Schverer et al. 2018). At plasma membrane, neurosteroids modulate the activity of the GABA type A ($GABA_A$) receptors, as well as the glutamate receptors N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). In the cytoplasm, neurosteroids interact with sigma 1 receptors and microtubule cytoskeleton (Schverer et al. 2018).

In 1941, Selye demonstrated the anesthetic properties of steroid hormones. It was the first evidence that these molecules could alter brain function (Selye 1941). It is now well established that this effect is due to allopregnanolone, a metabolite of progesterone (Bitran et al. 1995). This neurosteroid acts on the $GABA_A$ receptors that are mainly involved in the inhibitory neurotransmission in the brain. Two sites for allopregnanolone were identified on this receptor: a direct activation site and a potentiation site, where allopregnanolone can enhance the activity of GABA (Fig. 1b). In addition, since allopregnanolone is inactive on NMDA and AMPA receptors of glutamate (Mellon 2007), this neurosteroid is therefore characterized as an inhibitory neurosteroid. Further studies have demonstrated that other neurosteroids such as pregnenolone sulfate, dehydroepiandrosterone, and dehydroepiandrosterone sulfate were able to, in contrast, inhibit the activity of $GABA_A$ receptors, hence leading to a decrease in the inhibitory neurotransmission (Fig. 1b).

On the other hand, the excitatory glutamatergic neurotransmission, supported by NMDA and AMPA receptors, was also shown to be under the influence of neurosteroids. AMPA receptors are involved in the initiation of the glutamatergic response and NMDA receptors are responsible for its maintaining (Shi et al. 1999). Pregnenolone sulfate can act directly on these receptors at sites that have not been identified yet. This neurosteroid was shown to inhibit the activity of AMPA receptors, and to have a dual effect on NMDA receptors, depending on the subunit composition. Indeed, NMDA receptors can be composed of either NR2A, NR2B, NR2C, or NR2D subunits. Pregnenolone sulfate can potentiate the activity of NR2A- and NR2B- subunits while having the opposite effect on NR2C- and NR2D-containing receptors. In brain regions having a high level of plasticity, such as the hippocampus and the prefrontal cortex, NR2A and NR2B subunits are the most present compared to the other two, and this explains why pregnenolone sulfate is often described as an "excitatory" neurosteroid (Fig. 1b). In addition, pregnenolone sulfate, dehydroepiandrosterone, and its sulfate conjugate can also activate sigma 1 receptors. They are endoplasmic reticulum resident chaperone proteins located in the cytoplasm of neurons and glial cells. Their activation results in the potentiation of NMDA receptor activity (Fig. 1b). Overall, due to the consequence of their action on their targets, pregnenolone sulfate, dehydroepiandrosterone, and dehydroepiandrosterone sulfate are considered as excitatory neurosteroids, whereas allopregnanolone is described as an inhibitory neurosteroid.

While these sulfated neurosteroids and allopregnanolone can modulate neurotransmission,

pregnenolone, dehydroepiandrosterone, progesterone, and estrogen were shown to interact with the microtubule cytoskeleton (Fig. 1b). Pregnenolone and dehydroepiandrosterone bind to the microtubule-associated protein 2 (MAP 2) (Schverer et al. 2018). MAP 2 is strongly involved in the regulation of microtubule dynamics by enhancing tubulin assembly and promoting microtubule stability (Gordon-Weeks and Fournier 2014). In addition, pregnenolone can bind to the cytoplasmic linker protein of 170 kDa (CLIP-170), which is also a microtubule-associated protein (Gordon-Weeks and Fournier 2014). Acting through this pathway, pregnenolone was shown to promote microtubule polymerization, leading to neurite outgrowth in vitro (Schverer et al. 2018). It has also been reported that estrogen and progesterone were both able to alter microtubule function, although no target has been identified yet (Camacho-Arroyo et al. 2011). Since the cytoskeleton is a critical actor of neuroplasticity (Gordon-Weeks and Fournier 2014), these data are the direct evidence that these neurosteroids can modulate structural plasticity (Fig. 1b).

Behavioral Consequences

An increasing body of evidence have suggested the positive activity of neurosteroids on neuroplasticity through modulation of their nuclear, cytoplasmic, and plasma membrane targets. Studies conducted in adult healthy humans and rodents showed that administration of some neurosteroids could alter the behavior.

Anxiolytic and Sedative Properties

Neurosteroids are long known for their anxiolytic and sedative properties. Indeed, administration of progesterone produced sedative and anxiolytic effects in men and women (Söderpalm et al. 2004). Allopregnanolone, a metabolite of progesterone, was shown to be the active compound responsible for such effects (Bitran et al. 1995). This neurosteroid was identified to be a positive allosteric modulator of the $GABA_A$ receptor, leading to the reinforcement of the inhibitory neurotransmission in the brain. This mechanism of action could explain the behavioral effects associated to allopregnanolone.

Effects on Memory

The biological substrate for memory processes is neuroplasticity, and as seen before, neuroplasticity involves gene expression regulation, and the excitatory and the inhibitory neurotransmissions involving glutamate and GABA receptors, respectively. It is now well established that neurosteroids can modulate memory processes, and their effects are summarized in Table 1. Numerous studies have demonstrated a potent effect of estrogens on memory processes (Hojo et al. 2008). This could rely on the genomic action of estrogen on the estrogen receptor, since it leads to improved neuroplasticity in the rodent hippocampus (Hojo et al. 2008). Progesterone effects were also investigated in rodents and humans, and a growing body of evidences reported that this neurosteroid has a negative impact on memory processes (Barros et al. 2015). The mechanism of action of progesterone is still under investigation, but it seems that it could interfere with long-term potentiation mechanisms, which are critical for memory function (Barros et al. 2015). Regarding testosterone, although it was shown to improve memory in rodents (Wagner et al. 2018), it did not improve age-related memory impairments in men (Resnick et al. 2017), suggesting that this neurosteroid does not have a beneficial role in memory processes. Pregnenolone and dehydroepiandrosterone were both shown to improve memory in rodents, probably through their action on cytoskeleton leading to neuroplasticity enhancement (Schverer et al. 2018). Their sulfated conjugates have also shown a positive impact on memory in rodents, since they can increase the excitatory neurotransmission mediated by NMDA and AMPA receptors (Schverer et al. 2018). In addition allopregnanolone, via its action on $GABA_A$ receptors, is also able to reverse memory alterations in a rodent model (Schverer et al. 2018). Overall, it seems that most of the neurosteroids could act as memory enhancers.

Neurosteroids, Table 1 Neurosteroid effects on memory processes

Neurosteroid	Effect on memory	Target	Reference
Estrogen	Enhancement (in rodents)	Estrogen receptor	Hojo et al. (2008)
Progesterone	Alteration (in rodents and humans)	Unknown	Barros et al. (2015)
Testosterone	Enhancement in rodents	Unknown	Resnick et al. (2017)
	No effect in humans		Wagner et al. (2018)
Pregnenolone	Enhancement (in rodents)	Cytoskeleton modulation	Schverer et al. (2018)
Dehydroepiandrosterone	Enhancement (in rodents)	Cytoskeleton modulation	Schverer et al. (2018)
Pregnenolone sulfate	Enhancement (in rodents)	NMDA receptor, sigma 1 receptor	Schverer et al. (2018)
Dehydroepiandrosterone sulfate	Enhancement (in rodents)	NMDA receptor, sigma 1 receptor	Schverer et al. (2018)
Allopregnanolone	Enhancement (in rodents)	$GABA_A$ receptor	Schverer et al. (2018)

Potential Use in Therapeutic

Neurosteroids have shown potential therapeutic activity in diverse pathological conditions and summarized in Table 2, and this could be directly due to the action on their targets leading to neuroplasticity modulation.

Central Nervous System Injuries

Injuries to the central nervous system include retinal degeneration, stroke, spinal cord and traumatic brain injury. They are all characterized by axon damage, leading to an environment that is not in favor of regeneration, explaining the limited capacity of self-repair in the central nervous system. Axonal regeneration is therefore a challenging field of research. In this context, neurosteroids have demonstrated regenerative properties. This is the case of progesterone, which showed a protective effect on traumatic brain injury in several models, through various mechanisms (Wei and Xiao 2013). Indeed, progesterone promotes axonal regeneration by having anti-inflammatory and neurotrophic properties, but is also by being protective against excitotoxicity, apoptosis, and lipid peroxidation (Wei and Xiao 2013). A synthetic derivative of pregnenolone also showed a protective effect in three animal models of spinal cord injury (Duchossoy et al. 2011). Indeed, it improved the recovery of locomotor function, and this correlated with preservation of MAP 2 at the injury site and of dendritic arborization of motoneurons caudally to the injury site (Duchossoy et al. 2011). Therefore, both progesterone and pregnenolone seems to have a positive

Neurosteroids, Table 2 Therapeutic effects of neurosteroids

Neurosteroid	Therapeutic properties	References
Progesterone	Regenerative	Wei and Xiao (2013)
Testosterone	Antidepressant	Walther et al. (2019)
Pregnenolone	Regenerative	Duchossoy et al. (2011)
	Antidepressant	Brown et al. (2014)
Allopregnanolone	Antiseizure	Pieribone et al. (2007), Sperling et al. (2017) and Yawno et al. (2017)
	Antidepressant	Gunduz-Bruce et al. (2019) and Meltzer-Brody et al. (2018)

impact on axonal regeneration in preclinical studies, although these promising results still need to be confirmed in humans.

Epilepsy

Epilepsy is a spectrum of neurological disorders, with a common point being an altered brain activity, causing unpredictable seizures or periods of unusual behavior or sensations. One strategy to decrease the seizure strength and frequency is to use molecules that decrease the intensity of neurotransmission. This is the reason why the allopregnanolone derivative ganaxolone was tested, because of its ability to increase the inhibitory neurotransmission through a potentiation of $GABA_A$ receptor activity. Ganaxolone showed anti-seizure properties in neonates (Yawno et al. 2017), pediatric, and adolescent patients with refractory epilepsy (Pieribone et al. 2007) and in adults with uncontrolled partial-onset seizures (Sperling et al. 2017).

Stress-Related Disorders

Stress-related disorders are chronic diseases such as anxiety, depression, posttraumatic stress disorder, which can occur after a chronic or intense stress exposure. Chronic stress is known to disrupt neuroplasticity, causing a plethora of symptoms including altered mood and cognitive deficits, such as alterations in memory or attention processes. Since neurosteroids are positive modulators of neuroplasticity, they were tested in patients with stress-related disorders. Testosterone and pregnenolone were found to be efficient to reduce depressive symptoms in humans (Brown et al. 2014; Walther et al. 2019). This was corroborated in preclinical studies using a pregnenolone derivative that cannot be metabolized, showing that this antidepressant activity could actually be due to pregnenolone and not to its metabolites (Schverer et al. 2018). The synthetic derivatives of allopregnanolone ganaxolone, brenanolone, and SAGE-217 were also tested in patients with stress-related disorders. Indeed, ganaxolone was given to patients with posttraumatic disorder. In this study, ganaxolone did not show any improvement of symptoms, which could be due to the dose that was not high enough to reach the brain and exert its beneficial effects on neuroplasticity (Rasmusson et al. 2017). Brexanolone, another allopregnanolone derivative, has recently been approved by the US Food and Drug Administration for the treatment of postpartum depression (Zorumski et al. 2019). This was based on several clinical studies showing that this molecule induces a reduction of the depressive symptoms in women (Meltzer-Brody et al. 2018). Finally, SAGE-217 was successfully tested in patients with major depression (Gunduz-Bruce et al. 2019). Altogether, these studies clearly show the potential of using the neurosteroid allopregnanolone to reduce depressive symptoms in humans.

Conclusion

Neurosteroids are steroids synthesized de novo in the central nervous system. Due to their mechanism of action involving diverse targets located in the plasma membrane or in the cytoplasm, they are able to modulate neuroplasticity. Therefore, neurosteroids could be innovative strategies for some pathological conditions in which both neuroplasticity and cognitive processes are altered.

Cross-References

▶ GABAergic System

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Neurotransmission

► Synaptic Transmission

Neurotrophic Factors

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Synonyms

Neurotrophins

Definition

Neurotrophic factors are peptides or protein molecules that support cell survival and cell death in neural and nonneural tissues. In this way, these molecules affect the development, maintenance, and plasticity of the nervous system during the development and in the adulthood (Sampaio et al. 2017).

Herein, the neurotrophic factors are grouped in families, such as the neurotrophin (NT) family, the glial cell-line-derived neurotrophic factor (GDNF) family ligands (GFLs), the neurokine family, fibroblast growth factor (FGF) family, and transforming growth factor-β (TGF-β) family. Moreover, the novel neurotrophic factors, cerebral dopamine neurotrophic factor (CDNF) and mesencephalic astrocyte-derived neurotrophic factor (MANF), are also explained in this text. Table 1 summarizes their main components and receptors involved in their mechanism of action and function.

Mechanism of Action

NT Family

The nerve growth factor (NGF), a neurotrophic factor essential for peripheral nervous system, was the first discovered member of the n eurotrophin family. Currently, NTs are a family of growth factors that comprises four structurally related neurotrophins: NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4, also known as NT-4/5). Indeed, neurotrophins regulate the neuronal survival, proliferation, differentiation, neuritis growth, synaptic function, and synaptic plasticity (Saragovi et al. 2019).

Neurotrophins are initially synthesized as precursors named pro-neurotrophins that undergo maturation by enzymatic cleavage. The pro-neurotrophins and mature neurotrophins act in opposite ways by binding to different receptors (Sampaio et al. 2017). Pro-neurotrophins interact only with p75 neurotrophin receptor ($p75^{NTR}$), which triggers apoptosis through activation of Rac and Jun N-terminal kinase (JNK) during development and pathological situations (Di Liberto et al. 2019).

Mature neurotrophins can bind both to $p75^{NTR}$ and tropomyosin receptor kinase (Trk) receptors. There are three different Trk receptors described for mammals – TrkA, TrkB, and TrkC. NGF preferentially binds to TrkA, BDNF, and NT4 to TrkB and NT3 to TrkC (Sampaio et al. 2017). Trk receptors modulate synaptic strength and plasticity by regulating the expression and activity of ion channels and neurotransmitter receptors. Moreover, Trk receptors activation recruits intracellular signaling pathways, such as PI3K, AKT, Ras, and Erk signaling (Di Liberto et al. 2019).

GFL Family

GFL family is represented by GDNF, neurturin (NRTN), artemin (ARTN), and persephin (PSPN). GFLs are synthesized, secreted, and activated by a variety of tissues. This family began with the GDNF discovered as a potent neurotrophic factor for dopamine neurons survival. Due to preclinical evidence of its neuroprotective effects, GDNF is

Neurotrophic Factors, Table 1 Summary of neurotrophic factors' families and their main components, receptors, and functions

Family	Neurotrophic factors	Receptors	Functions
NT	NGF BDNF NT-3 NT-4, NT-4/5	$p75^{NTR}$ TrkA TrkB TrkC	Neuronal survival, proliferation, differentiation, neuritis growth, synaptic function, and synaptic plasticity
GFLs	GDNF NRTN ARTN PSPN	GFRα 1 to 4/RET complex	GDNF mediates the dopamine neuron survival and shows protective effects for sympathetic, parasympathetic, sensory, and motor neurons
Neurokine	IL-6, IL-11, IL-27, IL-31 CNTF CT-1 CLCF1 LIF OSM NPN	Neurokine receptors formed by α- and β-receptor subunits	Inflammatory process and immune homeostasis. Neurokines also act on the embryonic development, behavior, metabolism, hematopoiesis, tissue integrity, and aging
FGF	FHF subgroup: FGF11, 12, 13, and 14 Mitogenic FGF subgroup: FGF1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 16, 17, 18, 20, and 22 Endocrine FGF subgroup: FGF19 (mouse FGF15), 21, and 23	Mitogenic FGF subgroup: FGFR-1 to 4 Endocrine FGF subgroup: KL or KLB/FGFR complex	Mitogenic FGF subgroup: cellular proliferation and population growth Endocrine FGF subgroup: metabolic homeostasis
TGF	TGF-α and TGF-β	EGFR	Acting on the fibroblasts, regulating their cellular proliferation and differentiation
MANF and CDNF	MANF CDNF	Not identified	MANF: maintenance of endoplasmic reticulum homeostasis and UPR pathways CDNF: dopaminergic neurons survival

one of the most largely investigated neurorestorative approach for Parkinson's disease (Sampaio et al. 2017). Additionally, GDNF was later described as a protective agent in the periphery, for sympathetic, parasympathetic, sensory, and motor neurons.

GFLs signal selectively by binding to each one of the four members of GDNF family receptor α (GFRα1 to 4). Additionally, glycosylphosphatidylinositol (GPI) is necessary to anchor the GFRα to plasma membrane. In its turn, GFL-GFRα complex has high affinity by the receptor tyrosine kinase RET, inducing survival and regeneration of neurons through the PI3K/Akt, Ras/Erk, Src, and PLCg signaling pathways (Saragovi et al. 2019).

Neurokine Family

Also known as neuropoietic cytokine family, neurokine family consists of interleukin (IL)-6, IL-11, IL-27, IL-31, ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine factor 1 (CLCF1), leukemia inhibitory factor (LIF), oncostatin M (OSM), and neuropoietin (NPN). Although these secreted proteins have a crucial role in the inflammatory process and immune homeostasis, they also mediate the embryonic development, behavior, metabolism, hematopoiesis, tissue integrity, and aging (Jones and Jenkins 2018).

CNTF was originally isolated due to its ability to sustain ciliary neurons in culture; however, it is now also known to play roles in glial differentiation. CNTF supports the survival and differentiation of sensory, sympathetic, and motor neurons. Its trophic effects also act on the oligodendrocytes and mesenchymal cells, including adipocytes, osteoblast, and muscle cells. CNTF is expressed by glial cells of the spinal cord, astrocytes,

Schwann cells, and skeletal muscles and released in response to injury (Pasquin et al. 2015).

Neurokine receptors display similar multisubunit structure and share overlapping polypeptide components. In general, neurokines have a low affinity or α-receptor subunits that require gp130 (an additional transmembrane component) for functional responsiveness. In the presence of ligand, the monomeric subunits associate to form the functional receptor complexes (Nathanson 2012). In addition, membership of neurokine family shows pleiotropic properties due to interaction with the shared signal-transducing receptor β-subunit. The bonded neurokine-β-receptor subunit activates several intracellular signaling pathways; among them Janus tyrosine kinase signal transducer and activator of transcription (Jak/STAT) pathway are highlighted (Jones and Jenkins 2018).

FGF Family

FGF family has a common ancestor, FGF13-like, that expands into multiple members. During the evolution, FGF13-like ancestor molecule bifurcated into FGF-homologous factor (FHF) subgroup (FGF11, 12, 13, and 14) and FGF4-like molecule. In your turn, FGF4-like is the precursor for two major functional subgroups: mitogenic and endocrine FGFs. Additionally, FGFs signal through four transmembrane receptor tyrosine kinases known as FGFR-1 to FGFR-4 with multiple splicing variants (Li 2019).

Mitogenic FGF subgroup includes FGF2, 5, 8, 9, and 10 and promotes cellular proliferation and population growth. This subgroup is involved in the development of ectodermic, mesodermic, and endodermic cells. As a result, mitogenic FGFs influence several systems (Li 2019). Of note, FGF2 has a key role in the central nervous system, promoting neurogenesis, survival, migration, differentiation, neuroregeneration, and synaptic plasticity, mainly during the development. Regarding the action mechanism, mitogenic FGFs binding to FGFRs activate three major pathways: PI3K/AKT, Ras/MAPK, and PLC/PKC (Di Liberto et al. 2019).

Otherwise, endocrine FGF subgroup comprises the FGF19 (mouse FGF15), 21, and 23 and regulates the metabolic homeostasis, affecting bile acids, glucose, lipid, and minerals metabolism, without direct proliferative activity. However, their functions seem to be important for cell survival. Lastly, endocrine FGFs act via a binary complex composed by transmembrane nonkinase accessory coreceptor, the α-Klotho (KL) or β-KL (KLB), and FGFRs (Li 2019).

TGF Family

TGFs were discovered as secreted products from cancer cells functionally related to epidermal growth factor (EGF). Subsequently, these growth factors have been found in normal cells. TGFs can transform fibroblasts, regulating their cellular proliferation and differentiation. TGFs divide themselves into TGF-α and TGF-β in accord with the ability to bind to EGF receptors (EGFR) (Moses et al. 2016).

In this sense, TGF-α binds to EGF receptors. This ligand can be found as a soluble protein or anchored to the plasma membrane as a biologically active precursor (Rayego-Mateos et al. 2018). Oppositely, TGF-β requires the EGF presence for phenotypic transformation, but it does not bind to EGF receptors (Moses et al. 2016). TGF-β is a growing family of secreted, dimeric growth factors and cytokines. Their functions include induction/specification of distinct cells and tissue phenotypes, homeostasis in the adult and development during the embryonic phase.

MANF and CDNF Family

MANF and CDNF are a novel neurotrophic factor family, homologous between themselves, functionally different from the classical secreted neurotrophic factors (Lindahl et al. 2017). They are mostly retained intracellularly and localized in the endoplasmic reticulum under normal conditions, being secreted from neurons only after calcium depletion (Poyhonen et al. 2019).

Although both MANF and CDNF display neurotrophic action, there are no transmembrane receptors identified for them. Evidence demonstrate that MANF is linked to the maintenance of endoplasmic reticulum homeostasis and unfolded

protein response (UPR) pathways (Lindahl et al. 2014). MANF mRNA is detected in astrocytes, microglia, and – in lower levels – in neurons from human and rodents. On the other hand, CDNF expression has not been investigated in detail. Currently, it is known that CDNF is a growth factor with a high specificity and activity to promote dopaminergic neuron survival (Lindahl et al. 2017).

Clinical Use

Alterations in the neurotrophic factor levels/expression and their signaling are involved in the development and/or progression of neurodegenerative and psychiatric disorders. Although neurotrophic factors play essential roles in the central and peripheral nervous system of vertebrates throughout development as well as in adult life, the therapeutic potential of these molecules is still inconsistent.

The therapeutic promise of neurotrophic factors for neurodegenerative disorders has been researched in the last decades. Even though these proteins show promising results in the preclinical tests, they failed in the clinical trial due to their pharmacokinetic properties. Moreover, serious side effects were observed after administration of different neurotrophic factors. Nevertheless, the new drug delivery approaches, such as gene therapy vectors, attempt to optimize the clinical benefit in reducing the side effects observed in patients. Consequently, the safety and efficacy of neurotrophic factors as a therapeutic strategy for neurodegenerative and psychiatric disorders still need to be elucidated.

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Neurotrophins

► Neurotrophic Factors

NFAT

► NFAT Family of Transcription Factors

NFAT Family of Transcription Factors

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Synonyms

NFAT; NFAT5; NFATc1; NFATc2; NFATc3; NFATc4

Definition

The NFAT family of transcription factors includes five different members: NFATc1 (also known as NFAT2 or NFATc), NFATc2 (NFAT1 or NFATp), NFATc3 (NFAT4 or NFATx), NFATc4 (NFAT3), and NFAT5 (TonEBP or OREBP). The gene encoding for the first NFAT protein was cloned more than 20 years ago and termed NFATp, as it was shown to be "preexistent" in the cytosol of resting T cells but to translocate into the nucleus and interact with the transcription factors Fos and Jun in activated T cells, where it bound in a trimeric complex to specific sites in the *Il2* gene promoter to induce the expression of interleukin (IL-)2 (Jain et al. 1993). NFAT proteins were shown to be regulated by calcineurin-mediated dephosphorylation in response to T cell receptor (TCR)-induced calcium signaling, which identified them as targets of the immunosuppressive effects of the calcineurin inhibitor cyclosporin A (Clipstone and Crabtree 1992; Jain et al. 1993). Initial studies focused on characterizing the functions of NFAT proteins in T cells; however, it soon became evident that members of this ubiquitously expressed family of transcription factors played essential roles in the regulation of programs of development and differentiation in a wide array of different cell types and tissues.

Basic Characteristics

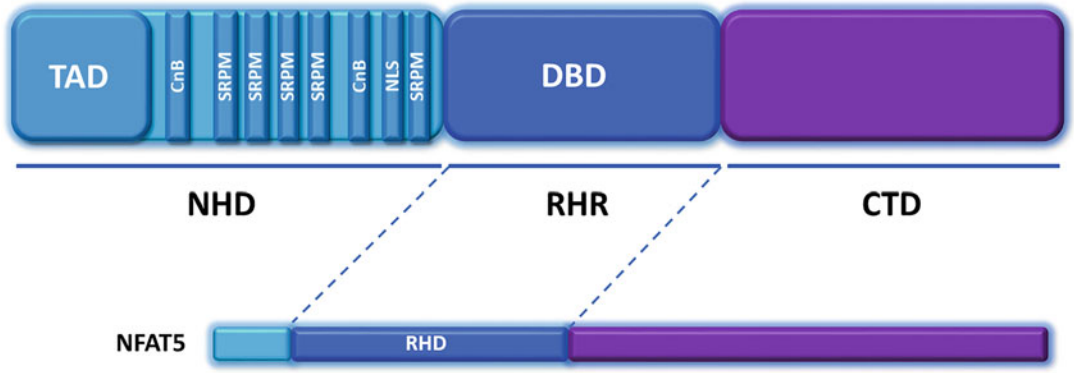
Structure of NFAT Proteins

All NFAT proteins share a conserved DNA-binding domain, which has structural homology with the Rel domain found in members of the NF-κB family of transcription factors. This domain is not only responsible for site-specific DNA binding, but it also mediates interactions with transcriptional partners, including Fos and Jun (Chen et al. 1998). With the exception of NFAT5, NFAT proteins also have an N-terminal regulatory domain, known as the NFAT homology domain (NHD), that contains the main transactivation domain as well as several regulatory regions. These include interaction sites for calcineurin and for NFAT kinases, which regulate NFAT activation through phosphorylation of multiple serine-containing motifs also present in the NHD (Fig. 1) (Hogan et al. 2003; Okamura et al. 2000). As all other NFAT family members, NFAT5 also has a Rel domain that contains the conserved DNA-binding region and a dimerization domain, but it lacks an NHD (Lopez-Rodriguez et al. 2001). The C-terminal regions of NFAT proteins are not conserved and may contain sites that allow interactions with other proteins.

Regulation of NFAT Activation

NFATc1, NFATc2, NFATc3, and NFATc4 are regulated by the calcium/calmodulin-activated phosphatase calcineurin (Macian 2005). NFAT5, which was also identified as the tonicity-responsive enhancer-binding protein (TonEBP), regulates the expression of osmoprotective genes in response to osmotic stress, though it can also modulate gene expression under isosmotic conditions in response to inflammatory stimuli (Aramburu and López-Rodríguez 2019).

NFAT activation is mainly controlled by its subcellular localization. The net result of the rate of nuclear import and export of NFAT proteins, which responds to their phosphorylation status,

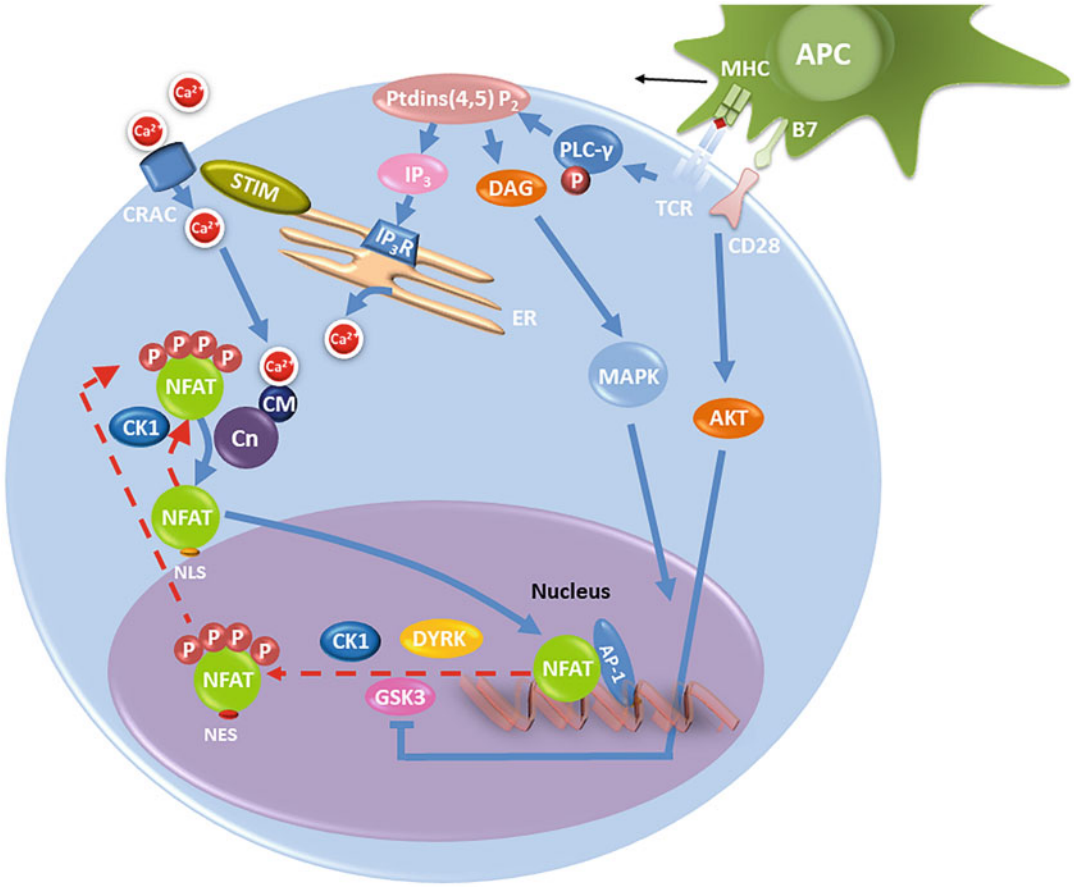


NFAT Family of Transcription Factors, Fig. 1 ***Structure of NFAT proteins.*** NFATc1, c2, c3, and c4 have a regulatory domain (NHD, NFAT-homology domain), which comprises a transactivation domain (TAD), several serine-rich phosphorylation motifs for NFAT kinases (SRPM), calcineurin-binding sites (CnB), and one or more nuclear localization signals (NLS). A conserved DNA-binding domain (DBD or Rel-homology region, RHR) follows the NHD and also contains residues required to interact with Fos and Jun proteins. NFAT5 shares the conserved Rel-homology (RHD) DNA-binding domain but differs in the rest of its structure from the other NFAT family members. The C-terminal domain (CTD) is not conserved among NFAT proteins

determines the overall level of NFAT that is present in the nucleus and, therefore, able to regulate gene expression. Other mechanisms have also been described, though, that may contribute to fine-tune the regulation of the transcriptional activity of NFAT.

Calcium and calcineurin: Engagement of calcium-coupled receptors induces the activation of the calcium/calmodulin-dependent phosphatase calcineurin, which binds NFAT proteins and directly dephosphorylates them, inducing their translocation into the nucleus. In T cells, where this complex regulation has been well characterized, recognition of antigen by the TCR induces activation of the phospholipase C γ, which hydrolyzes phosphatidylinositol 4,5-bisphosphate into inositol-1,4,5-triphosphate (IP_3) and diacylglycerol. IP_3 binds IP_3 receptors in the endoplasmic reticulum (ER), which causes calcium release from intracellular ER calcium stores. STIM proteins sense this release and interact with ORA1, an integral component of the calcium release-activated calcium (CRAC) channels in the plasma membrane, causing a further increase in the intracellular calcium levels (Feske et al. 2006; Feske et al. 2015; Zhang et al. 2005). In response to the increase in the intracellular calcium concentration, activated calcineurin binds to and dephosphorylates NFAT proteins, which are heavily phosphorylated and localized in the cytosol in resting cells (Fig. 2). At least 13 different phosphorylation sites located in serine-rich motifs and SPxx repeat motifs in the regulatory domain are dephosphorylated by calcineurin. This causes a conformational change in NFAT that exposes its nuclear localization signal, allowing NFAT import into the nucleus. There, it binds specific sites and cooperates with other transcription factors to activate the expression of distinct sets of genes (Hogan et al. 2003; Okamura et al. 2000).

NFAT kinases: Phosphorylation of NFAT proteins is required to promote nuclear export and to maintain cytosolic localization in resting cells. Several kinases have been reported to phosphorylate different serine-containing motifs. Casein kinase 1 (CK1) bids to the NHD and regulated NFAT nuclear export and cytosolic retention through phosphorylation of a serine-rich motif (Okamura et al. 2004). Glycogen synthase kinase 3 (GSK3) also phosphorylates serine-proline motives in NFATc2 and NFATc1, promoting NFAT nuclear export. These phosphorylation sites require previous priming by cyclic-AMP-dependent protein kinase-mediated phosphorylation of NFAT (Beals et al. 1997). The dual-specificity tyrosine-(Y)-phosphorylation-regulated kinases DYRK1A and DYRK2 also regulate



NFAT Family of Transcription Factors, Fig. 2 ***NFAT regulation by calcium/calcineurin and NFAT kinases in T cells.*** Pathways involved in the activation of NFAT by nuclear import and modulation of its transcriptional activity are represented with solid blue arrows, whereas the pathways involved in NFAT nuclear export or cytosolic retention are depicted with red dashed arrows. *AP-1* activator protein 1; *APC* antigen-presenting cell; Ca^{2+} calcium; *CK1* casein kinase 1; *CM* calmodulin; *Cn* calcineurin; *CRAC* calcium release-activated calcium channel; *CRM1* exportin 1; *DAG* diacylglycerol; *DYRK* dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase; *ER* endoplasmic reticulum; *GSK3* glycogen synthase kinase 3; *IP3* inositol-1,4,5-triphosphate; *IP3R* inositol-1,4,5-triphosphate receptor; *MAPK* mitogen-activated protein kinases; *MHC* major histocompatibility complex; *NES* nuclear export signal; *NLS* nuclear localization signal; *P* phosphate; *PLC-γ* phospholipase C γ; *Ptdins(4,5)P2* phosphatidylinositol 4,5-bisphosphate; *STIM* stromal interaction molecule; *TCR* T cell receptor

NFAT nuclear export through phosphorylation of a serine/proline-rich motif that primes NFAT for subsequent phosphorylation by CK1 or GSK3 (Gwack et al. 2006). LRRK2 may also phosphorylate NFAT proteins to contribute to the regulation of NFAT activation (Liu et al. 2011). Interestingly, it has been shown that many of these kinases, including CK1 and GSK3, exist in a complex with the noncoding RNA repressor of NFAT (NRON) that inhibits nuclear import of NFAT by facilitating interactions of those kinases with NFAT (Sharma et al. 2011). Following a non-canonical calcineurin-independent IL-7-dependent pathway of activation, JAK3 has also been shown to phosphorylate NFATc1 in $CD4^{-}CD8^{-}$ thymocytes, to cause NFAT translocation into the nucleus in a calcineurin-independent manner (Patra et al. 2013).

Transcriptional regulation of NFAT: NFATc1/αA is an isoform of NFATc1 that is regulated at a transcriptional level by an autoregulatory loop controlled by an inducible promoter. This

promoter responds to constitutively expressed isoforms of NFATc1 and NFATc2 and other transcription factors that induce the expression and accumulation of the inducible NFATc1/αA isoform in activated cells (Serfling et al. 2006).

Posttranslational regulation: Several posttranslational mechanisms contribute to the regulation of NFAT activity. Sumoylation participates in NFAT nuclear retention and might also regulate NFAT transcriptional activity (Terui et al. 2004). Evidence also supports that poly(ADP-ribose) polymerase-1 (PARP-1) modifies NFATc1 and NFATc2, regulating nuclear export and transcriptional activity (Olabisi et al. 2008). Furthermore, NFATc2 can also be ubiquitinated by the E3 ubiquitin ligase HDM2 in breast cancer cells, leading to its degradation by the proteasome (Yoeli-Lerner et al. 2005).

Transcriptional Activity

NFAT proteins can cooperate with other transcription factors to form transcriptional complexes that activate or repress the expression of specific genes. Formation of those transcriptional complexes allows cells to integrate calcium signaling with other signaling pathways to regulate specific programs of gene expression. Initially identified as the nuclear component of the complex that directed the expression of IL-2 in activated T cells, activator protein 1 (AP-1) complexes are the best characterized NFAT partners. In response to the engagement of the TCR and co-stimulatory receptors, calcium signaling and the mitogen-activated protein kinase (MAPK) pathway converge in the activation of NFAT and the AP-1 proteins Fos and Jun (Jain et al. 1993). The DNA-binding domain of NFAT interacts with AP-1, forming a quaternary complex on DNA that induces the expression in T cells of many activation-induced genes, including numerous cytokines (Chen et al. 1998; Macian et al. 2001). The number of transcription factors that have been identified to cooperate with NFAT has grown with the years. In many cases, these interactions occur in specific cells of tissues and are responsible for the regulation of different programs of activation, differentiation, or development (Macian 2005; Wu et al. 2007). NFAT proteins are also able to form homodimers that bind to κB-like sites containing two tandem NFAT-binding sites separated by one or two bases (Jin et al. 2003). In addition, NFAT5 also forms homodimers that bind to DNA and regulate gene expression (Lopez-Rodriguez et al. 2001).

Functions

Adaptive immune system: Activation of effector cells is governed by the direct recognition of antigens by the B cell receptor (BCR) or peptides presented on major histocompatibility complex (MHC) molecules to cognate TCRs on T cells. NFAT functions have been extensively studied in T cells, although the participation of NFAT in the regulation of B cell responses has also been amply characterized. NFAT proteins are activated in B cells in response to BCR engagement and participate in the induction of programs of gene expression that regulate B cell activation and differentiation (Bhattacharyya et al. 2011; Venkataraman et al. 1994). In T cells, coordinated engagement of the TCR and co-stimulatory receptors (e.g., CD28) allows NFAT to cooperate with other transcription factors, such as Fos and Jun, to induce the expression of genes that are required to ensure effective T cell activation (Macian 2005; Muller and Rao 2010). However, in the absence of co-stimulation, TCR triggering still causes NFAT nuclear localization, which, without the opportunity to fully cooperate with Fos and Jun, forms homodimers that regulate the expression of genes that maintain T cells in a tolerant hyporesponsive state termed anergy (Macian et al. 2002; Baine et al. 2009). NFAT proteins also modulate immune tolerance through the modulation of regulatory T cell (Treg) function, a distinct population of T cells that express the transcription factor FoxP3 and have the capacity to suppress the activation of other effector immune cell populations. NFAT proteins not only regulate the expression of FoxP3 but also cooperate with this transcription factor to activate (*Ctla4*, *Cd25*, and *Gitr*) or repress (*Il2*) the expression of genes in Tregs and, therefore, control differentiation and function of these suppressor cells (Wu et al. 2006).

Interestingly, under conditions of chronic antigen stimulation, NFAT, in the absence of other transcriptional partners, controls the induction of exhaustion in T cells (Martinez et al. 2015).

T cell development is also regulated by NFAT. Immature thymocytes rearrange both the α- and β-chains of the TCR and mature from a double-negative ($CD4^-CD8^-$) thymocyte into a double-positive thymocyte that expresses both CD4 and CD8. NFATc1 has a crucial role in facilitating this process (Patra et al. 2013). Furthermore, genetic deletion of calcineurin activity in immature thymocytes leads to a positive selection block, whereas NFATc2 and NFATc3 regulate the thresholds of signal transduction that determine positive selection in the thymus (Gallo et al. 2007).

Mature CD4+ T helper cells can differentiate into different effector lineages, such as T_H1, T_H2, or T_H17 cells. These differentiation events have been shown to be regulated by NFAT that directs the expression of specific genes that help define the differentiation program of a particular T cell subtype. The ability of NFAT to direct distinct programs of differentiation relies on interactions with different transcriptional co-partners (Hermann-Kleiter and Baier 2010). As mentioned above, NFAT proteins also participate in the regulation of Treg function and have been implicated in the differentiation of follicular helper T cells (Martinez et al. 2016; Wu et al. 2006). NFAT proteins also participate in the orchestration of the $CD8^+$ T cell response, and NFATc1 controls programs of gene expression that confer cytotoxic function to these T cells (Klein-Hessling et al. 2017).

Innate immune system: NFAT proteins are expressed in many cells of the innate immune system and regulate their function, including eosinophils, neutrophils, mast cells, NK cells, dendritic cells, and macrophages. They serve a pivotal role in transforming molecular signals to expression of genes. Indeed, ligation of pattern recognition receptors (PRR) in myeloid cells can activate NFAT in response to the presence of pathogens (Fric et al. 2012). However, not only PRR but also other receptors can induce NFAT activation in cells of the innate immune system. For instance, ligation of the Fcε receptor in mast cells results in NFAT activation, which induces cytokine expression in those cells (Monticelli et al. 2004). Interestingly, not only calcineurin-regulated members of the NFAT family but also NFAT5 participates in the regulation of gene expression in response to pattern recognition receptors during the innate immune response (Buxadé et al. 2012).

Nonimmune tissues and organs: NFAT proteins are present in almost all tissues, although the expression of any individual NFAT protein can often be limited to specific cell types. For instance, whereas NFATc1, NFATc2, and NFATc3 are expressed in cells of the immune system, NFATc4 expression has not been reported in immune cells. An increasing number of studies have characterized the roles that different NFAT proteins play as regulators of development, differentiation, and function in many cell types and tissues (Wu et al. 2007). For instance, in *skeletal muscle,* the expression of specific NFAT proteins is developmentally regulated to control progression from immature precursors to mature myocytes and contribute to the specification of muscle fiber type (Calabria et al. 2009). NFAT also regulates *cartilage growth and bone remodeling*. The role that NFAT proteins play in osteoclast differentiation has been amply documented. In these cells, RANKL-mediated activation of NFATc1 directs the expression of a set of genes required for osteoclast differentiation (Takayanagi et al. 2002). Initial characterization of a mouse model that lacked NFATc1 unequivocally showed that NFATc1 played a key role in the formation of the heart's valves (de la Pompa et al. 1998). In the *adult heart*, NFAT proteins partner with members of the GATA and MEF2 families of transcription factors to control myocardial hypertrophy (Passier et al. 2000). NFATc3 and NFATc4 are expressed in perivascular mesenchymal cells and regulate the assembly of blood vessels during embryogenesis. Thus, mice that lack those two NFAT proteins present abnormal vascular development (Graef et al. 2001). Vascular endothelial growth factor (VEGF) is a major activator of NFAT proteins in *endothelial cells*, and

engagement of the VEGF receptor causes the expression of NFAT-dependent genes such as COX2 (Hernández et al. 2001). In the *nervous system*, NFAT regulates neuronal axon growth and is essential for neuronal development and the differentiation of Schwann cells (Graef et al. 2003; Kao et al. 2009). NFAT has also been shown to control beta cell growth in the *endocrine pancreas* and regulate insulin-signaling pathways and adipogenesis (Heit et al. 2006). NFATc1 has also been implicated in the maintenance of *stem cell* quiescence in the skin follicle by repressing the expression of the cell cycle kinase CDK4 (Horsley et al. 2008).

As mentioned above, NFAT proteins are central to the control of cell development and differentiation, in part by regulating proliferation and cell death. It is not surprising, therefore, that altered NFAT signaling occurs in *cancer cells* (Muller and Rao 2010). As expected, due to their pivotal role in lymphocyte development, dysregulated NFAT activity has been associated with several forms of B and T cell lymphoma and leukemia (Muller and Rao 2010). Furthermore, NFAT proteins regulate specific properties of different cancers. For instance, NFATc2 and NFAT5 positively modulate migration and invasion of breast cancer cells, while reports have shown that NFAT proteins promote tumor-associated angiogenesis and may directly activate the expression of oncogenes, such as *Myc* (Mancini and Toker 2009).

Drugs

Activation of NFAT is dependent on its dephosphorylation, and, therefore, the phosphatase calcineurin has been an important area of focus of research for NFAT inhibitor development. Pharmacological agents that inhibit calcineurin activity have been used to modulate NFAT activity and have become tremendously beneficial in the clinic since their introduction in the early 1980s. These drugs inhibit the activation of NFAT in immune cells, act as immunosuppressants, and are widely used for the treatment of autoimmune disease or the prevention of transplant rejection. Cyclosporine and FK506 are the most widely used and studied suppressors of NFAT activity. Both are calcineurin inhibitors whose mechanism of action is very similar. They bind separate intracellular peptidyl-prolyl isomerases (cyclophilin for cyclosporine; FKBP12 for FK506). These complexes then bind to distinct regions of calcineurin and inhibit its phosphatase activity. Nevertheless, the specificity of NFAT inhibition using calcineurin inhibitors is limited by the fact that NFAT is not their only target and they affect also other calcineurin targets in other tissues. This is a major mechanism that accounts for the significant toxic effects these drugs can have, including kidney dysfunction, hypertension, or neurotoxicity (Roy and Cyert 2020).

Due to the severe side effects that may occur after long-term administration of cyclosporine or FK506, finding new inhibitors with more specificity for NFAT has been pursued, which may result in drugs that could prevent most of the side effects of calcineurin inhibitors. Some studies have centered on targeting the interaction of NFAT and calcineurin. Calcineurin-binding sites on NFAT have been mapped to the N-terminal regulatory domain and include the amino acid sequence SPRIET. A closer analysis of this sequence among NFAT family members revealed the consensus-binding sequence PxIxIT, which laid the groundwork for the discovery using combinatorial libraries of the highly potent VIVIT peptide (Aramburu et al. 1998). This peptide has been successfully used in mouse models of graft rejection and tumor progression. The major benefit of the VIVIT peptide lies in its higher specificity for the inhibition of NFAT function; however, the PxIxIT sequence is also used by other calcineurin substrates (Roy and Cyert 2020). Other alternative approaches are also being explored including the design of compounds that may disrupt the ability of NFAT to bind DNA, enhance its nuclear export, or prevent interactions of NFAT and specific transcriptional partners (Lee et al. 2018; Mognol et al. 2019). The development of those new categories of drugs should lead to a higher degree of precision to specifically inhibit NFAT activity.

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NFAT5

▶ NFAT Family of Transcription Factors

NFATc1

▶ NFAT Family of Transcription Factors

NFATc2

▶ NFAT Family of Transcription Factors

NFATc3

▶ NFAT Family of Transcription Factors

NFATc4

▶ NFAT Family of Transcription Factors

NF-κB, Molecular Target

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Synonyms

Rel proteins

Definition

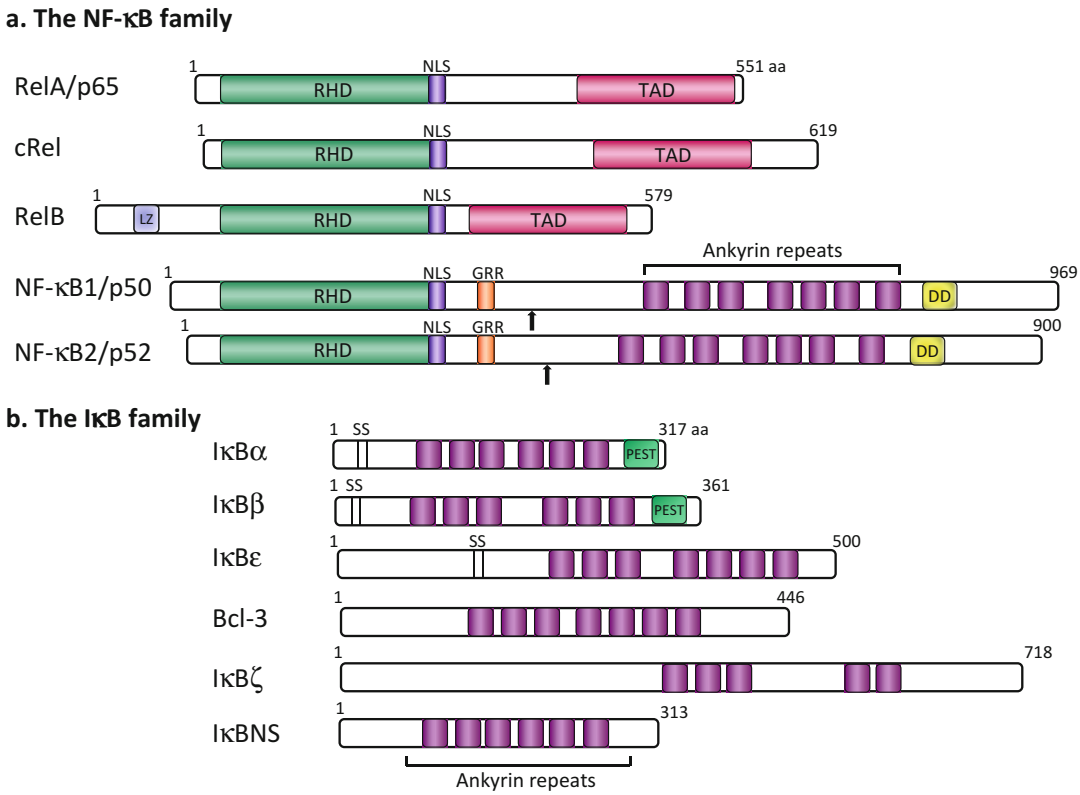
Nuclear factor kappa B (NF-κB) is the generic term for a family of dimeric eukaryotic transcription factors with a critical role in major cellular functions including the immune and inflammatory responses, cell proliferation and differentiation, and the control of cell survival. NF-κB is composed of members of the Rel family of DNA-binding proteins and includes the mammalian proteins RelA (or p65), RelB, cRel, p50, and p52 and the Drosophila proteins Dorsal, Dif, and Relish. These proteins form homo- or heterodimers that bind with different affinities to a consensus DNA sequence motif (called the κB site) consisting of the sequence 5'-GGGRNWYYCC-3' in which R is a purine, W is an adenosine or a thymine, Y is a pyrimidine, and N is any base.

Basic Characteristics

NF-κB Structure

NF-κB is a dimeric, ubiquitously expressed and evolutionary conserved transcription factor originally discovered as an inducible protein binding to a specific DNA sequence in the nuclei of activated B lymphocytes (Sen and Baltimore 1986). Over 30 years of research on NF-κB has revealed that NF-κB has a lot more functions in many biological systems (Zhang et al. 2017). In mammals, the NF-κB family consists of five members: RelA (p65), RelB, cRel, NF-κB1 (p105/p50), and NF-κB2 (p100/p52). Structurally, all five NF-κB proteins share a highly conserved 300 amino acid **Rel homology domain** (RHD) that encompasses a sequence-specific DNA-binding domain, a dimerization domain, and a nuclear translocation signal (NLS) (Fig. 1a). RelA, cRel, and RelB contain a transcription activation domain (TAD) and act as transcriptional activators. NF-κB1 and NF-κB2 are synthesized as large precursors, p105 and p100, that are posttranslationally processed to generate the mature forms, p50 and p52, which lack a TAD.

NF-κB proteins are principally regulated by subcellular localization, whereby the inactive cytoplasmic forms become transcriptionally active proteins upon translocation to the nucleus. In most resting cells, NF-κB is maintained inactive in the cytoplasm by binding to members of the "inhibitor of κB" (IκB) family (Fig. 1b) (Hayden and Ghosh 2008, 2012). The IκB proteins IκBα, IκBβ, IκBε IκBζ, IκBNS, and Bcl-3 contain 5–7 **ankyrin repeats**. These 33 amino

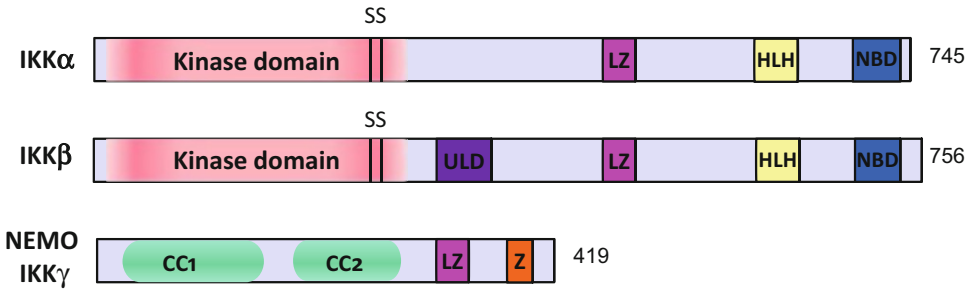


NF-κB, Molecular Target, Fig. 1 Schematic representation of the mammalian NF-κB proteins and their inhibitors, the IκB proteins. (**a**) NF-κB proteins share a highly conserved amino-terminal Rel homology domain (RHD), which includes a DNA-binding domain, a dimerization domain, and a nuclear localization signal (NLS). RelA (p65), cRel, and RelB contain in their carboxy-terminal part a nonhomologous transcriptional activation domain (TAD). RelB has an additional amino-terminal leucine zipper (LZ) motif. NF-κB1/p50 (or p105/p50) and NF-κB2/p52 (or p100/p52) contain a glycine-rich region (GRR), carboxy-terminal ankyrin repeats that are also present in IκB proteins, and a dead domain (DD). Proteolytic processing of NF-κB1/p50 and NF-κB2/p52 (cleavage sites indicated by arrows) generates their respective mature NF-κB proteins, p50 and p52. The number of amino acids (aa) is indicated for each protein. (**b**) The inhibitor of NF-κB (IκB) family includes Iκbα, IκBβ, IκBε, IκBζ, IκBNS, and Bcl-3. They are characterized by the presence of multiple ankyrin repeats that are essential for binding of IκBs to the RHD of NF-κB proteins. Iκbα, IκBβ, and IκBε, present in their amino-terminal region two critical serine residues (depicted SS) that once phosphorylated, target the IκBs for degradation. IκBα and IκBβ contain in their carboxy-terminal region a PEST domain rich in the amino acids proline, glutamic acid, serine, and threonine that acts as a targeting signal for protein degradation

acid helical structures are able to bind to the RHD of NF-κB and mask the NLS preventing NF-κB nuclear translocation. Ankyrin repeats are also found in the precursors NF-κB1 (p105) and NF-κB2 (p100), enabling them to function as IκBs and retain Rel proteins in the cytoplasm of unstimulated cells.

NF-κB Activation

There are two major signaling pathways leading to nuclear translocation and thereby activation of NF-κB proteins (Zhang et al. 2017; Hayden and Ghosh 2008, 2012). The most frequent, observed in all cell types, is the classical or canonical NF-κB activation pathway (Fig. 3). This pathway is rapidly activated by pro-inflammatory cytokines (► Cytokines) such as tumor necrosis factor-α (TNFα) (Tumor Necrosis Factor) and interleukin-1 (IL-1), as well as pathogen-associated molecular patterns (**PAMPs**). These effector molecules act through different receptors belonging to the tumor necrosis factor receptor (TNF-R) and



NF-κB, Molecular Target, Fig. 2 IKK complex. The IKK complex is composed of two kinases IKKα (or IKK1) and IKKβ (or IKK2) and a regulatory subunit NEMO (NF-κB essential modulator) or IKKγ. Both kinases contain a kinase domain sharing two conserved serine residues (depicted SS) necessary for the activation of the kinases through phosphorylation, a leucine zipper (LZ) motif allowing dimerization, an helix-loop-helix (HLH) region, and a Nemo-binding domain (NBD). IKKβ also contains an ubiquitin-like domain (ULD). NEMO contains two coiled-coiled domains (CC1 and CC2), a leucine zipper (LZ), and a zinc finger (ZF) at the carboxy-terminal region of the protein

Toll-like receptor (TLR)/interleukin-1 receptor (IL-1R) superfamilies (Toll-Like Receptors) and through assembly of large signaling complexes, activate a multiprotein complex, the IκB kinase (**IKK**) complex (Fig. 2). The most common form of this complex consists of two catalytically active protein kinases, IKKα (or IKK1) and IKKβ (or IKK2), forming heterodimers, which are associated with at least two molecules of the IKKγ regulatory subunit (also called NEMO). Though much has still to be learned about how IKK is activated, IKK is recruited to membrane bound multiproteins signaling complexes (called signalosomes) in which the linear ubiquitin assembly complex (LUBAC) (Iwai 2014) attaches a linear ubiquitin chain to IKKγ. This in turn leads to the activation of the catalytic IKK through autophosphorylation of two critical serines residues in their kinase domain.

The function of the activated IKK complex is to catalyze the rapid **phosphorylation** of the IκBs at two serine residues located in their amino-terminal regulatory domain (Fig. 1b) triggering their ubiquitin-mediated degradation by the 26S proteasome (▶ Ubiquitin/Proteasome Regulation). NF-κB dimers (predominantly RelA/p50 dimers) are released, they translocate to the nucleus where they bind to DNA, and activate gene transcription (▶ Transcriptional Regulation).

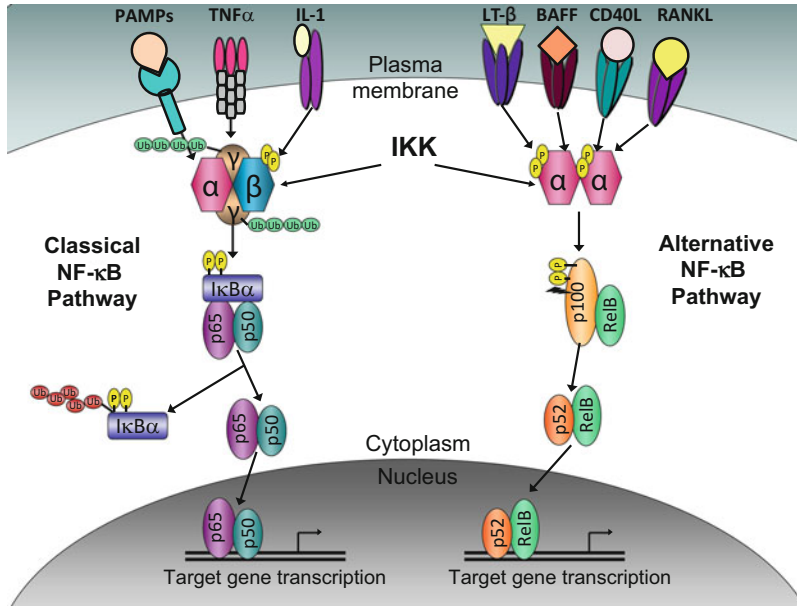
A second NF-κB activation pathway (Fig. 3), called the alternative or noncanonical pathway has a slower kinetics of activation. It responds to a subset of cytokines that bind to members of the TNF receptor superfamily. Cytokines such as lymphotoxin-β (LTβ), CD40 ligand (CD40L), the B-cell-activating factor BAFF (or Blys), and the receptor activator of NF-κB ligand (RANKL) (Fig. 3) turn on the alternative NF-κB pathway that strictly depends on the activation of IKKα homodimers, which phosphorylate NF-κB2 at two serine residues located at the carboxy-terminal end of the protein (Fig. 1b). Phosphorylation of NF-κB2 induces its ubiquitin-dependent processing to generate the mature p52 protein (amino-terminal portion of NF-κB2), while the carboxy-terminal part is degraded by the 26S proteasome. As NF-κB2 mainly associates with RelB, its processing leads to the formation of active p52/RelB heterodimers.

While the canonical NF-κB pathway is implicated in most cellular events associated with an immune response, the alternative pathway has more restrictive functions in the adaptive immunity such as the development of secondary lymphoid organs and the maturation and survival of B lymphocytes.

Cellular Functions

NF-κB and Immunity

NF-κB regulates both innate and adaptive immune responses (Immune Defense). Understanding the function of NF-κB in the

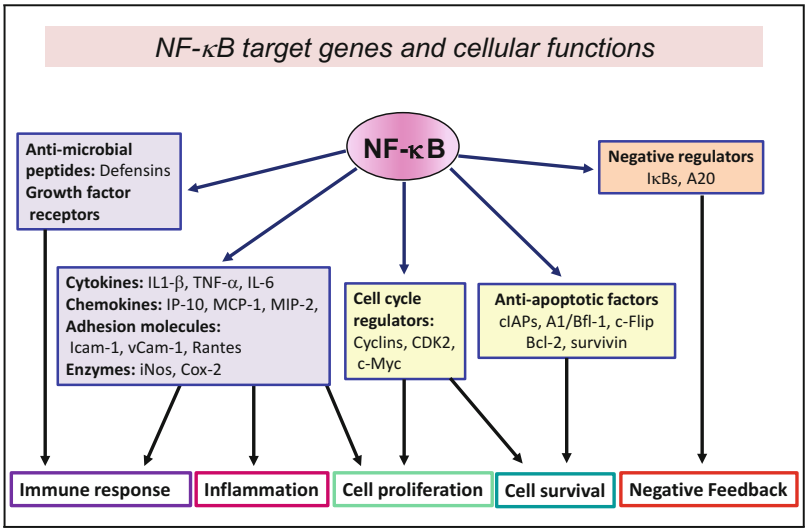


NF-κB, Molecular Target, Fig. 3 Classical and alternative NF-κB activation pathways. The classical (or canonical) NF-κB pathway is activated by a variety of stimuli including the pro-inflammatory cytokines, TNFα, and IL-1 and diverse pathogen-associated molecular patterns (PAMPs) that act through Toll-like receptors (TLRs). Binding of the ligand to its receptor induces assembly of large intracellular complexes to the receptor tail that ultimately recruit the classical IKK complex (composed of IKKα, IKKβ, and IKKγ). IKKγ undergoes nondegradative linear ubiquitination which induces IKK activation through autophosphorylation. Activated IKK phosphorylates the IκB proteins leading to their polyubiquitinylation and degradation. This leads to liberation and nuclear translocation of NF-κB dimers (mainly p65/p50) and coordinated expression of multiple inflammatory and innate immune genes. The alternative or noncanonical NF-κB pathway results in the nuclear translocation of p52/RelB dimers after phosphorylation-dependent processing of p100 (NF-κB2). It is strictly dependent on IKKα dimers that are activated by certain cytokines including lymphotoxin-β (LTβ), CD40 ligand (CD40L), and the B-cell-activating factor BAFF/Blys. Activation of the alternative pathway turns on a more limited gene expression program required for the activation and maintenance of the adaptive immunity

development, maintenance, and activation of cells from the immune system (including hematopoietic cells, macrophages, dendritic cells, B and T lymphocytes) has greatly benefited from the analysis of knockout mice in which individual NF-κB family members were defective (Zhang et al. 2017).

The innate immune response is initiated once the host detects the presence of foreign pathogens. This recognition step is performed by specialized cells of the innate immune system, the macrophages, and the dendritic cells. These cells express at their surface or intracellularly a number of pattern recognition receptors (**PRRs**), each of which has the ability to recognize specific pathogen-derived substances called pathogen-associated molecular patterns (**PAMPs**) characteristics of various classes of microbes including bacteria, viruses, and parasites. Activation of the classical NF-κB signaling pathway is essential to this acute phase of antimicrobial defense. Defective NF-κB activity in mice and humans is often associated with susceptibility to microbial infections. Indeed, activation of NF-κB dimers (mainly RelA/p50) induces transcription of genes encoding cytokines (► Cytokines), chemokines, adhesion molecules, and antimicrobial peptides (**defensins**), all of which are essential to build the innate immune response, clear the pathogen, and initiate the adaptive immune response (Figs. 3 and 4).

NF-κB is also crucial for the proper functioning of the adaptive immune system not only by acting on the immune cells themselves but also by participating in the development and organization



NF-κB, Molecular Target, Fig. 4 NF-κB cellular functions and target genes. NF-κB contributes to the induction of multiple target genes that can be divided into five major functional classes, which specify diverse cellular functions. NF-κB controls its own activity by inducing the expression of its regulators, the IκB proteins, and establishing a negative feedback regulatory loop. NF-κB induced multiple genes whose products are mediators of inflammation such as pro-inflammatory cytokines (TNFα, IL-1 and IL-6), adhesion molecules (VCAM-1, ICAM-1, E-selectin, RANTES), and the inflammatory enzymes, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). NF-κB has critical immunoregulatory functions both in the innate immune defense against pathogens and in the development, maintenance, and activation of the adaptive immunity. The anti-apoptotic function of NF-κB requires the activation of genes whose products are implicated in the inhibition of caspase activity such as the cellular inhibitors of apoptosis (cIAPs) and the cellular FLICE-inhibitory protein (cFLIP) or are negative regulators of the mitochondrial cell death program such as the Bcl-2 family protein members, Bfl-1/A1 and Bcl-X_L. NF-κB plays a role in cell proliferation and differentiation in particular by regulating the expression of the genes encoding the cell cycle regulator, cyclin D1, and the oncogene c-Myc (Yang et al. 2016)

of the secondary lymphoid organs (lymph nodes, spleen, and Peyer's patches), in which both B and T lymphocytes undergo maturation and activation. NF-κB proteins have an important role in lymphocyte development and homeostasis, primarily through regulation of the balance between cell proliferation and cell death (Fig. 3). The anti-apoptotic function of NF-κB is required during early development and expansion of B cells, which occurs in the bone marrow, and T cells, which takes place in the thymus. Different genetic approaches have uncovered the involvement of NF-κB proteins in T-cell activation and function. For instance, NF-κB is involved in the production of interleukin-18 (IL-18) and interferon-γ (INF-γ) (▶ Interferons), two cytokines required to develop the cellular or T-helper 1 (T_H1) immune response. Activation of both the canonical and noncanonical NF-κB pathways controls B-cell maturation and function such as immunoglobulin class switching and cytokine production. Analysis of the contribution of individual NF-κB family members to the development of secondary lymphoid organs led to the identification of NF-κB target genes specifically activated in different cell types (stromal, dendritic, and lymphoid) and implicated in the development and maintenance of the secondary lymphoid organs.

NF-κB, Inflammation and Inflammatory Diseases

Inflammation (▶ Inflammation) is a physiological protective mechanism by which the body defends itself against infection or tissue damage. This process is normally transient, well controlled, and responsible for tissue repair and regeneration. In certain circumstances however, the inflammatory response is chronically activated and leads to

the development of chronic inflammatory diseases (Liu et al. 2017).

NF-κB is one of the most important regulators of inflammatory responses. Pro-inflammatory stimuli such as those elicited by the cytokines TNFα and IL-1 are the most potent activators of NF-κB and trigger expression of pro-inflammatory genes encoding pro-inflammatory cytokines (TNFα, IL-1, and IL-6), chemokines, adhesion molecules, inflammatory enzymes (cyclooxygenase-2, COX-2 and inducible nitric oxide synthase, iNOS), and **matrix metalloproteinases** (MMPs) (▶ Matrix Metalloproteases) (Figs. 3 and 4). Massive production of pro-inflammatory mediators (TNFα and IL-1) amplifies the inflammatory response despite the activation of negative feedback mechanisms, for instance, the induced expression of the NF-κB inhibitors (IκBs) that is supposed to limit the duration of NF-κB activation. Recruitment of inflammatory cells such as macrophages and inflammatory T cells to the site of inflammation occurs quite rapidly after NF-κB activation. Cellular homeostasis is disturbed and tissue destruction occurs. High NF-κB activity is observed at the center of inflammation in several diseases affecting different tissues or organs including rheumatoid arthritis (▶ Inflammation and Rheumatoid Arthritis), systemic lupus erythematosus (SLE), atherosclerosis, inflammatory bowel diseases (IBDs), asthma, and psoriasis.

NF-κB is also implicated in the regulation of inflammasomes, in particular the NLRP3 inflammasome (Taniguchi and Karin 2018) (▶ Inflammasome). These multiprotein complexes assemble intracellularly upon infections or tissue damage and activate inflammatory caspases (Caspases) such as caspase-1. Activated caspase-1 cleaves pro-IL-1β and pro-IL-18 into their mature forms which are released and mediate inflammation. NF-κB induces the expression of pro-IL-1β and upregulates the amount of NLRP3, a pattern recognition receptor (PRR) which belongs to the NOD-like receptor (NLR) family and that sense pathogens or intracellular tissue damage. Excessive inflammasome activation is however prevented by autophagy, a key process that removes cellular waste. Autophagy is also controlled by NF-κB which appears as a key factor to regulate proper activation of inflammasomes and a well-controlled inflammatory response.

NF-κB, Cell Survival, Cell Proliferation, and Cancer

NF-κB controls the expression of genes that prevent programmed cell death (PCD) through apoptosis (Apoptosis) or necrosis. The classical pathway is responsible for inhibition of PCD triggered by activation of several receptors including type I and type II TNF receptors (TNF-RI and TNF-RII), Toll-like receptor 4 (TLR4), and T- and B-cell receptors, which are able to generate both survival and death signals once activated. NF-κB favors survival by inducing the expression of several anti-apoptotic genes (Fig. 4) whose products act at different steps along the receptor-mediated or the mitochondrial pro-apoptotic cascades. The first clear evidence that NF-κB is an effective inhibitor of apoptosis came from the analysis of RelA-deficient mice that die during embryonic development from a massive liver apoptosis. Defective NF-κB activation is unable to protect the hepatocytes from the toxic effects caused by the massive release of TNFα that occurs in the liver of a developing embryo. A similar phenotype is observed in mice defective in IKKβ and IKKγ, the two components of the IKK complex that are crucial for the activation of the anti-apoptotic program mediated through the classical NF-κB pathway. The alternative pathway also supports an anti-apoptotic program important for the survival of premature B cells and development of secondary lymphoid organs as described above (Zhang et al. 2017).

This protective effect of NF-κB against apoptosis has a dramatic side effect in cancer cells as it antagonizes the killing potential of most anti-cancer drugs that also activate NF-κB. In addition, aberrant NF-κB activity is observed in the large majority of human cancers and plays a central role in tumor development and progression to malignancy. Genetic mutations affecting the NF-κB or IκB subunits are relatively rare. Mutations leading to constitutive activation of NF-κB in certain malignant cells are found in signaling components that normally prevent excessive NF-κB

activation. However, the major cause of NF-κB activation relies on chronic inflammation in the tumor microenvironment (Taniguchi and Karin 2018; Didonato et al. 2012). Activated NF-κB in the tumor microenvironment is responsible for the massive production of pro-inflammatory cytokines that promote tumor cell growth. Therefore NF-κB is a major factor in the development of inflammation-associated cancers (▶ Cancer). Considering this central role of NF-κB in cancer, targeting NF-κB represents an attractive therapeutic approach in cancer therapy.

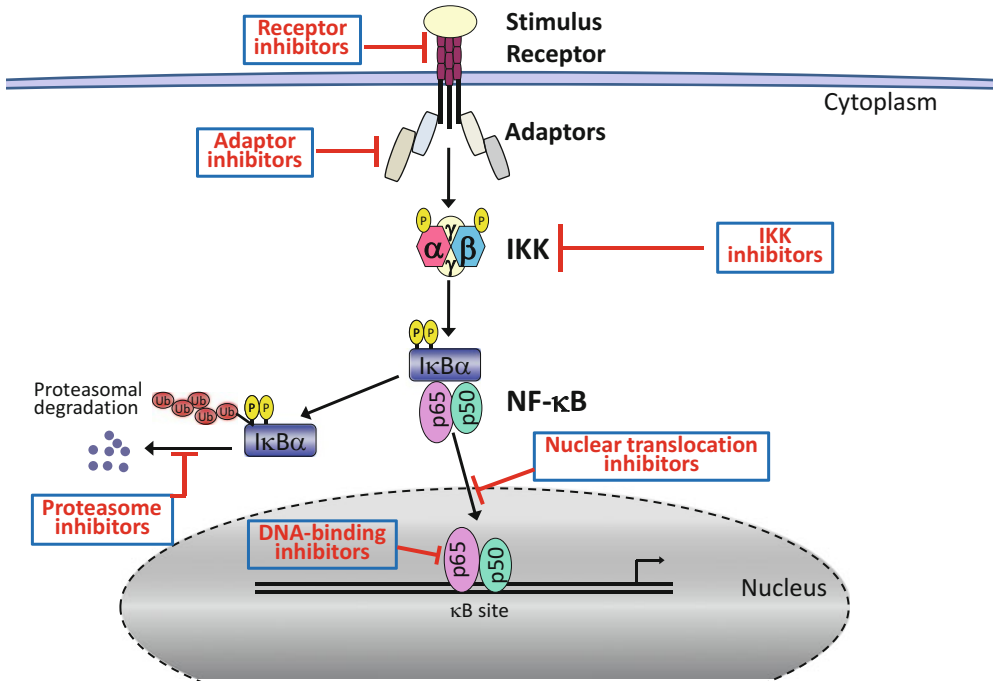
Drugs

There are several hundred reported NF-κB inhibitors (partially listed at www.nf-kb.org). These inhibitors include natural products, chemicals, metabolites, and synthetic compounds. A large majority of these products, in particular commonly used anti-inflammatory drugs such as corticosteroids and the nonsteroidal anti-inflammatory drugs (NSAIDs), aspirin, sulindac, ibuprofen, and sulfasalazine, have the ability to partially inhibit NF-κB activity in cell culture. However, the precise mechanism of action and the specific molecular target of most of these inhibitors remain unclear (Begalli et al. 2017; Durand and Baldwin 2017).

Several strategies targeting different steps of the signaling cascade can be used to inhibit NF-κB activation and function (Fig. 5). One general approach is to interfere with NF-κB nuclear activity by altering dimerization, nuclear translocation, binding to DNA, or interaction with the transcriptional machinery. This includes the use of decoy oligonucleotides that have κB sites and compete NF-κB dimers out of their target promoters or cell-permeable peptides able to block specifically the nuclear translocation of NF-κB.

A second approach to target NF-κB is to interfere with its activation by blocking the activation of the IKK complex or the degradation of IκBs (Didonato et al. 2012; Begalli et al. 2017; Durand and Baldwin 2017). Among the molecules tested are proteasome inhibitors, many of which inhibit IκB degradation and thereby NF-κB nuclear translocation. One proteasome inhibitor, bortezomib, is used for the treatment of solid and hematologic tumors. It is still unclear whether its therapeutic effect in cancer treatment is entirely or partially due to inhibition of NF-κB as the proteasome is involved in the degradation of many different polyubiquitinylated proteins and has no specificity for NF-κB signaling. A more promising approach to selectively inhibit NF-κB activation was to target IKKβ which drives NF-κB activation in response to a large number of stimuli and in many pathological conditions. The pharmacology industry came up with numerous small molecule inhibitors of IKKβ, some of which were evaluated in animal models for inflammatory diseases or cancer and in early-phase clinical trials. One such study revealed that pharmacological inhibition of IKKβ increases IL-1β secretion by myeloid cells leading to inflammasome activation and endotoxin-induced septic shock during bacterial infections (Taniguchi and Karin 2018; Didonato et al. 2012). Despite sustained effort of the pharmaceutical industry, no NF-κB or IKKβ inhibitor has been clinically approved. This is largely due to dramatic side effects and toxicity associated with systemic blockade of NF-κB or IKKβ function as NF-κB is central in maintaining normal immune responses and tissue homeostasis.

What path to follow next? Effective NF-κB drug development will have to embrace the complexity of the regulatory networks controlling NF-κB activation in a particular cell type, in response to a specific signal or in a disease context. Several new lines of research can be explored and can count on the undefective efforts of the basic research to understand and dissect the molecular events associated with NF-κB dynamic regulation. One approach is to target the signaling cascade upstream of NF-κB by designing small molecule inhibitors targeting rate-limiting protein-protein interactions downstream of receptor activation. One example would be to limit the formation of the mature TNFR1 complex by interfering with the interaction between Traf2 and TRADD, two key adaptors required for the recruitment of IKK to the activated TNFR1 complex. Recent information on crystal structures of interacting domains within Traf2 and TRADD



NF-κB, Molecular Target, Fig. 5 Inhibition of NF-κB signaling. Many therapeutic approaches are developed to inhibit NF-κB signaling. Anti-TNFα and anti-IL-1 drugs which block the signaling cascade at the level of the receptor are currently used in the treatment of inflammatory and autoimmune diseases. Selective IKK inhibitors targeting either the catalytic activity of IKK (ATP analogs) or the IKK complex formation or activation (allosteric IKK inhibitors and NEMO-binding domain peptide) preventing IκB phosphorylation have been characterized but none of these inhibitors is clinically approved. Proteasome inhibitors such as Bortezomib and lactacystin are not specific for NF-κB but are able to block the degradation of ubiquitinylated IκBs. Inhibition of NF-κB nuclear translocation using specific drugs or the IκBα super-repressor (nondegradable form of IκBα) is another way to inhibit NF-κB signaling. Glucocorticoids can interfere with the DNA-binding and transcriptional activity of NF-κB. Inhibitors targeting adaptor proteins may be of interest to specifically block IKK and NF-κB activation by a specific cytokine or receptor ligand

(Napetschnig and Wu 2013) make it realistic to design selective inhibitors that would block the TNFR1 pathway while leaving IKK-NF-κB activation intact in pathways that do not require Traf2 or TRADD. In the same line, precious information on IKK complex formation are provided by structural studies already available on IKKβ and IKKγ (Napetschnig and Wu 2013). In addition, IKK signaling is also regulated by ubiquitinylation/deubiquitinylation of IKKγ. This should be explored to design specific inhibitors that could modulate IKK activation in a specific pathway instead of blocking it completely. Another line of research is a system biology approach combined with transcriptomic analysis. Screening of small drug inhibitors targeting components of the NF-κB signaling networks can be performed in vivo by examining the gene expression profile associated with the disruption of a specific pathway. The Library of Integrated Network-Based Cellular Signatures (LINCS) Program launched by the National Institute of Health (NIH) (www.lincsproject.org) is creating a network-based understanding of biology by analyzing changes in gene expression and other cellular processes that occur when cells are exposed to diverse stimuli. This public database also contains the transcriptional profiles of genetic knockdowns of proteins involved in NF-κB signaling. In some case, effective inhibition could be similar to gene

knockdown. This transcriptional analysis might help in identifying bioactive compounds that disrupt a specific pathway within the complex NF-κB signaling network.

Glossary

Ankyrin Repeat The ankyrin repeat motif is one of the most common protein-protein interaction domains. Ankyrin repeats are modules of about 33 amino acids repeated in tandem. They are found in a large number of proteins with diverse cellular functions such as transcriptional regulators, signal transducers, cell-cycle regulators, and cytoskeletal proteins.

Defensins Defensins are a group of antimicrobial and cytotoxic peptides made by immune cells. There are seven defensins in humans, six alpha-defensins, and one beta-defensin, which are involved in the innate immune defense at the surface of epithelia from the respiratory tract, the intestinal tract, or the urinary tract.

IKK Complex The IκB kinase (IKK) complex is a high-molecular-weight (600–900 Kd) multisubunit complex present in the cytosol of most cell types. It contains two highly homologous catalytic subunits, IKKα (or IKK1) and IKKβ (or IKK2) that are serine/threonine protein kinases. They are structurally related and contain an amino-terminal kinase domain, a central leucine zipper region required for their dimerization and a carboxyl-terminal helix-loop-helix domain, which mediates protein interaction. The catalytic subunits interact through their carboxyl-terminal end with the third subunit, IKKγ (or NEMO for NF-κB essential modulator). IKKγ is the regulatory subunit of the IKK complex. It is structurally composed of protein-protein interaction motifs including two coiled-coiled domains, a leucine-zipper domain and a zinc-finger domain. IKKγ is essential for the structural organization and stability of the complex. IKKγ also has a regulatory function as it connects the IKK complex to potential upstream activators.

Matrix Metalloproteinases Matrix metalloproteinases (MMPs) also called metalloproteases, zinc endopeptidases, or matrixins are the largest and most diverse of the four groups of proteases. They are zinc-dependent, calcium-activated proteases synthesized as inactive precursors (or zymogens), which are proteolytically cleaved to generate the active enzyme. All matrixins contain an N-terminal peptide and a zinc-binding catalytic active site. The N-terminal peptide, which is cleaved during the activation step, contains a conserved motif, the cysteine switch whose cysteine residue chelates the zinc of the active site, rendering the enzyme inactive. MMPs degrade components of the extracellular matrix such as collagen and participate in several cellular processes including tissue remodeling, wound healing, angiogenesis, and tumor invasion.

PAMPs Pathogen-associated molecular patterns (PAMPs) are microbial components derived from pathogens such as bacteria, viruses, fungi, and parasites. PAMPs are specifically recognized by the Toll-like receptors (TLRs), which are expressed by cells of the innate immune defense such as dendritic cells and macrophages. PAMPs comprise for very diverse components including lipopolysaccharide (LPS) from Gram-negative bacteria, lipoproteins and lipopeptides, flagellin, and bacterial and viral unmethylated CpG DNA or double-stranded RNA (dsRNA) produced by most replicating viruses.

Phosphorylation Phosphorylation is the reversible process of introducing a phosphate group onto a protein. Phosphorylation occurs on the hydroxyamino acids serine and threonine or on tyrosine residues targeted by Ser/Thr kinases and tyrosine kinases, respectively. Dephosphorylation is catalyzed by phosphatases. Phosphorylation is a key mechanism for rapid posttranslational modulation of protein function. It is widely exploited in cellular processes to control various aspects of cell signaling, cell proliferation, cell differentiation, cell

survival, cell metabolism, cell motility, and gene transcription.

PRRs Pattern recognition receptors (PRRs) are receptors expressed by cells from the innate immune system acting as sensors to rapidly detect invading pathogens. PRRs recognize conserved pathogen-associated molecular patterns (PAMPs) and distinguish foreign organisms such as bacteria, viruses, fungi, or parasites, from cells of the host. PRRs are divided into three families. The most studied family is the Toll-like receptors (TLRs). TLRs are membrane proteins anchored in the plasma membrane or at the surface of endosomes. TLRs are characterized by a common ligand-binding domain, which is composed of leucine-rich repeats (LRRs). Their recognition of either extracellular pathogens or PAMPs present in endosomes activates the innate and adaptive immune responses through signaling cascades controlling selective activation of NF-κB and other inducible transcription factors. The other two families of PRRs, the NOD-like receptors (NLRs) and the RIG-like helicases (RLHs) are soluble receptors present in the cytosol and act as sensors to detect a variety of viral and bacterial products. NOD1 and NOD2 (two NLRs) detect bacterial peptidoglycan, while the retinoic acid-inducible gene-1 (RIG-1) and the melanoma differentiation-associated gene-5 (MDA-5) are RNA helicases that sense viral double-stranded RNA (dsRNA).

Rel Homology Domain The Rel homology domain (RHD) is an evolutionarily conserved domain found in some eukaryotic transcription factors, including NF-κB, the nuclear factors of activated T cells (NFATs), and the Drosophila proteins Dif and Relish. Some of these transcription factors form multiprotein DNA-bound complexes. Phosphorylation of the RHD appears to play a role in the regulation of the activity of some of these transcription factors and modulation of expression of their target genes. Structurally, the RHD is composed of two immunoglobulin-like-beta-barrel subdomains that grip the DNA in the major groove. The amino-terminal portion of the RHD contains a recognition loop that directly interacts with DNA bases. The carboxyl-terminal portion of the NF-κB RHD contains the site for interaction with the IκBs.

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NGS

▶ Gene Expression Analysis and Next-Generation Sequencing

Niacin

▶ Lipid-Lowering Drugs

Nicotinic Acetylcholine Receptor

▶ Nicotinic Receptors

Nicotinic Receptors

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Synonyms

Nicotinic acetylcholine receptor

Definition

The nicotinic receptor (nAChR) comprises a family of receptor subtypes that respond to the neurotransmitter acetylcholine (ACh) and the tobacco alkaloid nicotine (Zoli et al. 2018).

nAChR are found in:

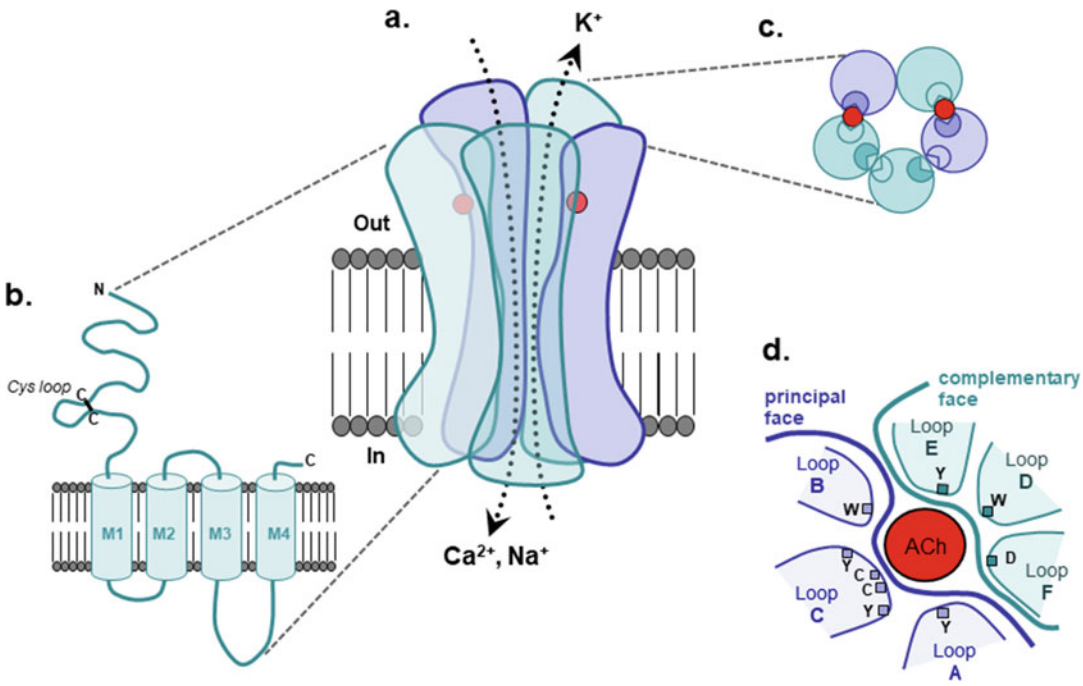
1. Skeletal muscle (*muscle nAChR*, comprised of α1-, β1-, γ- or ε-, and δ-subunits. The γ-subunit is expressed during development and is replaced by ε in nAChR in mature neuromuscular junctions). Also present in electrogenic tissues of electric fish, notably rays and eels of the genera *Torpedo* and *Electrophorus*
2. Sympathetic, parasympathetic, and sensory neurons of the peripheral nervous system (PNS), (*ganglionic nAChR*, typically comprised of combinations of α3-,α5-,β2-, and β4-subunits, also homomeric α7-nAChR composed of α7-subunits only). Also present in chromaffin cells and cell lines of PNS or chromaffin origin (notably SH-SY5Y and PC12 cells). Sensory (dorsal root ganglion) neurons may also express α9- and α10-subunits.
3. CNS neurons (in mammals: heteromeric nAChR comprised of α/β-combinations from α2-, α3-, α4-, α5-, α6-, β2-, β3-, and β4-subunits or homomeric α7-nAChR; also nAChR containing α7 in combination with β2-subunits). α4β2∗-nAChR (where ∗ indicates the possible inclusion of additional subunits in the pentamer) and α7-nAChR are the most prevalent subtypes in the mammalian brain. Avian species express an additional subunit, α8, that is most closely related to the α7-subunit and can form α7α8 heteromeric nAChR.
4. Non-neuronal cells, including astrocytes; endothelial cells of the vascular system; epithelial cells of the skin, lungs, and gastrointestinal tract; immune cells; macrophages; and muscle cells (express various populations of neuronal nAChR subunits; also expressed in some disease states, e.g., cancer cells and activated microglia). Mechanosensory hair cells are exceptional in expressing α9- and α10-subunits only.
5. Invertebrate nervous systems (e.g., insects) and muscle (e.g., nematodes) (an evolutionarily distinct portfolio of α- and β-like subunits constitute various *invertebrate nAChR*)

All subtypes of vertebrate nAChR except muscle nAChR are collectively referred to as *neuronal nAChR*.

The muscle nAChR is the prototype of the cys-loop family of ligand-gated ion channels (LGIC) that also includes $GABA_A$, $GABA_C$, glycine, $5HT_3$ receptors, and invertebrate glutamate-, histamine-, ACh-, and 5HT-gated chloride channels. These receptors are more distantly related to prokaryotic LGICs that lack the cys-loop (Nemecz et al. 2016).

Basic Characteristics

The nAChR is comprised of five subunits, each of which spans the lipid bilayer to create a water-filled pore or channel (Fig. 1a). Each subunit consists of four transmembrane segments; the second transmembrane segment (M2) lines the ion channel (Fig. 1b). Agonist binding leads to channel opening and the passage of cations. The extracellular N-terminal domain of every subunit contains a "cys-loop" that is the signature sequence of the



Nicotinic Receptors, Fig. 1 nAChR structure. (**a**) Schematic representation of a heteromeric nAChR. Five subunits span the lipid bilayer to create a water-filled central channel that is permeable to cations when opened in response to agonist binding. Binding of agonist molecules (represented by red spheres) to two orthosteric binding sites is required for channel opening. (**b**) Structure of a single subunit. Each subunit has a large extracellular domain containing the "cys-loop" and traverses the membrane four times; the second transmembrane domain M2 lines the ion channel. There is a variable intracellular loop between transmembrane spans M3 and M4 that contains putative phosphorylation sites; this region is likely to be important for regulation and trafficking of nAChR and interacts with chaperone, cytoskeletal, and scaffolding proteins. (**c**) Top view of the nAChR. Five subunits surround the central pore. The two orthosteric binding sites with ACh bound (red spheres) are found at subunit interfaces. Unorthodox and noncanonical binding sites involving the fifth subunit are also indicated. (**d**) The orthosteric (agonist) binding site. Agonist (red sphere) binds to a site at the interface between two subunits. The principal (α) subunit contributes amino acids in three loops (A, B, C) to bind the agonist, supported by additional hydrophobic residues in three loops (D, E, F) from the complementary face of the adjacent subunit (γ, ε, or δ in muscle nAChR, α or β in neuronal nAChR) (Changeux 2018). Amino acids are indicated by their single-letter codes

LGIC family: 2 cysteine residues, separated by 13 amino acids, form a disulfide bond to create a loop (Fig. 1b).

The *orthosteric binding site* for agonists, including ACh and nicotine, occurs at the interface between two adjacent subunits (Fig. 1c), in the extracellular part of the receptor created by the N-terminal domains of its subunits (Figs. 1a and 2). The principal face of the binding site is found on α-subunits that possess a pair of adjacent (vicinal) cysteine residues and a number of key hydrophobic residues organized in three loop structures (A, B, C) that coordinate the agonist (Fig. 1d). All α-subunits except for α5 and α10 can contribute to the orthosteric binding site. The adjacent subunit (γ, ε, or δ in muscle nAChR; β in neuronal heteromeric nAChR; α in homomeric α7-nAChR; or α10 in α9/α10-nAChR) contributes the complementary face of the orthosteric binding site, providing three conserved loops (D, E, F) containing predominantly hydrophobic residues (Fig. 1d). In muscle nAChR and αβ-heteromeric neuronal nAChR, there are two orthosteric binding sites, and both sites must be occupied by agonist for effective opening of the ion channel. Homomeric α7-

nAChR present five orthosteric binding sites (Bouzat et al. 2018).

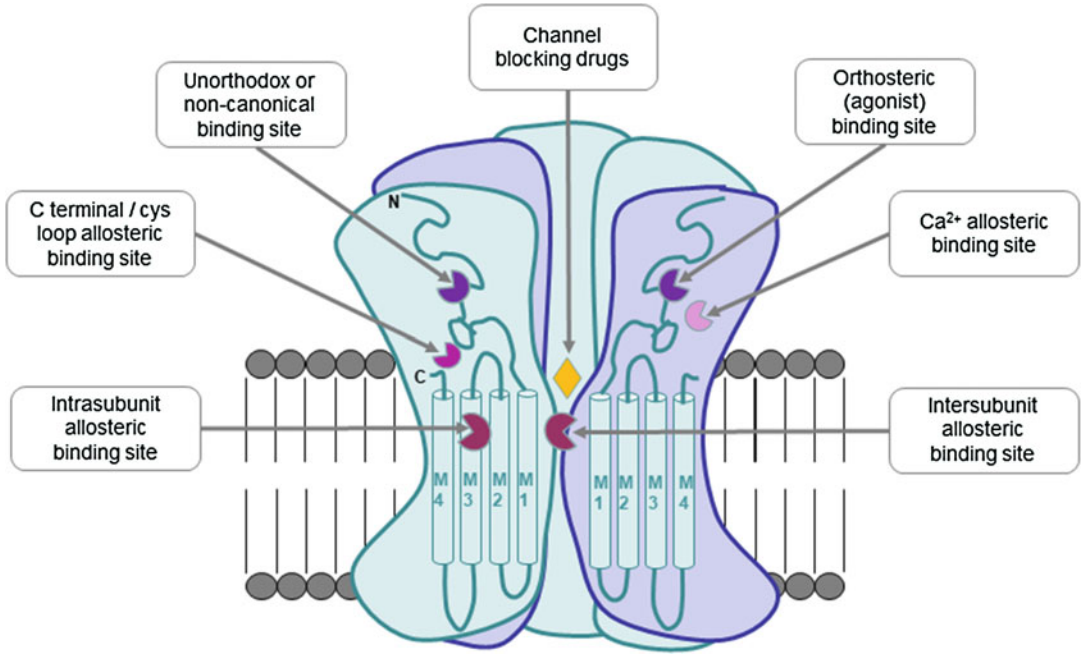
Heteromeric neuronal nAChR containing three α-subunits (e.g., $\alpha4_3\beta2_2$) have an additional *"unorthodox" agonist binding site* for ACh at the α/α-interface (Fig. 1c): agonist binding to such sites is not sufficient to cause the ion channel to open but can modify nAChR function dictated by the orthosteric sites (Wang and Lindstrom 2018).

nAChR function: in the resting (closed) state, the ion channel is occluded by a "hydrophobic girdle" that constitutes a barrier to ion permeation. Agonist binding to the orthosteric sites promotes a conformational change that results in a rotational movement of the M2 helices lining the pore, widening the pore by ~3 Å (Nemecz et al. 2016). This results in an influx of Na^+ and Ca^{2+} (the relative permeability of these two cations depends on nAChR subtype) and an efflux of K^+ under normal physiological conditions. Despite the presence of agonist, the nAChR channel closes within seconds to minutes, to enter a desensitized state. In this condition, the nAChR is refractory to activation. Multiple desensitized states have been proposed to exist (Nemecz et al. 2016). The rates of desensitization and recovery are nAChR subtype dependent and are also influenced by agonist concentration.

At the muscle end plate, nAChR activation produces depolarization that triggers muscle contraction. In sympathetic ganglia, postsynaptic nAChR mediate synaptic transmission, resulting in the depolarization of postganglionic neurons and the increased likelihood of generating an action potential. In the vertebrate CNS, it is uncommon for nAChR to have a postsynaptic role in mediating synaptic transmission. nAChR are more typically found at presynaptic and extrasynaptic locations on brain neurons where they exert a modulatory influence on synaptic activity and brain function (Zoli et al. 2018). This is achieved by alteration in excitability and/or increases in intracellular Ca^{2+}. Thus presynaptic nAChR promote the release of various neurotransmitters in different brain regions; extrasynaptic somatodendritic nAChR can influence local excitability and cell signaling and may produce long-term changes by altering gene expression. For example, α7-homomeric nAChR have exceptionally high relative permeability to Ca^{2+} (comparable to that of the NMDA receptor). Their association with glutamate synapses supports a contribution to long-term potentiation (LTP), through presynaptic facilitation of glutamate release and extrasynaptic enhancement of excitability. Postsynaptic α7-nAChR can activate Ca^{2+}-dependent cell signaling pathways, leading to activation of ERK, CREB, and immediate early genes such as cFos.

Allosteric modulation: in addition to the orthosteric and unorthodox binding sites, nAChR present multiple *allosteric binding sites* (Changeux 2018; Wang and Lindstrom 2018) (Fig. 2). Ligands targeting these sites typically have no intrinsic activity but may positively or negatively modulate agonist-induced responses. The various allosteric sites are well placed to modulate the conversion of agonist binding into channel opening. Most attention has focused on positive allosteric modulators (PAMs).

1. *Extracellular noncanonical binding sites*: analogous to the orthosteric and unorthodox binding sites but lacking the ability to bind ACh (primarily because they lack key amino acids in the principal binding face). For example, an $\alpha4_2\beta2_3$-nAChR presents β/α- and β/β-binding sites: although insensitive to ACh, other ligands (e.g., the anticholinesterase inhibitor galantamine) may bind to these noncanonical sites and hence influence receptor function (Wang and Lindstrom 2018). The β/β-noncanonical binding site is analogous to the benzodiazepine site of $GABA_A$ receptors.
2. *Extracellular Ca^{2+} binding site*: located at subunit interfaces, below the orthosteric sites, and closer to the membrane (Changeux 2018).
3. *Intrasubunit transmembrane sites*: for example, PNU-120596 binds to the intrasubunit vestibule located between the transmembrane spans of a single α7-subunit; occupancy of four such binding sites per pentamer is



Nicotinic Receptors, Fig. 2 nAChR binding sites for ACh and drugs. Schematic representation of binding sites on the nAChR for ACh and other agonists, allosteric modulators, and channel-blocking drugs. Only one site per category is indicated, but multiple sites may exist on the pentameric protein

reported to be necessary for positive modulation by PNU-120596 (Bouzat et al. 2018).

4. *Inter-subunit transmembrane sites*: for example, the large macrocyclic lactone ivermectin binds between the M3 and M1 transmembrane domains of adjacent subunits, toward the extracellular face of the membrane, and in close apposition with the M2 domain that lines the ion channel. Hence it is able to stabilize the open state of the ion channel (Changeux 2018).
5. *Sites at or near the extracellular C-terminus*: for example, 17β-estradiol binds to the C-terminus of the α4-subunit, facilitating interaction with the cys-loop to enhance channel opening (Wang and Lindstrom 2018).

Irrespective of where they bind, PAMs have been classified as *Type I* (increase agonist peak responses without altering timecourse (desensitization)), *Type II* (increase agonist peak responses and prolong responses by decreasing or reversing desensitization), or *Type III* (have weak intrinsic agonist activity as well as potentiating agonist responses) (Wang and Lindstrom 2018).

Drugs

Nature has created a diverse array of plant and animal toxins that act at mammalian muscle and neuronal nAChR or invertebrate nAChR, because the critical physiological functions of nAChR make them prime targets for defensive or predatory strategies. More recently, the perceived validity of neuronal nAChR as therapeutic targets has prompted the generation of new synthetic agonists and allosteric modulators. Examples are listed in Table 1.

Agonists activate nAChR by binding to the orthosteric agonist-binding sites (Fig. 1). Agonist can remain bound (often with higher affinity) when the nAChR enters the desensitized state.

Competitive antagonists interact with the nAChR at, or close to, the agonist-binding sites, stabilizing the receptor in a conformation with the channel closed and preventing agonists from accessing their binding site. Inhibition by reversible competitive antagonists is surmountable with increasing agonist concentration, shifting the concentration response relationship to the right.

Nicotinic Receptors, Table 1 Some drugs that act at nAChR

	Drug	Source	Specificity
Agonists	ACh	Endogenous agonist	nAChR and muscarinic receptors
	(±)-Anatoxin-a	*Anabaena flos-aquae* (blue-green algae)	Potent, nonselective
	Carbamylcholine	Synthetic carbamate analogue of ACh	Muscarinic > nicotinic
	Choline	Endogenous precursor and breakdown product of ACh	α7-nAChR selective
	(−)-Cytisine	*Fabaceae* genera	Potent partial agonist at α4β2-heteromeric neuronal nAChR Weak full agonist at α7-nAChR
	Dimethylphenylpiperazinium (DMPP)	Synthetic	Classical ganglionic agonist
	(±)-Epibatidine	*Epipedobates tricolor* (South American frog)	Very potent, nonselective
	Imidacloprid	Synthetic chloro-nicotinyl insecticide	Insect CNS nAChR
	5-Iodo-A-85380	Synthetic	β2-containing nAChR > others
	Levamisole	Synthetic imidazothiazole	Nematode muscle nAChR
	Lobeline	*Lobelia inflata*	A nonclassical agonist, also has non-specific actions
	(−)Nicotine	*Nicotiana tabacum*	Definitive agonist for nAChR (but an antagonist at α9-containing nAChR)
	Sazetidine A	Synthetic	α4β2-selective, stoichiometry dependent: $\alpha4_2\beta2_3 \gg \alpha4_3\beta2_2$
	Trans-metanicotine (TC-2403)	Synthetic	β2-containing > others
	Varenicline	Synthetic	Potent partial agonist at α4β2-heteromeric neuronal nAChR Weak full agonist at α7-nAChR
Competitive antagonists	α-Bungarotoxin	*Bungarus multicinctus* (banded krait)	Muscle and α7-, α8-, α9-, and α10-nAChR; some invertebrate nAChR
	α-Conotoxin	*Conus* sp.	Various/subtype-specific
	Decamethonium	Synthetic	Muscle > ganglionic
	Dihydro-beta-erythroidine (DHβE)	*Erythrina* sp.	β2 > β4
	D-Tubocurarine	*Strychnos toxifera; Chondrodendron tomentosum*	Nonselective
	Methyllycaconitine (MLA)	*Delphinium brownii*	Selective for α7-, α8-, α9-, and α10-nAChR, some invertebrate nAChR; weaker inhibition of α6 > α4 > muscle nAChR

(continued)

Nicotinic Receptors, Table 1 (continued)

	Drug	Source	Specificity
Noncompetitive antagonists	Chlorisondamine	Synthetic	Ganglionic (long-lasting blockade of CNS nAChR)
	Hexamethonium	Synthetic	Ganglionic (does not cross the blood-brain barrier)
	Histrionicotoxin	*Dendrobates histrionicus*	Nonselective
	Mecamylamine	Synthetic	Ganglionic: (terminology from historical clinical use; relatively non-selective for all neuronal heteromeric nAChR; crosses the blood brain barrier)
Positive allosteric modulators	LY2087101	Synthetic	Type I/intramembrane site $\alpha7 > \alpha4\beta2$-nAChR
	PNU-120596	Synthetic	Type II/intramembrane site α7-nAChR selective
	Galantamine	*Galanthus caucasicus*	Type III/noncanonical binding site
Negative allosteric modulators	Anesthetics (e.g., halothane, ketamine)	Synthetic	Transmembrane sites on α4β2-nAChR selectivity unclear

Noncompetitive antagonists do not compete for binding to the agonist-binding sites, but interact with distinct sites that modify nAChR function. Their inhibition is not surmountable with increasing agonist concentration. Some noncompetitive antagonists are "channel blockers" that act by either blocking the entrance to the nAChR channel or entering the lumen of the channel (Fig. 2). The action of such drugs can be "state-dependent," requiring prior activation of the nAChR to allow access to the channel. Many compounds that have other primary targets can also act as noncompetitive antagonists of nAChR, typically by blocking the ion channel. Examples include antagonists of voltage-operated Ca^{2+} channels, NMDA channel blockers, antidepressants, steroids, and β-amyloid peptide.

Positive allosteric modulators (PAMs) potentiate responses to nicotinic agonists by acting at a site distinct from the agonist-binding site (Fig. 2). Nonselective and subtype-selective PAMs have been characterized.

Negative allosteric modulators (NAMs) potentially act at the same sites as PAMs but have the opposite effect and diminish agonist responses (Zoli et al. 2018). Fewer examples have been reported, but both volatile and intravenous general anesthetics fall into this category. Drugs that act by occluding the nAChR pore (channel blockers) are not NAMs. However, drugs can display multiple actions that may differ in their concentration dependence.

Therapeutic or Agrochemical Applications

Historically ganglionic nAChR have been targets for treating hypertension. Muscle nAChR are inhibited by muscle relaxants such as atracurium besilate during surgery. The discovery of a large family of nAChR subtypes in the CNS, coupled with observations that nicotine has antinociceptive, neuroprotective, and procognitive effects, has made neuronal nAChR potential targets for treating a wide range of disorders. These include Alzheimer's and Parkinson's diseases, schizophrenia, attention-deficit hyperactivity disorder (ADHD), and pain. nAChR in peripheral cells are of interest in treating inflammation in different scenarios including ulcerative colitis and wound healing. Nicotine, cytisine (Tabex), and varenicline (Chantix, Champix) are in use for smoking cessation.

Imidacloprid is a widely used neonicotinoid insecticide that kills pests and other insects by targeting their central nAChR. Levamisole is used to kill nematodes by acting on nAChR in the worm's muscles.

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Nitric Oxide

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Synonyms

Nitrogen monoxide; Nitrogen(II) oxide

Definition

Nitric oxide (NO) is a colorless gas. It occurs in the atmosphere as a by-product of fossil fuel combustion. NO is also produced in mammalian and nonmammalian cells as well as in plants by enzymes called NO synthases (NOS). Being a radical, its half-life is in the range of seconds. Nevertheless, NO acts as an endogenous signaling molecule based on its ability to cross cell membranes. By activation of its receptor, NO-sensitive guanylyl cyclase, as well as by covalent reaction, e.g., with oxygen species or amino acid residues, NO is able to convey physiological and pathophysiological functions.

Although having been present in the earth's atmosphere for millions of years, nitric oxide was first identified by Joseph Priestly in 1772. Its physiological role became obvious only about 50 years ago. In 1982, Furchgott and Zawadzki (Furchgott and Zawadzki 1980) discovered that stimulation of endothelial cells with acetylcholine led to the release of a vasorelaxing factor, termed "endothelium-derived relaxing factor" (EDRF). In the subsequent years, EDRF was identified as NO by several independent research groups. Based on its presumed chemical instability and its radical character, a biological role of NO was first dismissed; however, subsequent work led to the identification of NO-producing enzymes (NO synthases, NOS) and the description of an NO receptor, namely, NO-sensitive guanylyl cyclase (NO-GC). Following the establishment of NO as physiological mediator along with its up- and downstream effectors, research focused on this novel pathway and led to the development of various enzymes involved in NO signaling. NO, initially identified in the vascular system, was found to regulate many different organs and tissues, including central and peripheral nervous system, but also to modulate various responses such as angiogenesis, apoptosis, immune response, or cell proliferation (Moncada et al. 1991). On the basis of their initial groundbreaking discoveries, Robert F. Furchgott, Louis Ignarro, and Ferid Murad were awarded the Nobel Prize in 1998.

Basic Mechanisms

Enzymatic NO Synthesis

Three isoforms of the NO synthase (NOS; EC 1.14. 13.39) are known (Förstermann and Sessa 2012), which were originally named after the

tissues in which they were first identified: neuronal NOS (nNOS; NOS type 1), inducible NOS (iNOS; NOS type 2; identified first in macrophages), and endothelial NOS (eNOS; NOS type 3). It was soon realized that NOS activity requires Ca^{2+}. nNOS and eNOS are both constitutively expressed enzymes that are regulated by intracellular Ca^{2+}/calmodulin. In contrast, iNOS is not constitutively expressed but rather induced by immunological stimuli; this isoform is independent of cytosolic Ca^{2+} as it constantly binds the Ca^{2+}/calmodulin complex.

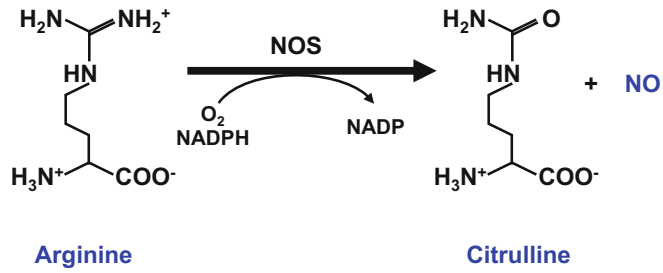
NO production results from a complicated redox reaction with arginine being transformed to citrulline in the presence of oxygen (Figs. 1 and 2). Necessary cofactors for all three isoforms include tetrahydrobiopterin (BH4), heme, NADPH, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN).

NO synthesis results from the biochemical conversion of arginine to citrulline. All NOS isoforms require BH4, heme NADPH, FAD, and FMN as cofactors. Arginine analogs such as N^G-monomethyl-l-arginine, N^G-nitro-l-arginine, and asymmetric dimethylarginine act as inhibitors of NOS.

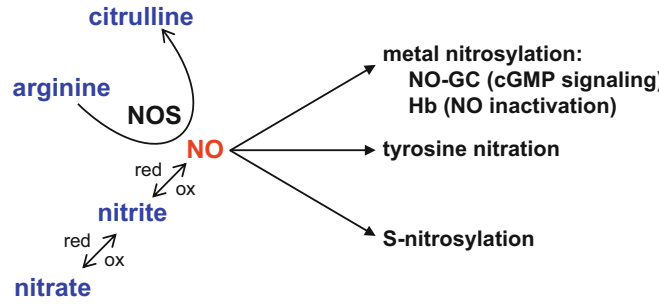
Nonenzymatic NO production

Nitric oxide may also be produced in an NOS-independent way under physiological conditions. Nonenzymatic NO generation was first shown to occur in the human stomach under acidic conditions. This pathway involves circulating nitrate which is reduced to nitrite by oral bacteria followed by further reduction under acidic conditions to form NO or other nitric oxide species (Fig. 2). Later, this nitrate-nitrite-NO pathway was shown to occur throughout the body in order to regulate blood flow, intercellular signaling, and responses to hypoxia. It is believed that the serial reduction of nitrate/nitrite serves as a backup system under hypoxic/ischemic or acidic conditions which impede oxygen-dependent NOS activity.

NO is produced either enzymatically by NOS or nonenzymatically after reduction of nitrate and nitrite. Possible reactions of NO include S-nitrosylation, tyrosine nitration, or metal nitrosylation. Nitrosylation of Fe-containing heme proteins such as NO-sensitive guanylyl cyclase (NO-GC) and hemoglobin (Hb) is thought to represent the physiological signaling and inactivation pathways, respectively.



Nitric Oxide,
Fig. 1 Catalytic reaction of NO synthases



Nitric Oxide,
Fig. 2 Generation and reactions of NO

NO Donors

Several compounds have been known for a long time to release NO or an NO-like species. Most of them belong to the group of organic nitrates and nitrites. For over 100 years, glyceroltrinitrate (GTN) has been exploited in the therapy of coronary heart disease. GTN releases NO after biotransformation, e.g., by mitochondrial aldehyde dehydrogenase (ALDH-2). Thereby, GTN effectively lowers the preload in patients. Other therapeutically used NO donors which do not need biotransformation but release NO spontaneously are molsidomine and sodium nitroprusside. Besides, a plethora of NO-releasing substances, e.g., NONOates, which exhibit varying release kinetics and stoichiometries have been introduced for research purposes.

NO Inactivation

Despite ample information on the mechanism of NO synthesis, much less information is available on how NO is inactivated and degraded. Binding to the heme moiety of hemoglobin yielding nitrate and methemoglobin is probably the most important physiological way of NO inactivation. Thereby, the dense capillarization of most tissues limits the range of NO diffusion and thus its half-life. Another important way to inactivate NO is the diffusion-limited reaction with superoxide ions under formation of peroxynitrite (Pacher et al. 2007). Being a very powerful and toxic oxidant, peroxynitrite may have pathophysiological importance based on its reaction with protein thiols, iron/sulfur centers, or zinc fingers. As superoxide concentrations are limited by the action of superoxide dismutase, the inactivation of NO to peroxynitrite will be favored under conditions that allow the formation of high concentrations of each compound.

Further chemical ways of NO inactivation include the oxidation to nitrite/nitrate which are excreted with the urine. The direct reaction between NO and oxygen, however, appears to be of minor importance due to the low concentrations of the reactants in vivo. This reaction may, however, be favored under conditions in which the concentrations of NO and oxygen are enriched such as in hydrophobic cell membranes.

NO Receptor

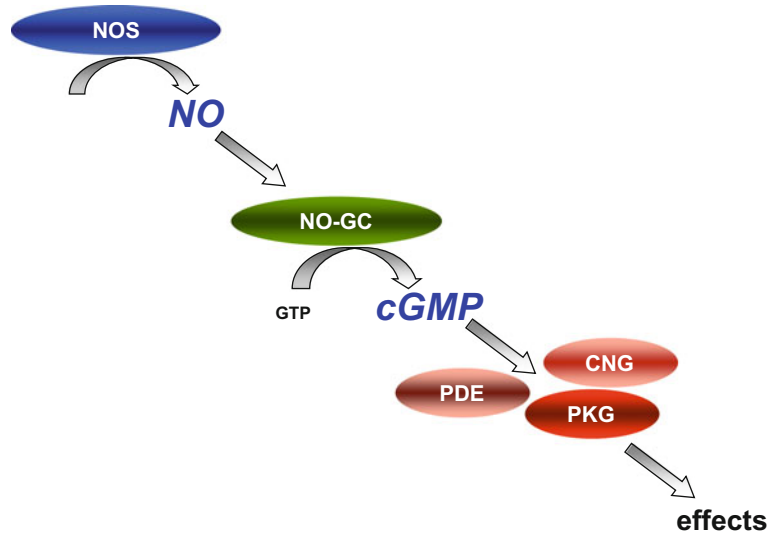
The commonly accepted receptor mediating the physiological effects of NO is NO-sensitive guanylyl cyclase (NO-GC; Fig. 3). Binding of NO to the enzyme's prosthetic heme group leads to a conformational change of the dimeric enzyme which dramatically increases its catalytic activity. As a consequence, the substrate guanosine triphosphate (GTP) undergoes a cyclization reaction resulting in the synthesis of cyclic guanosine monophosphate (cGMP). cGMP acts as intracellular second messenger by activating target enzymes such as cGMP-dependent protein kinase G (PKG) and cGMP-regulated ion channels (CNG). In addition, cGMP modulates the activity of several phosphodiesterases (PDE). Depending on the subtype of PDE, cGMP may either increase or decrease cAMP or cGMP levels. The resulting crosstalk is of paramount importance in various cell types such as cardiomyocytes, smooth muscle cells, or platelets.

NO from either of the three NOS activates NO-GC by binding to its prosthetic heme to increase the production of cGMP. cGMP acts on three different effector molecules: whereas cGMP-dependent protein kinase G (PKG) and cGMP-regulated ion channels (cyclic nucleotide-gated channels, CNG) are activated by cGMP to increase phosphorylation and ion permeability, respectively, cGMP either activates or inhibits PDE depending on the enzyme subtype. This may result in increased or decreased levels of either cGMP or cAMP in a given cell.

Physiological Effects of NO

Endothelial NO

Although NO synthesis in endothelial cells can be induced by various substances such as acetylcholine, substance P, bradykinin, or histamine, the main stimulus for NO production is vascular shear stress. Thereby, NO and the subsequent cGMP cascade exerts an important role in



Nitric Oxide, Fig. 3 NO/cGMP signaling cascade

maintaining basal blood flow. Shear-induced NO release results in vasodilatation, thus leading to decreased vascular resistance and reduction of blood pressure. Long-term effects of endothelial NO include the inhibition of smooth muscle proliferation which helps to prevent atherosclerosis. Whereas these effects are mediated by NO diffusion to the medial layer of the blood vessel, NO also diffuses in luminal direction, and physiological NO effects are also mediated by NO-GC-expressing blood cells. NO thus induces the inhibition of platelet aggregation and adhesion as well as inhibition of leukocyte adhesion and transmigration.

Neuronal NO

NO can be released from neurons of the central (CNS) and peripheral nervous system as well as the enteric nervous system (ENS).

In the CNS, NO acts mainly as neuromodulator: NO has been shown to modulate synaptic signaling, thus influencing learning and memory processes. Here, long-term regulation of synaptic transmission rather than acute neurotransmission is mediated by nNOS. Retrograde communication across synaptic junctions is thought to involve NO-GC as target, thus involving cGMP and downstream targets. In several animal models, NOS inhibitors have shown to impair learning and to induce amnesia. Similarly, blockade of nNOS can lead to hypertension indicating the involvement of nNOS-derived NO in the regulation of blood pressure. Furthermore, neuronal NO/cGMP is involved in the regulation of sleep, circadian clock, as well as pain processing.

The peripheral nervous system also contains nitrergic nerves that express nNOS. These nerves innervate vascular smooth muscle, and nNOS-derived NO is thus thought to participate in the regulation of vascular tone. In addition, penile erection is initiated by activation of nitrergic nerves leading to corpus cavernosum smooth muscle relaxation.

The importance of NO in the ENS is still not fully elucidated. In the ENS, the major inhibitory non-adrenergic, non-cholinergic (NANC) neurotransmitter is NO; accordingly, these neurons are called “nitrergic.” However, other transmitters are often co-released from nitrergic nerves including vasoactive intestinal peptide (VIP) or ATP.

In both the gastrointestinal (GI) and urogenital tract, NO from nitrergic nerves targets NO-GC. In contrast to vascular tissue in which NO-GC in SMC is the primary target of eNOS-derived NO, gastrointestinal smooth muscle contains at least

three NO-GC-expressing cell types, i.e., SMC, interstitial cells of Cajal, and fibroblast-like cells. As all three of them lie in close proximity to nitrergic varicosities, the inhibitory neurotransmission of NO is likely to influence smooth muscle tone of the GI tract through a concerted action on all three cell types. Besides the transport of chyme, nNOS-derived NO regulates the smooth muscle tone in ureter and bile ducts; as a consequence, NO is thought to modulate the passage of bile and urine.

Inflammatory NO

Inducible NOS is normally not present in tissues. Inflammatory stimuli such as lipopolysaccharides or cytokines are known to induce the expression of iNOS mainly in macrophages but also in other cells. In contrast to the constitutive forms of NOS (eNOS, nNOS), iNOS produces high concentrations of NO. As a major component of the immune response, NO exerts toxic effects on bacteria, parasites, and viruses. NO's mechanism of toxicity includes the inhibition of iron−/sulfur-containing enzymes and the induction of DNA damage in parasitic microorganisms but also in tumor cells. However, as a consequence of the high NO concentrations achieved in the immune response, the reaction with superoxide is favored which may also lead to detrimental effects within the host.

Pathophysiological Effects of NO

As stated above, the main toxic mechanism of NO is the superoxide-dependent formation of peroxynitrite. As this reaction is favored at high concentrations of the reactants, iNOS-derived NO is most likely to exert toxicity on the invading organism but also on healthy cells of the host organism. NO derived from eNOS and nNOS is less likely to cause pathophysiological effects based on the fact that lower NO concentrations are produced for signaling purposes and that cGMP is not involved in NO toxicity. However, excess stimulation of eNOS and nNOS was shown to contribute to neurodegenerative pathologies as well as cardiovascular diseases especially in patients with neuronal or cardiovascular risk factors.

Mechanistically, peroxynitrite promotes oxidation and tyrosine nitration reactions which will affect a plethora of biomolecules. In addition, nitrosylation of thiols and metals are also known to affect protein and nonprotein structures. Thus, these NO-dependent reactions may cause excitotoxicity, neuronal apoptosis, cytochrome oxidase inhibition, and energy depletion due to inhibition of mitochondrial respiration. As a consequence, excessive NO is involved in many different diseases: Harmful NO metabolites are involved in neurodegeneration seen, e.g., in stroke, multiple sclerosis, Alzheimer's, and Parkinson's disease. In the cardiovascular system, nitrosylation and nitration reactions participate in the development of atherosclerosis, myocardial infarction, or ischemia/reperfusion injury. Further diseases connected with excessive NO production include rheumatoid arthritis, inflammatory bowel disease, diabetes mellitus, and certain forms of cancer. In septic shock, massive expression of iNOS in the vascular wall may result in extreme fall of vascular resistance, hypotension, and vascular damage.

Pathophysiological effects also arise from reduced NO bioavailability. Endothelial dysfunction is commonly associated with a decrease in NO levels which may be explained either by reduced endothelial NO production or by increased inactivation of endothelial NO by reactive oxygen species. Endothelial dysfunction participates in serious cardiovascular and metabolic diseases such as hypertension, angina pectoris, myocardial infarction, stroke, renal failure, glaucoma, and diabetes mellitus.

Pharmacological Relevance

Since reduced NO production and excess NO release are linked to various diseases, reducing as well as increasing NO effects may be therapeutically reasonable. Options to increase NO levels are direct application of proper NO (as NO gas), substances that release NO (NO donors), or an

NO-like compound. In order to increase NO-induced effects, stimulation of downstream pathways (NO-GC stimulators or PDE inhibitors) is conceivable. In case of excess NO, several different NOS inhibitors are available.

Genuine NO can be applied as inhalative treatment. Inhaled NO has been used to treat pulmonary hypertension in newborns, postoperative pulmonary hypertensive crises, and acute hypoxemic respiratory failure in patients with underlying pulmonary hypertension. NO donors are a heterogeneous group with most therapeutically used substances being organic nitrates. Glyceroltrinitrate (GTN) has been used for over 100 years in the treatment of acute attacks of coronary heart disease. While the oxidative state of the NO released from nitrates is not entirely clear, other compounds such as sodium nitroprusside release radical NO. In addition, nitroxyl donors (HNO; e.g., Angeli's salt, CXL 1020) have been/are being developed in order to treat acute decompensated heart failure.

Riociguat was the first approved drug belonging to the group of NO-GC stimulators. These compounds do not release NO by themselves, but increase cGMP signaling by sensitizing NO-GC toward endogenously produced NO (Sandner et al. 2019). Riociguat can be used in patients with pulmonary hypertension, whereas other stimulators resembling riociguat structurally are aimed to treat patients with heart failure, diabetic nephropathy, achalasia, or sickle cell disease.

Inhibition of cGMP-degrading phosphodiesterases (PDE) is probably the best-known option to increase NO-induced cGMP-mediated effects. Prototypical substances are the PDE5 inhibitors sildenafil, vardenafil, and tadalafil. PDE5 inhibition in the corpus cavernosum blocks the degradation of cGMP which results from activation of nitrergic nerves followed by stimulation of NO-GC.

Excessive NO production by either NOS may be counteracted with isoform-specific NOS inhibitors. In the case of nNOS, suitable inhibitors could prevent excitotoxicity and neurodegeneration, whereas under septic conditions, iNOS-specific inhibitors might reduce cardiovascular symptoms. Arginine analogs (e.g., N^G-monomethyl-l-arginine, N^G-nitro-l-arginine, and asymmetric dimethylarginine) are potential candidates; however, due to moderate potency and poor selectivity, they are not a therapeutic option. Other iNOS inhibitors, e.g., 1400 W, failed in clinical trials because of inherent toxicity.

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Nitrogen Monoxide

► Nitric Oxide

Nitrogen(II) Oxide

► Nitric Oxide

NMR

► Nuclear Magnetic Resonance

NMR Spectroscopy

► Nuclear Magnetic Resonance

Nonhomologous End Joining

► DNA Damage Response

Nonreceptor Tyrosine Kinases

► Tyrosine Kinases

Non-selective Cation Channels

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Synonyms

Non-specific cation channels; NSCCs

Definition

Non-selective cation channels are macromolecular pores in the cell membrane that form an aqueous pathway enabling cations such as Na^+, K^+, or Ca^{2+} to flow rapidly, as determined by their electrochemical driving force, at roughly equal rates ($>10^7$ cations per channel pore and per second).

Basic Characteristics

In general, ion channels are multi-protein complexes residing in cellular membranes and allowing ions, mainly Na^+, K^+, Ca^{2+}, and Cl^-, to flow rapidly as determined by their electrochemical driving force in a thermodynamically downhill direction. The channel proteins are dynamic structures; they form channels that can exist in at least two conformational states, open and closed. In the closed state the channel can be inactive and/or is reluctant to be activated. Fast shifts in the submillisecond range between these states can be regulated by the membrane potential (voltage-gated ion channels), by ligands (ligand-gated ion channels) or agonists. In many cases these shifts can be modulated on a longer timescale (milliseconds to minutes) by hormones, neurotransmitters, drugs, and toxins.

The open channel has in most cases a selective permeability, allowing a restricted class of ions to flow, for example, Na^+, K^+, Ca^{2+}, or Cl^-, and, accordingly, these channels are called Na^+ channels, K^+ channels, Ca^{2+} channels, and Cl^- channels. In contrast, cation-permeable channels with little selectivity reject all anions but discriminate little among small cations. Different types of channels have different pore size dimensions, and ion discrimination is accomplished by a selectivity filter formed by channel-specific amino acid residues with characteristic arrangements of ion-binding sites in the pore region. Structural and dynamic differences were identified between selective and non-selective ion channels. Interactions of different ions within the channel significantly shape the landscape of free energy inside the pore, and it is suggested that structural plasticity within the selectivity filter and the selection of certain conformations by different ions are key molecular determinants in non-selective channels for a highly efficient permeation of different ions (Shi et al. 2018).

The first in-depth characterized non-selective cation channel was the nicotinic acetylcholine receptor (nAChR, see Nicotinic Receptors), which is a ligand-gated ion channel (see below) that does not select well among cations ($P_{Ca}/P_{monovalent\ cations}$ 4-7); the channel is even permeable to choline, glycine ethyl ester, and tris buffer cations. A number of other plasma membrane cation channels including certain ionotropic glutamate receptors, ionotropic purinergic (ATP) receptors (P2X receptors), cyclic nucleotide-gated channels (CNG channels), and the mechanosensitive Piezo channels are also called non-selective; they favor the flow of Ca^{2+} over monovalent cations with relative permeabilities of <1.5 (glutamate receptors of the AMPA/Kainate-type), <5 (glutamate receptors of the NMDA-type,

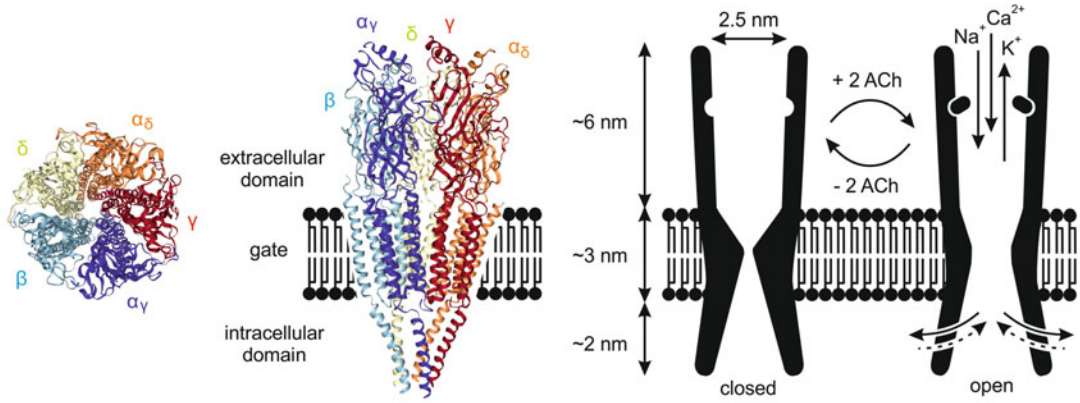
Piezo1/2), <12 (P2X receptors), and <90 (CNG channels) (Pankratov and Lalo 2014). Others, like hyperpolarization-activated and cyclic nucleotide-gated channels (HCN channels), conduct both K^+ and Na^+, but are impermeable to divalent cations, and the acid-sensing ion channel (ASIC) is mainly permeable to Na^+ with only minor permeability to Ca^{2+}. Another group of ion channels, which belong to the superfamily of transient receptor potential (TRP) channels (Nilius and Flockerzi 2014), have also been implicated to represent non-selective cation channels. Their permeability ratio for Ca^{2+} and monovalent cations varies widely with some TRP channels being highly selective for Ca^{2+} (TRPV5/6 $P_{Ca}/P_{Na} > 100$) and others being virtually Ca^{2+}-impermeable (TRPM4/5). Finally, besides Piezo, a subset of other channels activated by stretch have been implicated to be non-selective cation channels (Hille 2001). These channels appear to be ubiquitously expressed, but their structures are still unknown.

Non-selective cation channels are found in excitable and non-excitable cells. Driven by the electrochemical gradient, i.e., negative membrane potential and high extracellular levels of Na^+ and Ca^{2+} as compared to the cytoplasm, opening of non-selective cation channels in general leads to significant flux of Na^+ and Ca^{2+} into the cell. Subsequently, the resulting membrane depolarization and cytosolic Ca^{2+} increase triggers various cell-specific responses and functions. While the ligand-gated acetylcholine, glutamate, and ATP receptor channels directly transduce intercellular signals, CNG and some TRP channels indirectly deliver G protein- and/or receptor tyrosine kinase-mediated signaling as they are activated by intracellular second messenger molecules (cAMP, cGMP, Ca^{2+}, DAG). Other TRP channels, ASIC, and Piezo channels are involved in sensing chemical and physical conditions of the extracellular environment, and the activity of HCN channels contributes to the generation of rhythmic activity in heart and brain cells.

Due to recent methodological advances, within the last two decades detailed membrane structures of several non-selective cation channels, i.e., members of the ionotropic acetylcholine, glutamate, and ATP receptors as well as CNG, HCN, Piezo, ASIC, and TRP channels, have been identified by crystallographic analysis and/or cryo-electron microscopy. Like other ion channels, non-selective cation channels are composed of a certain number of subunits that are assembled in a predetermined arrangement and stoichiometry around a central, cation-selective pathway. The subunit compositions of the ion channels define their biophysical properties.

The structure of non-selective cation channels is extremely diverse: nACh receptor channels consist of five subunits (pentamer) with four transmembrane domains each; P2X receptor channels and ASICs are composed of three subunits with two transmembrane domains each; CNG, HCN, and TRP channels form tetramers with six transmembrane domains per subunit; ionotropic glutamate receptor channels are formed by four subunits with four transmembrane domains each; Piezo channels appear as trimers, predicted to encompass at least 26 transmembrane domains (Saotome et al. 2018) per subunit. Transmembrane segments are mainly formed by 19–21 amino acid residues that are folded into a α-helix. The α-helix is a rod-like structure, the tightly coiled polypeptide main chain of which forms the inner part of the rod, and the side chains extend outward in a helical array. Each residue is related to the next one by a rise of 0.15 nm along the helix axis and a rotation of 100°, which gives 3.6 amino acid residues per turn of helix. Accordingly, one turn of the helix is 0.54 nm, which is equal to 0.15 times the number of residues per turn. The hydrocarbon core of cellular membranes is typically 3 nm wide, which accordingly can be traversed by a α helix consisting of 19–21 residues.

For example, the pentameric nAChR, found in the membrane of vertebrate skeletal muscle at the synapse between nerve and muscle, also called the neuromuscular junction (muscle type nAChR), is cylindrical with a mean diameter of about 6.5 nm (Fig. 1). All five rod-shaped subunits ($\alpha_\gamma\gamma\alpha_\delta\delta\beta$) span the membrane. The receptor protrudes by <6 nm on the synaptic side of the membrane and by <2 nm on the cytosolic side. The pore of the channel is along its symmetry axis and includes an extracellular entrance domain, a



Non-selective Cation Channels, Fig. 1 The nicotinic acetylcholine receptor (nAChR) is localized within the cell membrane; above the cell membrane is the synaptic cleft, below the cytoplasm. Structure of the nAChR from top (left) and side view (middle; PDB 2BG9). The different colors code for the five subunits α_δ, α_γ, β, δ, and γ. Drawing (right) of the closed (left) and open (right) nAChR showing acetylcholine (ACh) binding to the α subunits and cation movement. Dimensions of the receptor were taken from reference (Corringer et al. 2000)

transmembrane domain and a cytosolic entrance domain. The diameter of the extracellular entrance domain is <2.5 nm, and it becomes narrower at the transmembrane domain. The pore is lined by five α-helices, one from each subunit, and adjacent extended loop regions (Corringer et al. 2000). If two ACh molecules bind to the receptor sites at the extracellular surface of the receptor, far from each other and from the pore, this pathway opens, allowing permeation of Na^+ (crystal radius 0.095 nm), K^+ (crystal radius 0.133 nm), and Ca^{2+} (crystal radius 0.099 nm), and initiates depolarization.

Monovalent or divalent cations, but not anions, readily flow through the open form of the nAChR channel. What makes the channel cation selective? The amino acid sequences of the pore-forming helices and the adjacent loop components contain three rings of negatively charged residues. One of them is located within the transmembrane region of the pore, and the other two flank the cytosolic entrance to the pore. Apparently, the upper part of the channel, namely, the α-helical components, acts as a water pore, whereas the lower loop components contribute to the selectivity filter of the channel (Corringer et al. 2000). Anions, such as Cl^-, cannot enter the pore because they are repelled by the negatively charged rings. Studies on the permeability of a series of organic cations differing in size, such as alkylammonium ions, triaminoguanidinium, histidine, and choline, indicate that the narrowest part of the open pore has the dimension of less than 0.65 × 0.65 nm square.

At the level of a single channel, addition of ACh is followed by transient openings of the channel. The current *i* flowing through an open nAChR channel is 4 pA at a membrane potential *V* of −100 mV. Since one ampere (A) represents the flow of 6.24×10^{18} charges per second, 2.5×10^7 Na^+ ions per second flow through an open channel. The conductance *g* of a plasma membrane channel is the measure of the ease of flow of current between the extracellular space and the cytosol or vice versa and is equal to *i/(V-Er)*, where *Er* is the reversal potential at which there is no ionic net flux; *g* is expressed in units of siemens (the reciprocal of an ohm), *i* in amperes, and *V* in volts. *Er* equals 0 mV for non-selective cation channels; thus, a current of 4 pA at a potential of 100 mV corresponds to a conductance of 40 pS. Non-selective cation channels reveal single-channel conductances of 10–170 pS with about 10 pS for HCN channels, 15–30 pS for P2X receptors and Piezo channels, 20–40 pS for NMDA receptors and CNG channels, 25–50 pS for nACh receptors, and 10–170 pS for TRP channels.

Drugs

While certain compounds activate specific non-selective cation channels (e.g., acetylcholine activates nACh receptors, ATP activates P2X receptors, capsaicin activates TRPV1, menthol activates TRPM8, etc.), most non-selective cation channels, mainly TRP channels, are commonly inhibited by trivalent cations such as Gd^{3+} and La^{3+}, 2-aminoethoxydiphenyl borate (2-APB), the imidazole derivative SKF 96365, and the polycation ruthenium red. The nonsteroidal anti-rheumatic drugs flufenamic and niflumic acid also inhibit some TRP channels. However, few TRPs are activated by 2-APB or flufenamic acid. Drugs to specifically target single members of the TRP channel family are still rare. The tarantula spider toxin GsMTx4 specifically inhibits mechanosensitive Piezo channels. Acid-sensing ion channels belong to the group of degenerin/epithelial Na^+ channels (DEG/ENaC) and are sensitive to amiloride, P2X receptors are inhibited by suramin and PPADS (pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid), and HCN channels are blocked by the verapamil derivative ivabradine (Nilius and Flockerzi 2014). The ionotropic glutamate receptors of the NMDA type are specifically inhibited by AP5 (R-2-amino-5-phosphonopentanoate) and certain anesthetics such as ketamine.

Cross-References

► TRP Channels

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Non-specific Cation Channels

► Non-selective Cation Channels

Nonspecific ChE

► Cholinesterases

Nonviral Peptidases

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Synonyms

Proteases; Proteinases; Proteolytic enzymes

Definition

Proteolytic enzymes catalyze the breaking of the carbon-nitrogen bonds between amino acids that are found in peptides and proteins. Most proteolytic enzymes are hydrolases, and hydrolysis of a peptide bond is a form of an acid-base reaction, in which a hydrogen ion (proton) is transferred. The terms “protease,” “proteinase,” and “proteolytic enzyme” are synonymous but strictly speaking can only be applied to peptidases that hydrolase bonds in proteins. Because there are many

peptidases that act only on peptides, the term "peptidase" is recommended. Peptidases are included in subclass 3.4 of enzyme nomenclature (Barrett et al. 2004; Puente et al. 2003). There is a group of enzymes that break peptide bonds by a different mechanism: an asparagine residue is induced to cyclize to form a succinimide, which breaks the peptide bond and releases a peptide. These kinds of enzymes are known as asparagine peptide lyases (Rawlings et al. 2011).

Basic Characteristics

Active Site Residues

Peptidases are found in all forms of life. There are about 1252 peptidases in humans, plus an additional 349 proteins that are homologous to peptidases, but either have no or different catalytic activity. This represents 3% of all the genes in the human genome.

Hydrolysis of a peptide bond is an example of a nucleophilic attack. The nucleophile in the reaction is either an activated water molecule or part of the side chain of an amino acid, and peptidases are described as having either a water nucleophile or a protein nucleophile. Peptidases with a water nucleophile either utilize one or two metal ions as ligands for the water molecule, in which case the peptidase generally acts at neutral pH, or the water is bound by two aspartic acid residues, in which case the peptidase usually has an acidic pH optimum. For peptidases with a protein nucleophile, the nucleophile is either the hydroxyl group from a serine or threonine residue or the thiol group from cysteine. Thus there are five major catalytic types: aspartic, metallo-, serine, threonine, and cysteine peptidases. A sixth type, glutamic peptidases, is known, but the only known examples are from fungi and viruses. There are still several peptidases for which the catalytic type is as yet undetermined.

A metallopeptidase binds a divalent metal ion tetrahedrally, with three ligands being amino acid residues and the fourth the water molecule that becomes the nucleophile in the reaction. The amino acid ligand residues are conserved. Most commonly, a zinc ion is bound, but peptidases that bind cobalt, manganese, nickel, and copper also exist. Metal ligands are most often histidine residues but can also be aspartic and glutamic acids and more unusually asparagine, glutamine, or lysine. When a peptidase binds two metal ions, five residues are involved, one of which ligates both ions. The metal ion is described as co-catalytic. Besides metal ligands, other residues are often essential for catalytic activity. The peptidases that are involved in the turnover of extracellular matrices, known as matrixins or matrix metallopeptidases (MMPs), are examples of metallopeptidases containing the motif HEXXH. In this motif, the two histidines are metal ligands while the glutamic acid is an active site residue. Glutamate carboxypeptidase binds two zinc ions via two histidines, an aspartic acid and two glutamic acids, and in addition has two active site residues, an aspartic and a glutamic acid. Aspartic peptidases bind and activate water via two aspartic acid residues.

Peptidases with a protein nucleophile often have an active site containing two or three residues. A second residue, most often a histidine (though lysine and arginine can substitute), is required to interact with the nucleophile and become a proton acceptor or general base in the catalytic reaction. If a catalytic triad exists, which is often the case when a histidine residue is the general base, then an aspartic or glutamic acid, asparagine, or glutamine is thought to orientate the imidazolium ring of the histidine side chain. In the cysteine peptidase cathepsin B, the order of the catalytic residues is Cys, His, Asn. In the serine peptidase chymotrypsin, the order is His, Asp, Ser.

For many serine and cysteine peptidases, catalysis first involves formation of a complex known as an acyl intermediate. An essential residue is required to stabilize this intermediate by helping to form the oxyanion hole. In cathepsin B, a glutamine performs this role and sometimes a catalytic tetrad (Gln, Cys, His, Asn) is referred too. In chymotrypsin, a glycine is essential for stabilizing the oxyanion hole.

Threonine peptidases (and some cysteine and serine peptidases) have only one active site residue, which is the N-terminus of the mature protein. Such a peptidase is known as an N-terminal

nucleophile hydrolase or Ntn-hydrolase. The amino group of the N-terminal residue performs the role of the general base. The catalytic subunits of the proteasome are examples of Ntn-hydrolases.

The order and nature of the active site residues and metal ligands is conserved between homologous peptidases. All the peptidases in a family will have the same catalytic type, with only two exceptions where the nucleophile can be either a serine, threonine, or cysteine.

Classification and Nomenclature

Peptidases have been classified by the *MEROPS* system since 1993 (Rawlings and Barrett 1993), which has been available via the *MEROPS* database and website (http://www.ebi.ac.uk/merops) since 1996 (Rawlings et al. 2018). The classification is based on sequence and structural similarities. Because peptidases are often multidomain proteins, only the domain directly involved in catalysis, and which bears the active site residues, is used in comparisons. This domain is known as the peptidase unit. Peptidases with statistically significant peptidase unit sequence similarities are included in the same family. To date, 262 families of peptidase have been detected (plus an additional 10 families of asparagine peptide lyases). Examples from 86 of these families are known in humans. A family is named from a letter representing the catalytic type ("A" for aspartic, "G" for glutamic, "M" for metallo, "C" for cysteine, "S" for serine, "T" for threonine, "P" for mixed catalytic type, "N" for asparagine peptide lyase, and "U" for unknown catalytic type) plus a number. Examples of family names are shown in Table 1. There are 76 families of metallopeptidases (33 in human), 16 of aspartic peptidases (4 of which are found in human), 96 of cysteine peptidases (30 in human), 56 of serine peptidases (22 in human), 6 of threonine peptidases (3 in human), 3 of glutamic peptidases (none in human), 2 of mixed catalytic type (1 in human), and 9 families for which the catalytic type is unknown (none in human). It should be noted that within a family, not all of the members will be peptidases. Usually non-peptidase homologues are a minority and can be easily detected because not all of the active site residues are conserved.

All peptidases within a family will have a similar tertiary structure, and it is not uncommon for peptidases in one family to have a similar structure to peptidases in another family, even though there is no significant sequence similarity. Families of peptidases with similar structures and the same order of active site residues are included in the same clan. A clan name consists of two letters, the first representing the catalytic type as before and the second assigned sequentially. A clan may contain peptidases of more than one catalytic type. So far this has only been seen for peptidases with protein nucleophiles, and these clans are named with an initial "P." Only five such clans are known. Clan PA includes peptidases with a chymotrypsin-like fold, which besides serine peptidases such as chymotrypsin also includes some cysteine peptidases from viruses. Clan PB contains the Ntn-hydrolases, which can be threonine, cysteine, or serine in type. Clan PC includes the cysteine peptidase gamma-glutamyl hydrolase and the serine peptidase alpha-aspartyl dipeptidase. Clan PD contains the self-processing proteins, including the cysteine peptidase hedgehog, and three families of intein-containing asparagine peptide lyases. Clan PE contains ornithine acetyltransferase precursor, which is a self-processing threonine peptidase and DmpA aminopeptidase, a serine peptidase. There are 56 clans of peptidases, or in other words there are 56 independent origins of peptidases.

Peptidases that are thought to be species variants of the same protein and have similar properties are included in the same *MEROPS* identifier. The identifier consists of the three-character family name (padded with zeroes if necessary), a dot, and a unique three digit number. Examples of *MEROPS* identifiers are shown in Table 1. Two special kinds of identifier are used for non-peptidase homologues in the *MEROPS* database for proteins and pseudogenes. For proteins, the three digit number begins with a nine, and for pseudogenes the first digit is replaced by a "P." There are 4,354 different *MEROPS* identifiers.

With the onset of genomic biology, there are now many sequences derived from genome

Nonviral Peptidases, Table 1 Pathological conditions involving peptidases

Disease	Type	Peptidases (MEROPS ID)
15q13.3 microdeletion syndrome	g	Cezanne-2 peptidase (C64.002)
3MC syndrome 1	g	Mannan-binding lectin-associated serine peptidase-3 (S01.132); mannan-binding lectin-associated serine peptidase 1 (S01.198)
3-methylglutaconic aciduria 8	g	HtrA2 peptidase (S01.278)
46,XY sex reversal 7	g	Desert hedgehog protein (C46.004)
Acne inversa, familial, 3	g	Presenilin 1 (A22.001)
Acute coronary syndrome		PCSK9 peptidase (S08.039)
Adult respiratory distress syndrome		Elastase-2 (S01.131)
African trypanosomiasis	p	Rhodesain (C01.072)
Allergy		Matrix metallopeptidase-9 (M10.004)
Alzheimer's disease		BACE1 g.p. (*Homo sapiens*) (A01.004); cathepsin D (A01.009); cathepsin E (A01.010); BACE2 g.p. (*Homo sapiens*) (A01.041); stem bromelain (C01.005); cathepsin S (C01.034); cathepsin B (C01.060); calpain-1 (C02.001); legumain, animal-type (C13.004); angiotensin-converting enzyme peptidase unit 1 (M02.001); matrix metallopeptidase-13 (M10.013); ADAMTS4 peptidase (M12.221); neprilysin (M13.001); insulysin (M16.002); carboxypeptidase PM20D1 (M20.011); HtrA2 peptidase (S01.278); PCSK9 peptidase (S08.039)
Alzheimer's disease 3	g	Presenilin 1 (A22.001)
Alzheimer's disease 4	g	Presenilin 2 (A22.002)
Alzheimer's disease 18	g	ADAM10 peptidase (M12.210)
Amelogenesis imperfecta, hypomaturation type, 2A1	g	Kallikrein-related peptidase 4 (S01.251)
Amelogenesis imperfecta, hypomaturation type, 2A2	g	Matrix metallopeptidase-20 (M10.019)
Amebic dysentery	p	Histolysain (C01.050)
Amyotrophic lateral sclerosis		Glutamate carboxypeptidase II (M28.010)
Anadysplasia 1, metaphyseal	g	Matrix metallopeptidase-13 (M10.013)
Anadysplasia 2, metaphyseal	g	Matrix metallopeptidase-9 (M10.004)
Analgesia		Aminopeptidase N (M01.001)
Angioedema induced by ACE inhibitors	g	Aminopeptidase P2 (M24.005)
Angioedema, hereditary 3	g	Coagulation factor XIIa (S01.211)
Ankylosing spondylitis		Endoplasmic reticulum aminopeptidase 1 (M01.018); ERAP2 aminopeptidase (M01.024)
Antigen-induced bronchoconstriction		Tryptase alpha (S01.143)
Arthritis		Membrane-type matrix metallopeptidase-1 (M10.014)
Arthritis, rheumatoid		Matrix metallopeptidase-3 (M10.005); granzyme B (*Homo sapiens*-type) (S01.010); elastase-2 (S01.131); proteasome subunit beta1c (T01.010); proteasome subunit beta2c (T01.011); proteasome subunit beta5c (T01.012); proteasome subunit beta1i (T01.013); proteasome subunit beta2i (T01.014); proteasome subunit beta5i (T01.015)
Arthrogryposis, distal, 5D	g	Endothelin-converting enzyme-like 1 endopeptidase (M13.007)
Aspartylglucosaminuria	g	Glycosylasparaginase precursor (T02.001)
Aspergillosis	F	Peptidase F (A01.026)

(continued)

Nonviral Peptidases, Table 1 (continued)

Disease	Type	Peptidases (MEROPS ID)
Asthma		Peptidase 1 (mite) (C01.073); matrix metallopeptidase-12 (M10.009); ADAM33 peptidase (M12.244); tryptase alpha (S01.143)
Atherosclerosis		Matrix metallopeptidase-9 (M10.004)
Atrial septal defect 6	g	Vertebrate tolloid-like 1 protein (M12.016)
Autoimmune blistering disease		Granzyme B (*Homo sapiens*-type) (S01.010)
Autoimmune lymphoproliferative syndrome 2A	g	Caspase-10 (C14.011)
Autoinflammation with arthritis and dyskeratosis	g	Family S79 unassigned peptidases (S79.UPW)
Autoinflammation, panniculitis, and dermatosis syndrome	g	OTULIN peptidase (C101.001)
Autoinflammatory syndrome, familial, Behcet-like	g	A20 peptidase (C64.003)
Batten disease	g	Tripeptidyl-peptidase I (S53.003)
Behcet's disease		Endoplasmic reticulum aminopeptidase 1 (M01.018)
Birdshot chorioretinopathy		Endoplasmic reticulum aminopeptidase 1 (M01.018); ERAP2 aminopeptidase (M01.024)
Blue diaper syndrome	g	PCSK1 peptidase (S08.072)
Bone marrow failure syndrome 4	g	Histone H2A deubiquitinase MYSM1 (M67.005)
Botulism	b	Bontoxilysin (M27.002)
Brachydactyly A1	g	Indian hedgehog protein (C46.003)
Bronchiectasis		Elastase-2 (S01.131)
Brooke-Spiegler syndrome	g	CylD peptidase (C67.001)
Burn		Stem bromelain (C01.005); ananain (C01.026)
Cancer		Cathepsin D (A01.009); cathepsin B (C01.060); caspase-6 (C14.005); ubiquitin-specific peptidase 7 (C19.016); ubiquitin-specific peptidase 20 (C19.025); otubain-1 (C65.001); aminopeptidase N (M01.001); aminopeptidase A (M01.003); matrix metallopeptidase-2 (M10.003); membrane-type matrix metallopeptidase-1 (M10.014); endothelin-converting enzyme 1 (M13.002); glutamate carboxypeptidase (M20.001); methionyl aminopeptidase 2 (M24.002); YME1L1 g.p. (*Homo sapiens*) and similar (M41.026); pappalysin-1 (M43.004); carboxypeptidase G3 (M9E.007); elastase-2 (S01.131); furin (S08.071); fibroblast activation protein alpha subunit (S09.007); peptidase Clp (type 3) (S14.003); proteasome subunit beta1c (T01.010); proteasome subunit beta2c (T01.011); proteasome subunit beta5c (T01.012); proteasome subunit beta1i (T01.013); proteasome subunit beta2i (T01.014); proteasome subunit beta5i (T01.015); 20 S constitutive proteasome peptidase complex (eukaryote) (XT01.001)
Cancer, anal	v	Ubiquitin-specific peptidase 46 (C19.052)
Cancer, bladder		SENP2 peptidase (C48.007)
Cancer, bladder, invasive		Matriptase (S01.302)
Cancer, breast		Cathepsin D (A01.009); cathepsin E (A01.010); impas 1 peptidase (A22.003); stem bromelain (C01.005); cathepsin B (C01.060); ubiquitinyl hydrolase-L5 (C12.005); legumain, animal-type (C13.004); ubiquitin-specific peptidase 14 (C19.015); ubiquitin-specific peptidase 7 (C19.016); ubiquitin-specific peptidase 1 (C19.019); ubiquitin-specific peptidase 15 (C19.022);

(continued)

Nonviral Peptidases, Table 1 (continued)

Disease	Type	Peptidases (MEROPS ID)
		ubiquitin-specific peptidase 17 (C19.023); ubiquitin-specific peptidase 22 (C19.035); USP17-like peptidase (C19.078); SENP2 peptidase (C48.007); matrix metallopeptidase-2 (M10.003); membrane-type matrix metallopeptidase-1 (M10.014); leucine aminopeptidase 3 (M17.001); aspartyl aminopeptidase (M18.002); kallikrein-related peptidase 14 (S01.029); transmembrane peptidase, serine 4 (S01.034); cathepsin G (S01.133); hepsin (S01.224); urokinase-type plasminogen activator (S01.231); dipeptidyl-peptidase IV (eukaryote) (S09.003); rhomboid-like protein 5 (S54.008); iRhom1 (S54.952); rhomboid domain containing 2 (S54.955)
Cancer, breast, basal-like		Ubiquitin-specific peptidase 21 (C19.034)
Cancer, breast, estrogen receptor alpha-positive		Cathepsin O (C01.035)
Cancer, breast, HER2-positive		Xaa-Pro dipeptidase (eukaryote-type) (M24.007)
Cancer, breast, triple-negative		Cathepsin D (A01.009); cathepsin S (C01.034); ubiquitin-specific peptidase 2 (C19.013); matrix metallopeptidase-9 (M10.004); membrane-type matrix metallopeptidase-1 (M10.014)
Cancer, breast invasive ductal carcinoma		Ubiquitin-specific peptidase 28 (C19.054)
Cancer, cervical	v	Ubiquitin-specific peptidase 46 (C19.052)
Cancer, cervical		OTUD5 peptidase (*Homo sapiens*) (C85.001); membrane-type matrix metallopeptidase-1 (M10.014); kallikrein-related peptidase 8 (S01.244)
Cancer, colon		Cathepsin D (A01.009); caspase-3 (C14.003); ubiquitin-specific peptidase 10 (C19.018); ubiquitin-specific endopeptidase 39 [misleading] (C19.972)
Cancer, colon		Membrane-type matrix metallopeptidase-1 (M10.014); neprilysin (M13.001)
Cancer, colorectal		Stem bromelain (C01.005); dipeptidyl-peptidase I (C01.070); caspase-8 (C14.009); ubiquitin-specific peptidase 5 (C19.001); ubiquitin-specific peptidase 22 (C19.035); ubiquitin-specific peptidase 47 (C19.055); ubiquitin-specific endopeptidase 39 [misleading] (C19.972); autophagin-1 (C54.003); aminopeptidase A (M01.003); matrix metallopeptidase-7 (M10.008); neprilysin (M13.001); carboxypeptidase M (M14.006)
Cancer, colorectal, gut microbiome-associated	b	Colibactin peptidase (*Escherichia*-type) (S12.010)
Cancer, epithelial defense against		ADAMDEC1 peptidase (M12.219)
Cancer, epithelial tumor		Fibroblast activation protein alpha subunit (S09.007)
Cancer, esophageal		Caspase-8 (C14.009), dipeptidyl-peptidase IV (eukaryote) (S09.003)
Cancer, esophageal		
Cancer, gastric		Cathepsin F (C01.018); caspase-8 (C14.009); ubiquitin-specific peptidase 7 (C19.016); ubiquitin-specific peptidase 3 (C19.026); ubiquitin-specific endopeptidase 39 [misleading] (C19.972); membrane-type matrix metallopeptidase-1 (M10.014); umbilical vein peptidase (S01.309); proteasome subunit beta5c (T01.012)
Cancer, gastric	g	Caspase-10 (C14.011)
Cancer, gastric	v	Epstein-Barr virus-type assemblin (S21.003)
Cancer, head and neck		Taspase-1 (T02.004)
Cancer, kidney clear cell renal cell carcinoma	g	Ubiquitinyl hydrolase-BAP1 (C12.004)

(continued)

Nonviral Peptidases, Table 1 (continued)

Disease	Type	Peptidases (MEROPS ID)
Cancer, liver	g	Spartan peptidase (M100.001)
Cancer, liver hepatocellular carcinoma		Ubiquitin-specific peptidase 27X (C19.075); aminopeptidase N (M01.001); dipeptidyl-peptidase IV (eukaryote) (S09.003)
Cancer, lung		Impas 1 peptidase (A22.003); caspase-3 (C14.003); ubiquitin-specific peptidase 22 (C19.035); ubiquitin-specific peptidase 24 (C19.047); ubiquitin-specific endopeptidase 39 [misleading] (C19.972); matrix metallopeptidase-12 (M10.009); membrane-type matrix metallopeptidase-1 (M10.014); HtrA3 peptidase (S01.284); proteasome subunit alpha 6 (T01.971)
Cancer, lung adenocarcinoma		Ubiquitin-specific peptidase 22 (C19.035)
Cancer, lung adenocarcinoma, primary		Napsin A (A01.046)
Cancer, lung, non-small cell		Presenilin 1 (A22.001); ubiquitin-specific peptidase 14 (C19.015); ubiquitin-specific peptidase 9X (C19.017); ubiquitin-specific peptidase 10 (C19.018); ubiquitin-specific peptidase 28 (C19.054); ubiquitin-specific peptidase 44 (C19.057); ubiquitin-specific peptidase 49 (C19.073); USP17-like peptidase (C19.078)
Cancer, nasopharyngeal carcinoma	v	Epstein-Barr virus-type assemblin (S21.003)
Cancer, oral		Cathepsin S (C01.034)
Cancer, oropharyngeal	v	Ubiquitin-specific peptidase 46 (C19.052)
Cancer, ovarian		Caspase-9 (C14.010); ubiquitin-specific peptidase 11 (C19.014); ubiquitin-specific peptidase 15 (C19.022); ubiquitin-specific endopeptidase 39 [misleading] (C19.972); membrane-type matrix metallopeptidase-1 (M10.014); methionyl aminopeptidase 2 (M24.002); StcE peptidase (M66.001); testisin (S01.011); kallikrein-related peptidase 5 (S01.017); kallikrein-related peptidase 6 (S01.236); kallikrein-related peptidase 8 (S01.244); kallikrein-related peptidase 4 (S01.251); kallikrein-related peptidase 7 (S01.300)
Cancer, ovarian, epithelial		Matrix metallopeptidase-3 (M10.005)
Cancer, pancreatic		Ubiquitin-specific peptidase 5 (C19.001); ubiquitin-specific peptidase 49 (C19.073); ubiquitin-specific endopeptidase 39 [misleading] (C19.972); desumoylating isopeptidase 2 (C97.002); kallikrein-related peptidase 7 (S01.300)
Cancer, pancreatic neuroendocrine tumor		Ubiquitinyl hydrolase-L1 (C12.001)
Cancer, prostate		Calpain-1 (C02.001); calpain-2 (C02.002); ubiquitinyl hydrolase-L5 (C12.005); caspase-3 (C14.003); ubiquitin-specific peptidase 14 (C19.015); ubiquitin-specific peptidase 9X (C19.017); ubiquitin-specific peptidase 25 (C19.041); USP17-like peptidase (C19.078); matrix metallopeptidase-9 (M10.004); matrix metallopeptidase-3 (M10.005); membrane-type matrix metallopeptidase-3 (M10.016); methionyl aminopeptidase 2 (M24.002); glutamate carboxypeptidase II (M28.010); STAM-binding protein-like 1 (M67.003); kallikrein-related peptidase 14 (S01.029); transmembrane peptidase, serine 4 (S01.034); kallikrein-related peptidase 15 (S01.081); kallikrein-related peptidase 3 (S01.162); hepsin (S01.224); urokinase-type plasminogen activator (S01.231); epitheliasin (S01.247); kallikrein-related peptidase 4 (S01.251); matriptase (S01.302); PCSK6 peptidase (S08.075)

(continued)

Nonviral Peptidases, Table 1 (continued)

Disease	Type	Peptidases (MEROPS ID)
Cancer, skin multiple self-healing palmoplantar carcinoma	g	Family S79 unassigned peptidases (S79.UPW)
Cancer, skin, uveal melanoma	g	Ubiquitinyl hydrolase-BAP1 (C12.004)
Cancer, thyroid, non-medullary, 5	g	Factor VII-activating peptidase (S01.033)
Candidiasis	f	Canditropsin (A01.037)
Cardiac disease		Chymase (*Homo sapiens*-type) (S01.140)
Cardiomyopathy, dilated 1U	g	Presenilin 1 (A22.001)
Cardiomyopathy, dilated 1V	g	Presenilin 2 (A22.002)
Cardiorenal disease		Neprilysin (M13.001)
Cardiovascular disease		Membrane-type matrix metallopeptidase-1 (M10.014); tryptase alpha (S01.143); subtilisin NAT (S08.044); dipeptidyl-peptidase IV (eukaryote) (S09.003); serine carboxypeptidase A (S10.002)
Caspase-8 deficiency	g	Caspase-8 (C14.009)
Cataract		Calpain-2 (C02.002)
Cavitary optic disc anomalies	g	Matrix metallopeptidase-19 (M10.021)
cerebral arteriopathy, autosomal recessive, with subcortical infarcts and leukoencephalopathy	g	HtrA1 peptidase (*Homo sapiens*-type) (S01.277)
Cerebral arteriopathy, autosomal dominant, with subcortical infarcts and leukoencephalopathy, 2	g	HtrA1 peptidase (*Homo sapiens*-type) (S01.277)
Cerebral small vessel disease	g	HtrA1 peptidase (*Homo sapiens*-type) (S01.277)
Ceroid lipofuscinosis, neuronal, 2	g	Tripeptidyl-peptidase I (S53.003)
Chagas disease	p	Cruzipain (C01.075); aminopeptidase AMZ1 (M54.003); oligopeptidase B (S09.010)
Charcot-Marie-Tooth disease 2T	g	Neprilysin (M13.001)
Childhood-onset neurodegenerative disorder	g	Cathepsin D (A01.009)
Cholestasis		Aminopeptidase N (M01.001)
Cholesteryl ester storage disease	g	PLN02872 protein (*Homo sapiens*-type) (S33.017)
Chronic obstructive pulmonary disease		Matrix metallopeptidase-12 (M10.009); elastase-2 (S01.131); cathepsin G (S01.133); myeloblastin (S01.134)
CODAS syndrome	g	PIM1 peptidase (S16.002)
Colorectal neoplastic disease		Presenilin 1 (A22.001)
Combined oxidative phosphorylation deficiency 31	g	Mitochondrial intermediate peptidase (M03.006)
Complement component 2 deficiency	g	Complement component C2a (S01.194)
Complement component C1s deficiency	g	Complement component activated C1s (S01.193)
Complement factor B deficiency	g	Complement factor Bb (S01.196)
Complement factor D deficiency	g	Complement factor D (S01.191)
Complement factor I deficiency	g	Complement factor I (S01.199)
Cone-rod dystrophy 9	g	ADAM9 peptidase (M12.209)
Congenital bilateral aplasia of the vas Deferens, X-linked	g	Gpr64 (*Mus musculus*)-type protein (P02.007)
Coronary heart disease		PCSK9 peptidase (S08.039)
Coronary heart disease 6	g	Matrix metallopeptidase-3 (M10.005)
Corticobasal syndrome	g	Presenilin 1 (A22.001)
Cushing disease	g	Ubiquitin-specific peptidase 8 (C19.011)
Cyclic hematopoiesis	g	Elastase-2 (S01.131)
Cylindromatosis, familial	g	CylD peptidase (C67.001)

(continued)

Nonviral Peptidases, Table 1 (continued)

Disease	Type	Peptidases (MEROPS ID)
Cystic fibrosis		Cathepsin S (C01.034); elastase-2 (S01.131)
Deafness, autosomal recessive, 8	g	Transmembrane peptidase, serine 3 (S01.079)
Demyelinating disease		ADAM10 peptidase (M12.210)
Dermatitis, atopic		Kallikrein-related peptidase 7 (S01.300)
DiGeorge syndrome	g	HtrA2 peptidase (S01.278)
Diabetes mellitus, non-insulin-dependent, 1	g	Calpain-10 (C02.018)
Diabetes, type 2		BACE2 g.p. (*Homo sapiens*) (A01.041); neprilysin (M13.001); dipeptidyl-peptidase IV (eukaryote) (S09.003)
Diabetic ulcer		Granzyme B (*Homo sapiens*-type) (S01.010)
Diffuse panbronchiolitis	b	Pseudolysin (M04.005)
Dysplasia, acrocapitofemoral	g	Indian hedgehog protein (C46.003)
Dysplasia, Beukes familial hip	g	UfSP2 peptidase (C78.002)
Dysplasia, mandibuloacral with type B lipodystrophy	g	Farnesylated protein-converting enzyme 1 (M48.003)
Dysplasia, metaphyseal, Spahr-type	g	Matrix metallopeptidase-13 (M10.013)
Dysplasia, spondyloepimetaphyseal, Di Rocco-type	g	UfSP2 peptidase (C78.002)
Dysplasia, spondyloepimetaphyseal, Missouri-type	g	Matrix metallopeptidase-13 (M10.013)
Early-onset mitochondrial syndrome	g	HtrA2 peptidase (S01.278)
East Coast fever	p	Papain homolog (*Theileria*-type) (C01.079)
Ehlers-Danlos syndrome, dermatosparaxis-type	g	Procollagen I N-peptidase (M12.301)
Ehlers-Danlos syndrome, periodontal type, 1	g	Complement component activated C1r (S01.192)
Ehlers-Danlos syndrome, periodontal-type, 2	g	Complement component activated C1s (S01.193)
Emphysema		Cathepsin K (C01.036); matrix metallopeptidase-12 (M10.009); elastase-2 (S01.131)
Encephalitis/encephalopathy, mild, with reversible myelin vacuolization	g	Myelin regulatory factor (S74.003)
Endophthalmitis	b	Lysostaphin (M23.004)
Endotoxic shock		Caspase-1 (C14.001)
Enterocolitis	b	DegP peptidase (S01.273)
Enterokinase deficiency	g	Enteropeptidase (S01.156)
Epilepsy		Kallikrein-related peptidase 8 (S01.244)
Epileptic encephalopathy, early infantile, 36	g	ALG13 g.p. (*Homo sapiens*) (C85.005)
Epileptogenesis		Matrix metallopeptidase-9 (M10.004)
Factor II deficiency	g	Thrombin (S01.217)
Factor VII deficiency	g	Coagulation factor VIIa (S01.215)
Factor X deficiency	g	Coagulation factor Xa (S01.216)
Factor XI deficiency	g	Coagulation factor XIa (S01.213)
Factor XII deficiency	g	Coagulation factor XIIa (S01.211)
Familial hypercholanemia	g	Acyl-coenzyme A amino acid N-acyltransferase 2 (S09.A50)
Familial juvenile hyperuricemic Nephropathy 2	g	Renin (A01.007)
Farber lipogranulomatosis	g	Acid ceramidase precursor (C89.001)
Fibrosis		Procollagen C-peptidase (M12.005); fibroblast activation protein alpha subunit (S09.007)
Frontotemporal dementia	g	Presenilin 1 (A22.001)
Galactosialidosis	g	Serine carboxypeptidase A (S10.002)
Gastric ulcer		Pepsin A (A01.001); gastricsin (A01.003)

(continued)

Nonviral Peptidases, Table 1 (continued)

Disease	Type	Peptidases (MEROPS ID)
Gastroenteritis	b	DegP peptidase (S01.273); EspP peptidase (*Escherichia coli*) (S06.002); Tsh peptidase (S06.003); Sat peptidase (S06.004); Pic peptidase (S06.005)
Giardiasis	p	Giardain (C01.094)
Glioma		Matrix metallopeptidase-9 (M10.004)
Glutathionuria	g	Gamma-glutamyltransferase 1 (mammalian-type) (T03.006)
Graft-versus-host disease		Granzyme B (*Homo sapiens*-type) (S01.010)
Haim-Munk syndrome	g	Dipeptidyl-peptidase I (C01.070)
Hairy leukoplakia	v	Epstein-Barr virus-type assemblin (S21.003)
Heart failure		Corin (S01.019)
Hemolytic uremic syndrome atypical 3	g	Complement factor I (S01.199)
Hemolytic uremic syndrome atypical 4	g	Complement factor Bb (S01.196)
Hemophagocytic lymphohistiocytosis	v	Epstein-Barr virus-type assemblin (S21.003)
Hemophilia B	g	Coagulation factor IXa (S01.214)
Hennekam lymphangiectasia-lymphedema syndrome 3	g	ADAMTS3 peptidase (M12.220)
Herniated intervertebral disk		Chymopapain (C01.002)
Herniated intervertebral disk		Collagenase G/A (*Clostridium histolyticum*) (M09.002)
Herpes	v	Ubiquitin-specific peptidase 7 (C19.016); assemblin (S21.001)
Heterotaxy, visceral, 7, autosomal	g	Matrix metallopeptidase-21 (M10.026)
Hirschsprung disease, cardiac defects, and autonomic dysfunction	g	Endothelin-converting enzyme 1 (M13.002)
Holoprosencephaly 3	g	Sonic hedgehog protein (C46.002)
Huntington's disease		Cathepsin E (A01.010)
Hypercholesterolemia, autosomal dominant, 3	g	PCSK9 peptidase (S08.039)
Hyperenzymemia		Bikunin (LI02.001)
Hypertension		Renin (A01.007); aminopeptidase A (M01.003); angiotensin-converting enzyme (XM02.001); neprilysin (M13.001); endothelin-converting enzyme 1 (M13.002); corin (S01.019)
Hypertrophic scarring		Granzyme B (*Homo sapiens*-type) (S01.010)
Hypophosphatemic rickets, X-linked dominant	g	PHEX peptidase (M13.091)
Hypoplasia or aplasia of tibia with polydactyly	g	Sonic hedgehog protein (C46.002)
Ichthyosis, congenital, autosomal recessive 11	g	Matriptase (S01.302)
Ichthyosis, congenital, autosomal recessive 12	g	Caspase-14 (C14.018)
IFAP syndrome with or without BRESHECK syndrome	g	Site 2 peptidase (M50.001)
Immunodeficiency 12	g	Paracaspase (C14.026)
Immunodeficiency 24	g	ctps g.p. (*Brachydanio rerio*) (C26.A36)
Infectious mononucleosis	v	Epstein-Barr virus-type assemblin (S21.003)
Inflammation		Leukotriene A4 hydrolase (M01.004); fibroblast activation protein alpha subunit (S09.007)
Inflammatory bowel disease		Leukotriene A4 hydrolase (M01.004)
Inflammatory skin and bowel disease, neonatal, 1	g	ADAM17 peptidase (M12.217)
Influenza	v	Granzyme B (*Homo sapiens*-type) (S01.010)
Intellectual disability syndrome	g	OTUD6B peptidase (*Homo sapiens*) (C85.009)
Intervertebral disc disease	g	Matrix metallopeptidase-9 (M10.004)

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Nonviral Peptidases, Table 1 (continued)

Disease	Type	Peptidases (MEROPS ID)
Intimal hyperplasia		Chymase (*Homo sapiens*-type) (S01.140)
Intracerebral hemorrhage	g	Angiotensin-converting enzyme (XM02.001)
IPEX-like syndrome	g	Paracaspase (C14.026)
Iron-refractory iron deficiency anemia	g	Matriptase-2 (S01.308)
Ischemia, cardiac		Cathepsin D (A01.009); PIM1 peptidase (S16.002)
Ischemia, cerebral		Matrix metallopeptidase-12 (M10.009); 20 S constitutive proteasome peptidase complex (eukaryote) (XT01.001)
Ischemia, myocardial		SENP3 peptidase (C48.003); lumbrokinase (S01.243)
Ischemic heart disease		Dipeptidyl-peptidase IV (eukaryote) (S09.003)
Keratitis	b	Arginyl peptidase (S01.281)
Keratolytic winter erythema	g	Cathepsin B (C01.060)
Keratosis follicularis spinulosa decalvans X-linked	g	Site 2 peptidase (M50.001)
Kidney disease		Angiotensin-converting enzyme-2 (M02.006)
KSHV inflammatory cytokine syndrome	v	Herpesvirus 8-type assemblin (S21.006)
Late-infantile Batten disease	g	Tripeptidyl-peptidase I (S53.003)
Laurin-Sandrow syndrome	g	Sonic hedgehog protein (C46.002)
Leishmaniasis	p	CPA peptidase (C01.076); leishmanolysin (M08.001)
Leishmaniasis, cutaneous	p	Granzyme B (*Homo sapiens*-type) (S01.010)
Lethal congenital contracture syndrome 9	g	GPR126 vascular inducible G protein-coupled receptor (P02.017)
Lethal tight skin contracture syndrome	g	Farnesylated protein-converting enzyme 1 (M48.003)
Leukemia		Matrix metallopeptidase-7 (M10.008)
Leukemia, acute myeloid		Transmembrane peptidase, serine 11F (S01.321)
Leukemia, adult T-cell	v	Retropepsin (human T-cell leukemia virus) (A02.012)
Leukemia, chronic lymphocytic		SENP2 peptidase (C48.007); 20 S constitutive proteasome peptidase complex (eukaryote) (XT01.001)
Lewy body disease		Endothelin-converting enzyme 1 (M13.002); endothelin-converting enzyme 2 (M13.003)
Liver cirrhosis	g	Matrix metallopeptidase-2 (M10.003); matrix metallopeptidase-9 (M10.004)
Lymphoma, Burkitt	v	Epstein-Barr virus-type assemblin (S21.003)
Lymphoma, familial non-Hodgkin	g	Caspase-10 (C14.011)
Lymphoma, Hodgkin's	v	Epstein-Barr virus-type assemblin (S21.003)
Lymphoma, primary effusion	v	Herpesvirus 8-type assemblin (S21.006)
Machado-Joseph disease		Calpain-1 (C02.001); ataxin-3 (C86.001)
Macular degeneration, age-related, 7	g	HtrA1 peptidase (*Homo sapiens*-type) (S01.277)
Macular degeneration, age-related, 13	g	Complement factor I (S01.199)
Macular degeneration, age-related, 14	g	Complement component C2a (S01.194); complement factor Bb (S01.196)
Malaria	p	Plasmepsin-1 (A01.022); plasmepsin-2 (A01.023); plasmepsin-5 (*Plasmodium* sp.) (A01.075); falcipain-2 (C01.046); falcipain-1 (C01.077)
MASP2 deficiency	g	Mannan-binding lectin-associated serine peptidase 2 (S01.229)
Medullary cystic kidney disease 1	g	MUC1 self-cleaving mucin (S71.001)
Meningitis	b	IgA1-specific serine peptidase (*Neisseria*-type) (S06.001)
Meningitis, aseptic	v	Coxsackievirus-type picornain 3C (C03.011)
Meningitis, neonatal	b	C5a peptidase (S08.020)

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Nonviral Peptidases, Table 1 (continued)

Disease	Type	Peptidases (MEROPS ID)
Meningitis, pneumococcal		Caspase-3 (C14.003)
Mental retardation, autosomal recessive 1	g	Neurotrypsin (S01.237)
Mental retardation, X-linked 99	g	Ubiquitin-specific peptidase 9X (C19.017)
Mesothelioma		Dipeptidyl-peptidase IV (eukaryote) (S09.003)
Mesothelioma, malignant	g	Ubiquitinyl hydrolase-BAP1 (C12.004)
Microcephaly-capillary malformation syndrome	g	STAMBP isopeptidase (M67.006)
Microcornea, myopic chorioretinal atrophy, and telecanthus	g	ADAMTS18 peptidase (M12.028)
Microphthalmia, isolated, with coloboma, 5	g	Sonic hedgehog protein (C46.002)
Microvascular complications of diabetes 3	g	Angiotensin-converting enzyme (XM02.001)
Multicentric osteolysis, nodulosis, and arthropathy	g	Matrix metallopeptidase-2 (M10.003)
Multiple familial trichoepithelioma 1	g	CylD peptidase (C67.001)
Multiple mitochondrial dysfunctions syndrome 6	g	Mitochondrial processing peptidase beta-subunit (M16.003)
Multiple myeloma		20 S constitutive proteasome peptidase complex (eukaryote) (XT01.001)
Multiple sclerosis		Matrix metallopeptidase-12 (M10.009); myelin basic protein autolytic or peptidase activity (S9G.105)
Muscular dystrophy, limb-girdle, autosomal dominant 4	g	Calpain-3 (C02.004)
Muscular dystrophy, limb-girdle, autosomal recessive 1	g	Calpain-3 (C02.004)
Muscular dystrophy-dystroglycanopathy Congenital with brain and eye anomalies A9	g	Dystroglycan (S72.001)
Muscular dystrophy-dystroglycanopathy limb-girdle C9	g	Dystroglycan (S72.001)
Musculoskeletal syndrome		Matrix metallopeptidase-13 (M10.013)
Myasthenia gravis		Cathepsin V (C01.009)
Myocardial infarction		Corin (S01.019); 20 S constitutive proteasome peptidase complex (eukaryote) (XT01.001)
Myocarditis	v	Cardiovirus picornain 3C (C03.009)
Myopathy, myofibrillar, 7	g	Kyphoscoliosis peptidase (C110.001)
Necrotizing enterocolitis	b	Aeruginolysin (M10.056)
Nelson's syndrome	g	Ubiquitin-specific peptidase 8 (C19.011)
Neonatal sepsis	b	C5a peptidase (S08.020)
Nephronophthisis-like nephropathy 1	g	Aminopeptidase P3 (M24.026)
Netherton syndrome	g	Kallikrein-related peptidase 5 (S01.017); kallikrein-related peptidase 6 (S01.236)
Netherton/Omenn-like syndrome	g	Paracaspase (C14.026)
Neutropenia, severe congenital 1, autosomal dominant	g	Elastase-2 (S01.131)
Nonalcoholic fatty liver disease		Ubiquitin-specific peptidase 4 (C19.010)
Obesity		Cathepsin B (C01.060)
Obstructive sleep apnea/hypopnea syndrome with ischemic stroke	g	Calpain-10 (C02.018)
Olmsted syndrome, X-linked	g	Site 2 peptidase (M50.001)
Optic atrophy 11	g	YME1L1 g.p. (*Homo sapiens*) and similar (M41.026)

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Nonviral Peptidases, Table 1 (continued)

Disease	Type	Peptidases (MEROPS ID)
Osteoarthritis		Stem bromelain (C01.005); matrix metallopeptidase-13 (M10.013); membrane-type matrix metallopeptidase-1 (M10.014); ADAM17 peptidase (M12.217); ADAMTS4 peptidase (M12.221); ADAMTS5 peptidase (M12.225)
Osteogenesis imperfecta 13	g	Procollagen C-peptidase (M12.005)
Osteogenesis imperfecta 19	g	Site 2 peptidase (M50.001)
Osteoporosis		Cathepsin K (C01.036)
Pancreatitis, acute		Cathepsin B (C01.060); trypsin-2 type A (S01.258)
Pancreatitis, hereditary	g	Cationic trypsin (*Homo sapiens*-type) (S01.127); chymotrypsin C (S01.157)
Papillon-Lefevre syndrome	g	Dipeptidyl-peptidase I (C01.070)
Parkinson's disease		Cathepsin E (A01.010); presenilin 1 (A22.001); ubiquitin-specific peptidase 30 (C19.060); HtrA2 peptidase (S01.278)
Parkinson's disease 5	g	Ubiquitinyl hydrolase-L1 (C12.001)
Parkinson's disease 7	g	DJ-1 putative peptidase (C56.002)
Parkinson's disease 13	g	HtrA2 peptidase (S01.278)
Partial gonadal dysgenesis with minifascicular neuropathy 46,XY	g	Desert hedgehog protein (C46.004)
Pathological tissue damage		Matrix metallopeptidase-1 (M10.001); matrix metallopeptidase-8 (M10.002); matrix metallopeptidase-2 (M10.003); matrix metallopeptidase-9 (M10.004); matrix metallopeptidase-3 (M10.005); matrix metallopeptidase-7 (M10.008); matrix metallopeptidase-12 (M10.009)
Periodontitis	b	Gingipain K (C25.002)
Periodontitis, aggressive, 1	g	Dipeptidyl-peptidase I (C01.070)
Peripheral artery disease		Dipeptidyl-peptidase IV (eukaryote) (S09.003)
Perrault syndrome 3	g	Peptidase Clp (type 3) (S14.003)
Pharyngitis	b	DegP peptidase (S01.273)
Pharyngoconjunctival fever	v	Adenain (C05.001)
Pick disease of the brain	g	Presenilin 1 (A22.001)
Plasminogen deficiency	g	Plasmin (S01.233)
Pneumococcal pneumonia	b	Arginyl peptidase (S01.281)
Pneumonia	b	Aeruginolysin (M10.056)
Pneumonia, neonatal	b	C5a peptidase (S08.020)
Polycystic kidney disease 1 with or without polycystic liver disease	g	Polycystin-1 (P02.036)
Polymicrogyria, bilateral frontoparietal	g	GPR56 (*Homo sapiens*)-type protein (P02.008)
Polymicrogyria, bilateral perisylvian, autosomal recessive	g	GPR56 (*Homo sapiens*)-type protein (P02.008)
Polyneuropathy, hearing loss, ataxia, retinitis pigmentosa, and cataract	g	Mername-AA196 putative peptidase (S09.054)
Preaxial polydactyly 2	g	Sonic hedgehog protein (C46.002)
Preeclampsia/eclampsia 5	g	Corin (S01.019)
Pregnancy loss, recurrent, 2	g	Thrombin (S01.217)
Premature aging	g	Spartan peptidase (M100.001)
Progressive supranuclear palsy		Cathepsin S (C01.034)
Prolidase deficiency	g	Xaa-Pro dipeptidase (eukaryote-type) (M24.007)
Proprotein convertase 1 deficiency	g	PCSK1 peptidase (S08.072)

(continued)

Nonviral Peptidases, Table 1 (continued)

Disease	Type	Peptidases (MEROPS ID)
Proteasome-associated autoinflammatory syndrome 1	g	Proteasome subunit beta5i (T01.015)
Proteasome-associated autoinflammatory syndrome 3	g	Proteasome subunit beta1i (T01.013); proteasome subunit beta 4 (T01.987)
Pseudo-TORCH syndrome 2	g	Ubiquitin-specific peptidase 18 (C19.030)
Psoriasis		Leukotriene A4 hydrolase (M01.004); endoplasmic reticulum aminopeptidase 1 (M01.018); ERAP2 aminopeptidase (M01.024); proteasome subunit beta1c (T01.010); proteasome subunit beta2c (T01.011); proteasome subunit beta5c (T01.012); proteasome subunit beta1i (T01.013); proteasome subunit beta2i (T01.014); proteasome subunit beta5i (T01.015)
Pycnodysostosis	g	Cathepsin K (C01.036)
Quebec platelet disorder	g	Urokinase-type plasminogen activator (S01.231)
Renal tubular dysgenesis	g	Renin (A01.007); angiotensin-converting enzyme peptidase (XM02.001)
Respiratory diseases	v	Coronavirus picornain 3C-like peptidase-1 (C30.001)
Restenosis		Matrix metallopeptidase-9 (M10.004)
Reticulate acropigmentation of Kitamura	g	ADAM10 peptidase (M12.210)
Retinitis pigmentosa 75	g	Cytosolic carboxypeptidase 5 (M14.025)
Ruijs-Aalfs syndrome	g	Spartan peptidase (M100.001)
Sarcoma, Ewing		Pappalysin-1 (M43.004)
Sarcoma, Kaposi's	v	Herpesvirus 8-type assemblin (S21.006)
Sarcoma, osteo		SENP2 peptidase (C48.007)
Schizophrenia		Human endogenous retrovirus K retropepsin (A02.011)
Sepsis	b	Dipeptidyl-peptidase I (C01.070); vibriolysin (M04.003); Aeruginolysin (M10.056); protein C (activated) (S01.218)
Severe fever with thrombocytopenia syndrome	v	Site 1 peptidase (S08.063)
Sjogren's syndrome		Kallikrein 13 (*Mus musculus*) (S01.173)
Solitary median maxillary central incisor	g	Sonic hedgehog protein (C46.002)
Soman poisoning		Xaa-Pro dipeptidase (eukaryote-type) (M24.007)
Spastic ataxia 5, autosomal recessive	g	Afg3-like protein 2 (M41.007)
Spastic paraplegia 7, autosomal recessive	g	Paraplegin (M41.006)
Spastic paraplegia 76, autosomal recessive	g	Calpain-1 (C02.001)
Spastic paraplegia 79, autosomal recessive	g	Ubiquitinyl hydrolase-L1 (C12.001)
Spermatogenic failure Y-linked 2	g	Ubiquitin-specific peptidase 9Y (C19.028)
Spinal muscular atrophy with progressive myoclonic epilepsy	g	Acid ceramidase precursor (C89.001)
Spinocerebellar ataxia 3	g	Ataxin-3 (C86.001)
Spinocerebellar ataxia 28	g	Afg3-like protein 2 (M41.007)
Spinocerebellar ataxia 43	g	Neprilysin (M13.001)
Spinocerebellar ataxia, autosomal recessive, 2	g	Mitochondrial processing peptidase non-peptidase alpha subunit (M16.971); mitochondrial processing peptidase subunit alpha unit 2 (M16.985)
Spinocerebellar ataxia, autosomal recessive, 7	g	Tripeptidyl-peptidase I (S53.003)
Spontaneous preterm delivery		Cystinyl aminopeptidase (M01.011)
Staphylococcal scalded skin syndrome	b	Exfoliatin A (S01.270)
Strabismus		Bontoxilysin (M27.002)
Streptococcal pharyngitis	b	Streptopain (C10.001)

(continued)

Nonviral Peptidases, Table 1 (continued)

Disease	Type	Peptidases (MEROPS ID)
Stroke		Calpain-1 (C02.001); calpain-2 (C02.002); corin (S01.019)
Stroke, ischemic	g	Angiotensin-converting enzyme (XM02.001); thrombin (S01.217)
Systemic sclerosis		Matrix metallopeptidase-1 (M10.001)
Tetanus	b	Tentoxilysin (M27.001)
Theileriosis	p	Papain homolog (*Theileria*-type) (C01.079)
Thrombophilia due to protein C deficiency, autosomal dominant	g	Protein C (activated) (S01.218)
Thrombophilia due to protein C deficiency, autosomal recessive	g	Protein C (activated) (S01.218)
Thrombophilia due to thrombin defect	g	Thrombin (S01.217)
Thrombophilia, X-linked, due to factor IX defect	g	Coagulation factor IXa (S01.214)
Thrombosis		Atroxase (M12.147); coagulation factor Xa (S01.216); thrombin (S01.217); t-plasminogen activator (S01.232)
Thrombotic thrombocytopenic purpura congenital	g	ADAMTS13 peptidase (M12.241)
Tick-borne encephalitis	v	Flavivirin (S07.001)
Triphalangeal thumb-polysyndactyly syndrome	g	Sonic hedgehog protein (C46.002)
Tumor predisposition syndrome	g	Ubiquitinyl hydrolase-BAP1 (C12.004)
Tylosis with esophageal cancer	g	iRhom2 (S54.953)
Type 2A von Willebrand disease	g	ADAMTS13 peptidase (M12.241)
Ulcerative colitis		Matrix metallopeptidase-9 (M10.004)
Vaginal candidiasis	f	Candidapepsin SAP1 (A01.014); candidapepsin SAP2 (A01.060)
Vibratory urticaria	g	EGF-like module containing mucin-like hormone receptor-like 2 (P02.001)
Vitiligo-associated multiple autoimmune disease 1	g	Family S79 unassigned peptidases (S79.UPW)
Vitreoretinopathy, neovascular inflammatory	g	Calpain-5 (C02.011)
Wegener's granulomatosis	a	Myeloblastin (S01.134)
Weill-Marchesani syndrome 1	g	ADAMTS10 peptidase (M12.235)
Weill-Marchesani syndrome 4	g	ADAMTS17 peptidase (M12.027)
Winchester syndrome	g	Membrane-type matrix metallopeptidase-1 (M10.014)
Wolman disease	g	PLN02872 protein (*Homo sapiens*-type) (S33.017)

Viral diseases and genetic abnormalities involving only non-peptidase homologues are omitted. The following abbreviations are used: *a* autoimmune disease, *b* bacterial infection, *f* fungal infection, *g* genetic abnormality, *p* parasite infection, *v* viral infection. Links are provided to the relevant summary pages in the *MEROPS* database

sequencing projects that are too divergent to be considered species variants of known peptidases. Of the 1,137,158 sequences in the *MEROPS* database, only 274,396 (24%) have been assigned to an identifier.

It is recommended that a well-characterized peptidase should have a trivial name. Although not rigidly adhered to, there is a different suffix for each catalytic type: metallopeptidases names end with "lysin," aspartic peptidases with "pepsin," cysteine peptidase with "ain," and serine peptidases with "in."

Compound and Complex Peptidases

Most peptidases have only one peptidase unit; however, there are some exceptions. A compound

peptidase has more than one peptidase unit within a single protein molecule. An example is the somatic form of the angiotensin-converting enzyme, which has two peptidase units. The testicular form of this enzyme contains only the second peptidase unit and is transcribed from the same gene but at an alternative initiating methionine. Each peptidase unit has a *MEROPS* identifier, but additionally there is a special identifier for each compound peptidase, which is XM02.001 in the case of angiotensin-converting enzyme.

A complex peptidase consists of several proteins in a complex. The 20S proteasome is a complex of 28 subunits, arranged in 4 stacked rings each containing 7 different subunits. The first and fourth rings contain the same seven (alpha) subunits, as do the second and third (beta subunits). Each subunit is the product of an individual gene, and the subunits are homologous to each other. Only three of the beta subunits are catalytically active, each conferring a different specificity (trypsin-like, chymotrypsin-like, or peptidylglutamyl peptide hydrolyzing, now often known as caspase-like). The proteins form a cylinder with the six active sites on the inside, and the substrate protein is threaded through one end and degraded to peptides that exit at the opposite end. The 28S proteasome contains additional subunits which form a cap and attach to one end of the 20S proteasome. These extra subunits are important for unfolding the substrate protein. Again, there is not only a *MEROPS* identifier for each alpha and beta subunit, but also identifiers for the complexes. The 20S proteasome is XT01.001 and the 28S proteasome is XT01.002.

Precursors and Zymogens

Most peptidases are not synthesized as active enzyme but as precursors or zymogens. The zymogen is transported to the site where it is needed and then activated, usually by the removal of an N-terminal propeptide by proteolysis. For some members of family S1, especially members of the blood coagulation pathway, a propeptide is not released but cleavage is still required for activity. The propeptide remains attached to the peptidase by a disulfide bridge, becoming the heavy chain in a two-chain complex. Cleavage generates a new hydrophobic N-terminal residue, and a molecular rearrangement then activates the peptidase. For some members of peptidase family M10, the propeptide acts by providing a fourth ligand for the catalytic zinc ion, and only on release of the propeptide does the zinc interact with water and the peptidase become active. Because this fourth ligand is a cysteine, this method of activation is known as a cysteine switch.

Specificity and Substrate-Binding

The specificity of a peptidase describes where in a peptide or protein sequence cleavage will occur. Some peptidases will only cleave near the amino- and carboxy-termini of the substrate, and these are termed exopeptidases. A peptidase that does not require free termini and can cleave at any acceptable site within a peptide is termed an endopeptidase. Most endopeptidases are able to cleave proteins, but there are a few that can only cleave short peptides, and these are known as oligopeptidases. Neprilysin is an oligopeptidase that degrades peptide hormones such as substance P, thereby switching off the physiological signal. Some endopeptidases are isopeptidases, cleaving nonstandard peptide bonds. An example is isopeptidase T, which releases ubiquitin from ubiquitinated proteins by cleaving the isopeptide bond between the C-terminal Gly of ubiquitin and the amino group of a nonterminal lysine on the protein targeted for degradation.

There are several different types of exopeptidases: aminopeptidases, carboxypeptidases, dipeptidyl-peptidases, tripeptidyl-peptidases, peptidyl-dipeptidases, dipeptidases, and omega peptidases.

Generally, a family of peptidases contains either exopeptidases or endopeptidases, but there are exceptions. Family C1 contains not only endopeptidases such as cathepsin L but also the aminopeptidase bleomycin hydrolase. Some members of this family can act as exopeptidases as well as endopeptidases. For example, cathepsin B also acts as a peptidyl-dipeptidase, and cathepsin H also acts as an aminopeptidase. Family S9 includes prolyl oligopeptidase, dipeptidyl-

peptidase IV, and the omega peptidase acylaminoacyl-peptidase.

The specificity of an endopeptidase is more difficult to describe than that of an exopeptidase. A peptidase with limited specificity will only cleave after one type of amino acid. Examples are trypsin, which cleaves proteins at lysyl or arginyl bonds, and chymotrypsin, which cleaves after hydrophobic residues. Granzyme B and caspase-3, which are involved in apoptosis or programmed cell death, cleave only after aspartyl bonds. However, even this can be simplistic; recent evidence has shown that granzyme B from mouse does not cleave Bid, whereas in human cleavage of this protein initiates the apoptotic pathway. The lysosomal endopeptidase legumain cleaves asparaginyl bonds but under acidic conditions can cleave aspartyl bonds; experiments with processing of the tetanus toxin have shown that only a few asparaginyl bonds are cleaved. Whether this is because only a few bonds are available to the peptidase because of the structure of the substrate or because the specificity of the peptidase is more complicated is unclear. Peptidases that cleave a variety of peptide bonds are described as having broad specificity.

Cleavage occurs at the scissile bond. Residues in the substrate toward the N-terminus are numbered P1, P2, P3, etc., whereas residues toward the C-terminus are numbered P1′, P2′, P3′, etc. Cleavage occurs between P1 and P1′. For a peptidase with limited specificity, only the residue in P1 or P1′ is important for specificity. A peptidase with an extended substrate-binding site will have a preference for residues in other positions. For example, cathepsin L prefers substrates with phenylalanine in P2 and arginine in P1. However, this is a preference only, and cathepsin L cleaves substrates after other amino acids. Caspase-3 has a preference for Asp in both P4 and P1, but it is unusual for substrate specificity to extend much further from the scissile bond. The peptidase with the most extended substrate specificity may be mitochondrial intermediate peptidase that removes an octapeptide targeting signal from the N-terminus of cytoplasmically synthesized proteins that are destined for import into the mitochondrial lumen.

A peptidase has a series of substrate-binding pockets to accommodate residues of the substrate. The S1 binding pocket accommodates residue P1, the S1′ binding pocket accommodates residue P1′, and so on. A binding pocket may contain several residues that interact with the substrate, and a single residue may take part in more than one binding pocket. Some peptidases bind the substrate at more than one binding site. A binding site away from the active site is described as an exosite. The presence of one or more exosites makes the peptidase very specific for a substrate, and it is not unusual for a peptidase with an exosite to cleave just one protein. Peptidases with such limited specificity include the blood coagulation enzymes. In some peptidases, additional substrate-binding sites are located on domains other than the peptidase unit. For example, the matrixins (family M10) interact with proteins of the extracellular matrix through the hemopexin-like domain that is C-terminal to the peptidase unit.

A proteolytic cascade occurs when one peptidase activates the next in a proteolytic pathway, and this in turn activates the next and so on. This is a mechanism to amplify the initial signal, because one peptidase molecule can activate many zymogen molecules. Examples of proteolytic cascades include blood coagulation, activation of digestive peptidases in the intestine, and apoptosis.

Drugs

The action of a peptidase can be neutralized by an inhibitor. Some inhibitors are very broad in their action and are capable of inhibiting many different peptidases, including peptidases of different catalytic types. Some inhibitors are assumed to be specific for a particular catalytic type but can inhibit peptidases of different types. Leupeptin, for example, is widely used as an inhibitor of serine peptidases from family S1, but it is also known to inhibit cysteine peptidases from family C1. Cysteine peptidase inhibitors such as

iodoacetic acid interact with the thiol of the catalytic cysteine. However, this reduction can occur on any thiol group and can affect other, predominantly intracellular, peptidases with a thiol dependency. One example is thimet oligopeptidase. Metal chelators such as EDTA can inhibit metallopeptidases but can also affect peptidases that have a requirement for metal ions that is independent of their catalytic activity, such as the calcium-dependent cysteine endopeptidase calpain 1.

Inhibitors which interact only with peptidases of one catalytic type include pepstatin (aspartic peptidases), E64 (cysteine peptidases from clan CA), diisopropyl fluorophosphates (DFP), and phenylmethane sulfonylfluoride (PMSF) (serine peptidases). Bestatin is a useful inhibitor of aminopeptidases.

Table 1 lists peptidases that are known or potential drug targets, alleviate disease symptoms, or aid disease identified from searching the literature from 2017 to 2019. In nearly all cases, the drug will be a synthetic peptidase inhibitor (Abbenante and Fairlie 2005).

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NPY

► Neuropeptide Y

NSCCs

► Non-selective Cation Channels

Nuclear Magnetic Resonance

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Synonyms

NMR; NMR spectroscopy

Definition

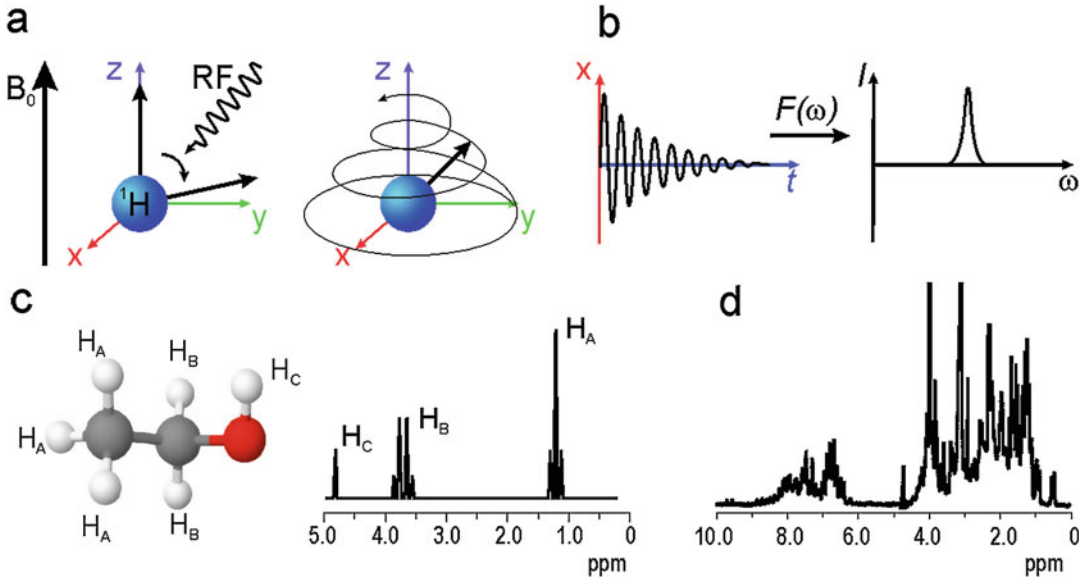
Nuclear magnetic resonance (NMR) is a spectroscopic technique detecting magnetic properties of atomic nuclei. All atoms that contain an odd number of protons and/or neutrons have a magnetic moment. This magnetic moment can be oriented and perturbed by an external magnetic field. In the presence of a magnetic field, the nuclear magnetic moment oscillates with a frequency (Larmor frequency) that depends on the external field strength as well as the gyromagnetic ratio of the nucleus, a fundamental property of atomic nuclei. Since the gyromagnetic ratio is constant for nuclei of the same type, the Larmor frequency can be used for highly precise measurement of the local magnetic field around nuclei and the magnetic coupling with nearby nuclei. Hydrogen nuclei are most commonly observed by NMR, but ^{13}C, ^{15}N, ^{31}P, and ^{19}F are also frequently used in life sciences, but in principle any nucleus with a magnetic moment can be observed. In some cases, the natural abundance of a magnetically active isotope is sufficient to measure its NMR signal. In other cases, specific, stable isotope enrichment is necessary. This is particularly useful for biomolecular NMR spectroscopy.

NMR spectroscopy is a nondestructive technique (for the sample) and does not rely on light or optical properties. Typically NMR is carried out in the solution state; however solid- and gaseous-state NMR is also possible.

Description

NMR techniques rely on polarizing the magnetic moments of the sample in a strong, external magnetic field. This is usually done by inserting the sample into a superconducting magnet or powerful electromagnet. The strength of the field determines the Larmor frequency of the nuclei. NMR spectrometers are characterized by the proton Larmor frequency (e.g., a 600 MHz spectrometer, 14.1 Tesla magnet, has a magnetic field that causes protons to oscillate at around 600 MHz). Depending on their gyromagnetic ratio, the frequencies of other nuclei will be different at the same field. For example, a 600 MHz ^{1}H spectrometer will have a ^{13}C frequency of 151 MHz. In the presence of an external magnetic field, the degeneracy of the nuclear spin energy levels is destroyed with a net population of spins slightly favoring the lower energy state(s), resulting in a net magnetic moment. The nuclear magnetization is diverted from its equilibrium state by intense radio-frequency pulses. The perturbed system returns to equilibrium, and a free induction decay (FID) signal is detected (Fig. 1a). A Fourier transform of the FID shows the resonance frequencies of nuclei present in the sample in an NMR spectrum (Fig. 1b). The resonance peaks in the spectrum have area proportional to the abundance of the nuclei. Therefore, peak integration can be used to measure relative abundance of particular nuclei in a small molecule. The position (chemical shift) of the resonance is usually given in ppm (parts per million) shift from a reference frequency. The advantage of a ppm scale over frequency units is that it is independent of the spectrometer frequency, therefore allowing easy comparison of data obtained on different spectrometers.

A molecule in NMR can be treated as "spin system"; this is a collection of magnetically



Nuclear Magnetic Resonance, Fig. 1 Principle of NMR. (**a**) Nucleus oriented along magnetic field B0 is diverted from equilibrium by an RF pulse. It then returns to equilibrium emitting decaying RF signal (FID). (**b**) The FID is recorded by a spectrometer. Its Fourier transformation gives a spectrum in frequency domain. (**c**) Ethanol molecule and its NMR spectrum. Each group of hydrogens resonates at different frequencies. Coupling between groups causes peak splitting. (**d**) Proton 1D spectrum of a small protein (SUMO). The resonances are highly overlapped making the interpretation difficult. Multidimensional NMR allows separating and identifying peaks overlapping in one-dimensional spectra

coupled nuclei. NMR allows measurement of through-bond coupling called scalar or J-coupling within the spin system. The magnitude of the J-coupling depends on the identities of the coupled nuclei as well as the bond geometry. Therefore, it provides essential information on molecular connectivity and bond properties. In addition, the nuclear Overhauser effect (NOE) is a through-space interaction and can be used to measure spin systems' proximity in space (Fig. 1c).

The possibility of magnetic excitation transfer between nuclei gives rise to multidimensional NMR spectroscopy. This allows the extraction of much more precise structural information from the sample. For example, in heteronuclear correlation experiments, one can observe connectivity between different nuclei (e.g., ^{1}H and ^{15}N). It is especially useful for the analysis of complex molecules like proteins (Fig. 1d). Such experiments can be coupled into multidimensional matrices allowing observation of multiple through-bond and space correlations.

Applications

Synthetic Chemistry Quality Control

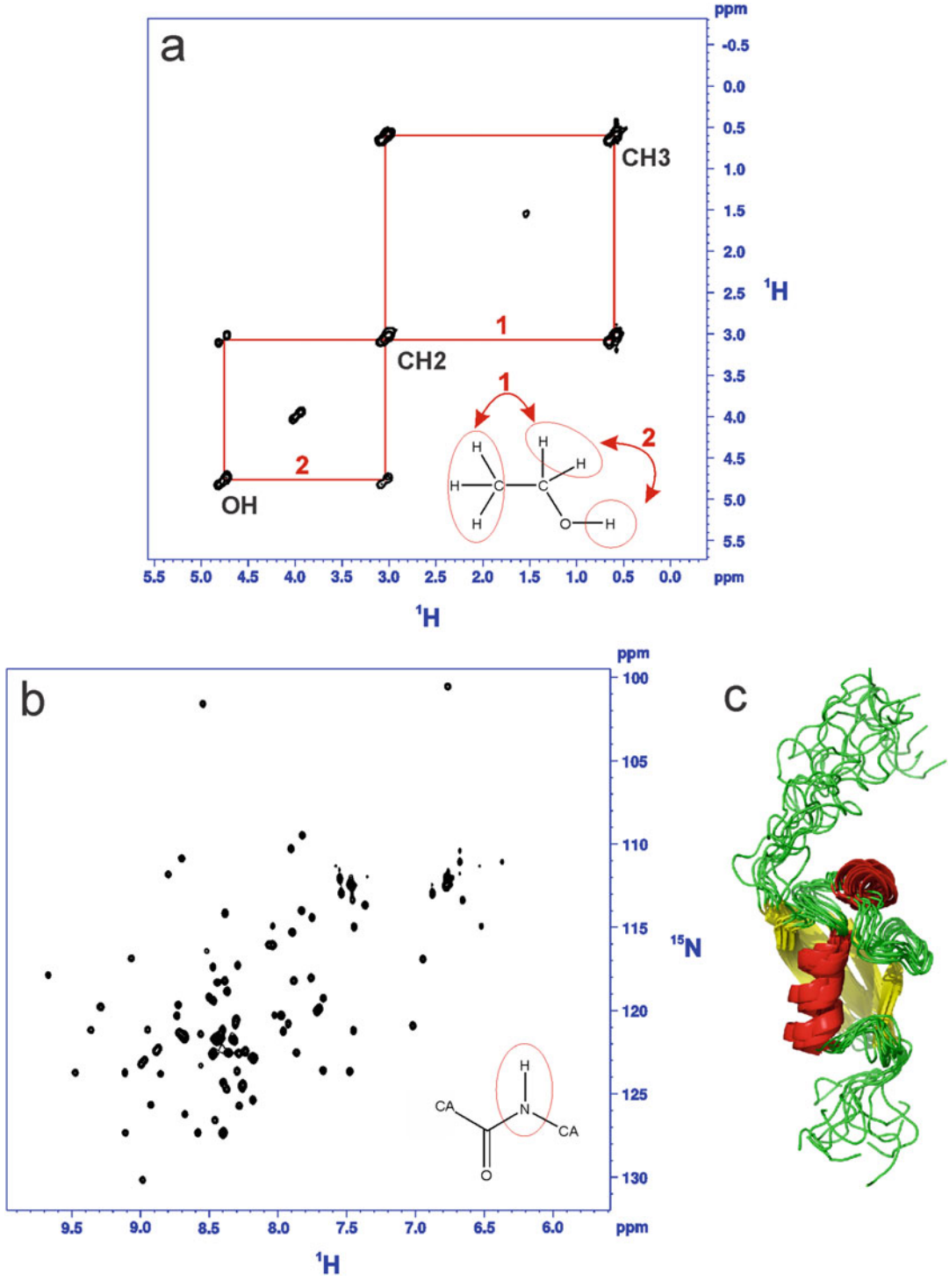
Synthetic organic chemistry and especially medicinal chemistry are the most popular areas for NMR application (Kiss et al. 2018). Simple one- or two-dimensional spectra give the chemist information about the identity and purity of the synthesized molecule (Pauli et al. 2014). Here, the principal information comes from proton, ^{1}H, and natural abundance, ^{13}C, spectra as stable isotope labeling is not feasible. In most cases distinct chemical moieties have resonances in different regions of the chemical shift scale (Fig. 1c). For example, protons belonging to aromatic systems have resonances located around 6–8 ppm, while saturated alkanes are visible between 0 and 2 ppm. Peaks in proton spectra have integrals that are directly proportional to the number of protons in each chemical environment, typically the number of hydrogens attached to one "heavy" atom. The adjacent groups of hydrogens in different chemical environments are coupled to each other; this causes splitting of the resonances within each group. The magnitude of the splitting is identical for both connected systems and characterizes bond type and geometry. To obtain more information about molecule connectivity, two-dimensional spectra can be used. This data usually allows the chemist to quantify the purity of a synthesized molecule and confirm its identity by comparing expected or predicted spectra with the measured result (Finch 2014). Most of the renowned medicinal chemistry journals require NMR characterization of the molecules reported. Usually at least assigned ^{1}H and ^{13}C spectra are required to prove compound identity.

Compound Identity Evaluation

NMR is one of the principal techniques for characterizing molecular identity. This is necessary especially for metabolite identification (Hamdam et al. 2013; Grillo 2015; Hollinshead et al. 2016; Nagana Gowda and Raftery 2015), characterization of degradation products, and evaluation of the identity of drugs and complex natural products, among other uses (Guleria et al. 2018; Beni et al. 2011; Holzgrabe and Malet-Martino 2011; Kiss et al. 2018; Rudszuck et al. 2019; Wang et al. 2013). Together with mass spectroscopy and sometimes elemental analysis, the chemist can analyze molecular connectivity and relative abundance. In most cases acquisition of multidimensional spectra is necessary to gather sufficient information to identify the molecule. The most useful spectra are as follows:

(i) COSY (COrrelated SpectroscopY) shows which signals arise from neighboring (up to four bonds) protons. The spectrum contains a proton 1D along its diagonal axis with cross-peaks (off-diagonal elements) appearing when there is spin-spin coupling between protons (Fig. 2a). COSY allows resolution of overlapping multiplets and tracing the carbon chain(s) of the molecule.

(ii) TOCSY (TOtal Correlated SpectroscopY) is similar to COSY but allows tracing coupling networks of any length as the intensity of cross-peaks is not related to the number of bonds connecting the protons. This experiment is particularly useful for large



Nuclear Magnetic Resonance, Fig. 2 Multidimensional NMR spectroscopy. (**a**) A COSY spectrum of ethanol. Peaks on spectrum diagonal are equivalent to 1D proton spectrum. Cross-peaks (outside diagonal) indicate through-bond coupling of protons. (**b**) An HSQC spectrum of a small protein (SUMO). Each peak relates to proton-nitrogen pair of the protein. The spectrum is unique to each protein and can be used as a "fingerprint" for

molecules containing multiple, separated coupling networks.

(iii) HSQC (Heteronuclear Single-Quantum Coherence) shows connectivity between two different nuclei. It is usually used to observe direct connectivity between ^{1}H-^{13}C and ^{1}H-^{15}N pairs. In these 2D spectra, one axis shows ^{1}H resonance frequency while the other ^{13}C (^{15}N) frequency. HSQC spectra allow determination of which hydrogen is directly bonded to which carbon (nitrogen) and therefore makes it possible to trace the connectivity between chemical groups that do not have direct hydrogen-hydrogen coupling (Fig. 2b).

(iv) NOESY (Nuclear Overhauser Effect SpectroscopY) is used for determining which resonances arise from protons that are close to each other (strength of the signal is proportional to the inverse sixth power of the distance between the nuclei) in space even if they are not bonded. A NOESY spectrum yields through-space correlations via spin-lattice relaxation. These spectra are usually recorded for hydrogen only. NOESY spectra can also detect chemical and conformational exchange. The 2D NOESY spectrum contains a 1D proton spectrum along its diagonal axis with cross-peaks indicating spatial proximity of resonances.

NMR is a nondestructive technique. This allows measuring the same sample multiple times. Measuring a sample after a certain period gives information about substance stability in solution when spectra taken at different time points are compared.

Protein Analysis

NMR experiments can provide significant information about protein properties (Zhuravleva and Korzhnev 2017). A one-dimensional proton spectrum of a protein allows estimation of how well folded the protein is, detection of aggregation, and the formation of higher-order complexes. The possibility of ^{15}N (or more seldom ^{13}C) labeling by recombinant production in isotopically enriched media allows heteronuclear correlation spectra (HSQC, Fig. 2b) of a protein to be recorded. HSQC spectra give a good measure of protein folding and allow easy observation of protein interactions with its ligand. The bigger the biomolecule, the more significant are the relaxation mechanisms leading to broadening of NMR resonance lines. This makes direct acquisition of spectra of a protein over approximately 40 kDa difficult. In such cases the use of higher field spectrometers (leading to better peak dispersion), selective labeling of a few atoms in a protein, deuteration, or special relaxation-insensitive experiments is necessary to obtain useful information. Correlation experiments can be extended into three-dimensional ^{1}H-^{15}N-^{13}C experiments. Those in turn enable tracing of residue connectivity and assignment of observed resonances to particular residues in a biomolecular sequence.

Investigation of Biomolecular Structure and Dynamics by NMR

One of the most prominent applications of NMR is structural investigation of biological macromolecules. While most of the published biomolecular structures were solved by X-ray crystallography (and, more recently cryo-EM), NMR structures complement this structural research. NMR has the advantage that it produces structures in solution without possible crystallization artifacts, can deliver structures of intrinsically disordered proteins or nucleic acids (Kellett et al. 2019; Schlundt et al. 2017), and can provide structures for biomolecules too small for cryo-EM techniques

Nuclear Magnetic Resonance, Fig. 2 (continued) identification and quality control. Folded proteins have good dispersion of resonances. When the protein is unfolded, peaks collapse toward single position around 8.5 ppm. (**c**) An example of NMR protein structure (SUMO). The calculated ensemble is converging where lots of NMR restraints are observed (structured elements). In the parts where little restraints are observed, the ensemble is dispersed (Bayer et al. 1998)

(Joshi and Vendruscolo 2015). The structural investigation of a biomolecule by NMR is a complex process (Arthanari et al. 2019). Initially a correlation spectrum is inspected for the presence of secondary structure and good resonance dispersion. Next 3D correlation experiments are recorded usually using a ^{13}C, ^{15}N double-labeled sample. This allows assignment of each resonance to an atom within a defined residue. When the assignment is complete, a set of experiments is selected that provides information on inter-atom distances (NOESY), bond angles (from coupling constants and/or chemical shifts), and orientation restraints (residual dipolar coupling). With all the structural data gathered, the structure is calculated by a software package that takes into account both the general geometry of biological macromolecules and experimental restraints. An ensemble of structures is generated (Fig. 2c). It converges when the experimental data is sufficient to determine a specific fold. Lack of measurable restraints will lead to significant dispersion of the ensemble and will indicate that the investigated molecule is flexible.

Both measured geometrical restraints and the general strength of through-space coupling (heteronuclear NOE experiment) can be used to evaluate biomolecule flexibility (Kovermann et al. 2016). It is also possible to measure relaxation of assigned atoms. Relaxation rates for different magnetization operators result from the intrinsic dynamic processes of the atoms under investigation and allow NMR to assess biomolecular dynamics across a range of timescales from ps to s or longer. Relaxation is faster in the case of rigid molecules and slower in the case of highly flexible molecules. Relaxation rates can thus report on the rigidity of biomolecules, the overall tumbling rate (correlation time), and local molecular dynamics. All these methods allow structural biologists to measure the degree of flexibility of parts of the molecule (e.g., loop closing an active site of an enzyme). These measurements can also be employed in evaluation of ligands causing allosteric or dynamic alterations of a biomolecule (Ambadipudi and Zweckstetter 2016; Skora and Jahnke 2015).

A very valuable tool for drug discovery but also a challenging task in NMR structural research is evaluation of protein-small-molecule structure (Erlanson et al. 2019). Quite often the size of the protein, complex abundance, or lack of access to stable isotope labeling will prevent acquisition of NOESY spectra giving distance restraints between ligand and protein. Several methodologies have been developed to solve this problem (attaching a paramagnetic atom to the proximity of a binding site (Nitsche and Otting 2018), per-atom analysis of STD-signal intensity, using chemical shifts to guide computational docking) (Ding et al. 2013; Sturlese et al. 2015; Wang et al. 2019). However, no generally accepted methodology exists at the moment.

Orthogonal Interaction Assays

NMR techniques are widely used for validation of hits produced by high-throughput screening (HTS) techniques (Li and Kang 2017; Wu et al. 2015). Most of the HTS assays involve an optical readout. Therefore, they are susceptible to false positives produced by highly absorbing or fluorescent compounds or impurities. Since NMR does not rely on optical properties but observes directly the magnetic field around atoms, it provides an excellent way to validate hits from other assays. Moreover, NMR observes protein in solution, without the need for immobilization and chemical labeling, in a buffer closer to the natural cellular environment. This eliminates several sources of erroneous measurement. NMR experiments usually require several minutes to several hours per sample. This makes them unsuitable to screen very large libraries, but primary hits from an HTS screen are usually in numbers suitable for NMR-based assays. This is especially true since the introduction of fully automated sample changers. Due to the fact that large biomolecules are difficult to observe by NMR (see Protein Analysis section), it is often easier to carry out ligand-observed experiments, where interaction with a larger biomolecule (protein) is inferred indirectly via the effect on the ligand signals. For smaller biomolecules (typically below 40 kDa), it is possible to observe interaction from both the receptor signals and the ligand signals. The experiments used for binding validation are identical to those used for NMR-based screening and are

described in the next section (Gossert and Jahnke 2016).

NMR-Based Screening

Until recently, NMR has been considered a low-throughput technique. This was caused by manual sample installation in most systems and the lengthy time required to prepare and execute experiments. Introduction of high-capacity sample changers (i.e., Bruker SampleJet), development of experiment automation software, and more time-efficient experiments have changed this situation (Sillerud and Larson 2012). It is now possible to measure several thousand experiments in a few days. This is still not sufficient for true HTS screening campaigns but satisfies the requirement of fragment and focused library screening (Harner et al. 2013).

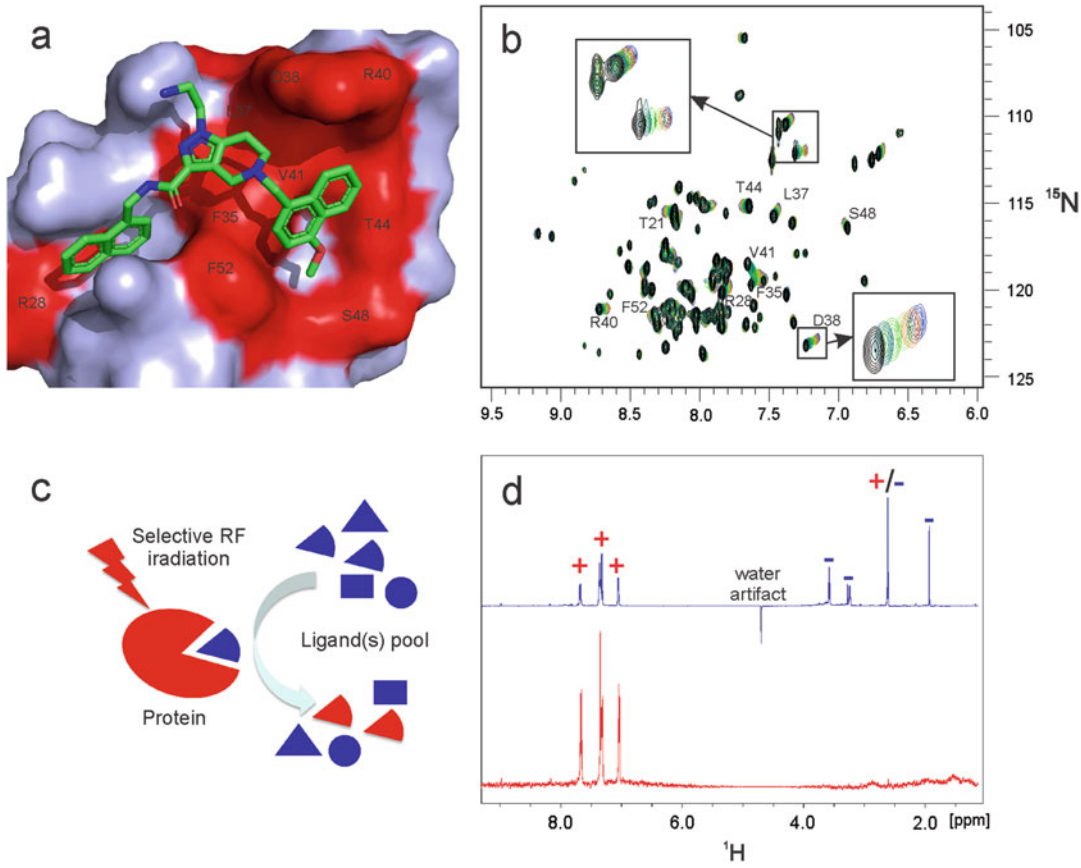
NMR is a method of choice for fragment screening (Kashyap et al. 2018). This is due to the fact that NMR experiments are sensitive to weak interactions in the high μM-low mM regime. Additionally, NMR experiments require relatively high concentrations of the observed molecule (tens to hundreds of μM); this is usually not a problem for small, highly soluble fragment molecules.

In order to reduce the number of experiments necessary to screen a library, the molecules are often pooled together into cocktails of up to tens of molecules. Molecules from cocktails evaluated as active are then measured individually. Some techniques allow direct identification of the active molecule in the cocktail by observation of its characteristic chemical shift.

In cases when direct observation of protein and ligand is not feasible because of low ligand solubility or lack of possibility for macromolecule labeling with stable isotopes, a reporter molecule that is easily detectable in NMR experiments can be used and displacement of the reporter from the molecule site of interest measured. Here, ^{19}F signals are often used as fluoride resonances, which are very easy to identify, and the ^{19}F spectrum does not contain any interfering signal from ligands, buffers, or the macromolecule.

The NMR experiments most commonly used for screening and orthogonal validation can be divided into three groups (Cala et al. 2014; Sugiki et al. 2018):

- Macromolecule-observed experiments – here the macromolecule (usually protein) is directly observed by NMR. Biomolecules in the native state contain only ^{1}H as an NMR-active nucleus. ^{1}H spectra of large molecules are usually heavily overlapped and difficult to interpret. Therefore, most biomolecule-observed experiments are conducted on stable isotope-labeled protein. ^{15}N or ^{13}C is usually used. The isotopes are introduced as components of defined media for recombinant expression. In the case of very large biomolecules, more sophisticated, selective labeling of certain residues or chemical moieties (i.e., methyls) can be applied using a suitable expression medium. The presence of two NMR-active nuclei allows recording of correlation spectra (HSQC), which are the most used for biomolecule-observed screening experiments. While it is theoretically possible to use ^{1}H-^{1}H experiments (COSY, NOESY) for screening, such experiments remain exceptional and are used primarily for DNA and RNA screening. Correlation spectra allow approximate mapping of the ligand binding site by comparing the position of peaks in the 2D spectrum for the biomolecule in the apo-form and after ligand addition (Fig. 3a, b). Spectral changes of resonances (shifts, intensities, splitting) indicate binding. If residue assignment is available, changes in the correlation spectra can be mapped onto the protein structure and allow approximate ligand placement. The biomolecule-observed experiments require a relatively large amount of materials. Typically, 0.5 mg of protein is required for a single experiment.
- In cases when isotopically labeled protein is not feasible or too expensive, NMR-based screening can be performed by observation of ligand. In this case only ligand hydrogen or fluorine nuclei are used and no special isotope labeling is necessary. Hydrogen-detected experiments are usually based on the altered magnetic properties of ligand that encountered the macromolecular receptor compared with



Nuclear Magnetic Resonance, Fig. 3 NMR of protein-ligand interactions. (**a**, **b**) A structure of a protein with inhibitor bound (Dawidowski et al. 2017). The residues in contact with the inhibitors have their resonances shifted in dose-dependent manner. Assigning of the peaks that change upon binding allows mapping of the binding site without crystal structure. (**c**, **d**) Principle of STD binding experiment. The ligand spectrum is observed as a standard proton 1D (blue) and STD (red). Protein is selectively excited by RF irradiation. The excitation is transferred to ligands (red) that bind briefly to the protein (+). Non-binding ligands (blue) give no signal on STD spectrum (−). The STD is one of a palette of ligand-observed binding experiments

ligand remaining free in solution. One of the most popular experiments is saturation transfer difference (STD, Fig. 3c, d), which uses selective magnetic excitation of protein only (Wagstaff et al. 2013). The excitation is transferred to a binding ligand. Fast exchange of the bound ligand with the pool of free ligand allows a pool of ligand, excited via binding to the protein, to build up. Then the 1H spectrum of the ligand under protein saturation conditions is compared to the one where the excitation signal does not "hit" the protein. The difference spectra show only those ligand resonances that come in contact with excited protein. Due to fast turnover of ligands in the binding site, the relative amount of protein to ligand can be very small (up to 1:1000), which makes the STD experiment particularly useful when only small amounts of protein are available. The WaterLOGSY (Dalvit et al. 2001) experiment is similar to STD; however excitation is directed toward water and then transferred to protein and ligand. The sign of magnetic polarization of the ligand is different for water-ligand and water-protein-ligand interactions. This difference can be measured and used for binding evaluation. When a small molecule contacts a macromolecule, it acquires

the relaxation properties of the macromolecule. This causes much faster relaxation of binding ligands when compared to non-binders, which translates to faster magnetization decay and broader resonance lines. Since linewidth is difficult to analyze directly, relaxation-sensitive experiments can be used to measure how fast the molecule is losing its magnetization due to contact with the protein. Similarly, an NMR sample can be placed in a magnetic field gradient and diffusion speed measured along the gradient. Bound ligand will diffuse much slower, and the difference can be observed.

- The interesting alternative to proton-observed screening experiments is ^{19}F NMR (Norton et al. 2016). Fluoride spectra are characterized by much large chemical shift dispersion (around 200 ppm compared to ~15 ppm for ^{1}H), much narrower spectral lines, and sensitivity comparable to ^{1}H spectroscopy. These factors make ^{19}F NMR attractive to screen mixtures on many compounds simultaneously, as there is little chance that the peaks will overlap. Direct identification of interacting ligand is possible simply by comparing the chemical shift of the "interacting" peak with the compound reference spectrum. Relaxation-sensitive experiments are usually chosen for screening. Of course, ^{19}F NMR requires special libraries where each compound contains at least one fluoride atom. Many vendors and pharmaceutical companies have already assembled such libraries with many thousands of compounds. In cases where the screened compounds do not contain fluoride, a fluorinated reporter molecule can be used. The requirements for such a molecule are specific interaction with the binding site of interest, at least one ^{19}F atom in the structure, and reasonably good solubility. It is also possible to introduce ^{19}F atom(s) into the protein structure in the proximity of the active site to observe ligand binding (Arntson and Pomerantz 2016).

Due to the versatility of experimental methodologies, NMR is one of the indispensable elements of target-oriented screening campaigns. It can also enable fast target identification for hits coming from phenotypic screening when the expected target macromolecule is available.

Pharmaceutical Relevance

NMR is one of the key techniques in the pharmaceutical sciences. It is indispensable for synthetic, medicinal chemistry, pharmacopoeic quality control, and metabolite analysis. Target-based drug discovery benefits from reliable, NMR-based orthogonal screening, binding site analysis, and structural biology data. The main drawback of NMR is the availability of sufficient sample quantities and stable isotope labeling. This is partially alleviated by increases in NMR spectrometer sensitivity (cryogenically cooled preamplifiers) and new experiment design. Development of new, sophisticated ligand binding evaluation approaches will certainly increase the role of NMR in structural analysis of biomolecule-ligand complexes. Development of in-cell NMR (Kang 2019; Luchinat and Banci 2016), while still in an early phase, offers opportunities to investigate biological systems in the native, cellular environment (Burz et al. 2019) and will enable phenotype-based cellular experiments by NMR.

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Nuclear Receptor Regulation of Hepatic Cytochrome P450 Enzymes

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Drug-metabolizing enzymes · Enzyme induction · Gene regulation · Receptor activation · Receptor pharmacology

Glossary Terms

Cytochrome P450 (CYP)	Family of hemeprotein monooxygenase enzymes that plays a central role in the oxidative metabolism of structurally diverse lipophilic steroids, fatty acids, drugs, and environmental chemicals. P450-catalyzed biotransformation of drugs is primarily carried out by 10–15 distinct human P450 enzymes, each encoded by a separate P450 gene.
Lithocholic acid	3α-hydroxy-5β-cholanoic acid, a hepatotoxic and cholestatic secondary bile acid which is formed by bacterial dehydroxylation of primary bile acids in the intestine.
Orphan nuclear receptor	Receptor protein belonging to the nuclear receptor superfamily whose physiological ligand has not been identified.
P450 induction	The process whereby cellular and tissue levels of one or more cytochrome P450 enzymes are increased in response to treatment of cells, or a whole organism, with certain drugs or environmental chemicals referred to as P450 inducers. P450 induction leads to an increase in the cell's capacity for P450-catalyzed oxidative metabolism of many xenochemicals, as well as endogenous steroidal and fatty acid P450 substrates.
Peroxisome proliferator chemicals	Chemicals that activate the nuclear receptor PPARα (peroxisome proliferator-activated receptor-α) and induce the enlargement and proliferation of liver peroxisomes in susceptible rodent species (e.g., rats and mice). Persistent exposure to peroxisome proliferator chemicals is closely linked to PPARα activation and liver tumor development in these species.
Phthalate esters	Di- and mono-esters of phthalic acid, an ortho-dicarboxylic acid derivative of benzene. These compounds are widely used as industrial plasticizers to coat polyvinylchloride surfaces of plastics used in food packaging and medical devices (intravenous drip bags, blood storage bags, etc.) and are common environmental contaminants. Several phthalate mono-esters are peroxisome proliferator chemicals and can activate the peroxisome proliferator-activated receptor PPAR.

Retinoid X receptor, RXR	Nuclear receptor that binds and is activated by certain endogenous retinoids, such as 9-*cis*-retinoic acid. RXR is the obligatory heterodimerization partner for a large number of non-classic steroid nuclear receptors, such as thyroid hormone receptor, vitamin D_3 receptor, peroxisome proliferator-activated receptor, and pregnane X receptor.

Definition

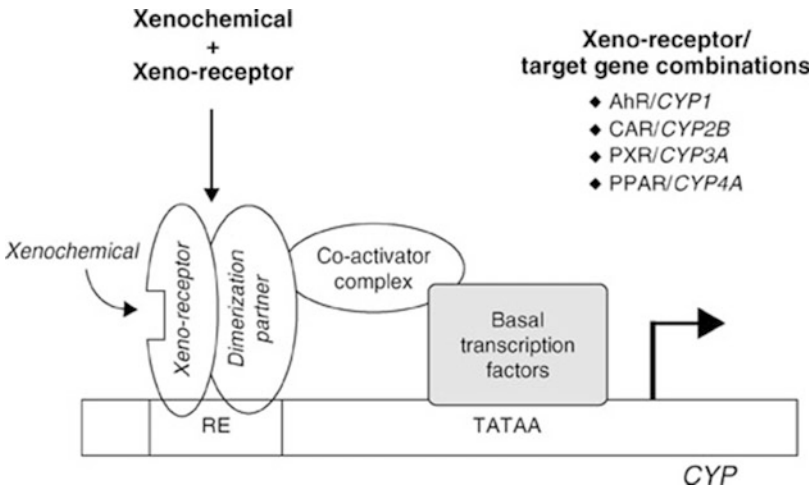
Cytochrome P450 (CYP) induction is the process whereby cellular or tissue levels of one or more P450 enzymes are increased as a result of de novo protein synthesis in response to treatment with certain drugs (e.g., phenobarbital and rifampicin) or exposure to environmental chemicals (e.g., dioxins and polychlorinated biphenyls), which are designated P450 inducers. This inductive response generally results from an increase in P450 gene transcription and leads to an increase in the capacity for P450-catalyzed oxidative metabolism of both xenochemicals (i.e., drugs and other foreign chemicals) and endogenous lipophilic substrates (i.e., steroid hormones, vitamins, fatty acids, and bile acids). Other drug-metabolizing enzymes, such as drug conjugation enzymes (e.g., glutathione *S*-transferases and UDP-glucuronosyltransferases), are also subject to induction by classic P450 inducers, whose pharmacological and toxicology effects are discussed here.

Basic Mechanisms

P450 induction can occur in many cell types and tissues but is most prominent in the liver, a major organ for metabolism of steroids, drugs, and environmental chemicals. Many of the inducible P450s are active catalysts of drug metabolism, and P450 induction typically enhances the capacity for chemical biotransformation, resulting in a shorter elimination half-life and more rapid clearance of the chemical from the body. Consequently, P450 induction can have a major impact on P450-dependent drug metabolism, pharmacokinetics, and drug-drug interactions; the toxicity and carcinogenicity of foreign chemicals; and the disposition and biological activity of endogenous steroids and certain other hormones. Although some P450 substrates also serve as P450 inducers, there is no necessary relationship between the ability of a chemical to induce a particular P450 enzyme and its ability to serve as a substrate for metabolism by that same P450.

At least 10 of the 57 known human P450s are subject to induction by xenochemicals. In most, but not all cases, the induction of P450 protein and enzyme activity occurs by a mechanism that involves increased transcription of the corresponding P450 gene. Members of four P450 gene families, *CYP* families 1, 2, 3 and 4, are induced by receptor-dependent transcriptional mechanisms (Fig. 1). P450 genes belonging to the *CYP1* gene family and the *CYP2S1* gene are induced by the aryl hydrocarbon receptor (AhR, also known as dioxin receptor), which is a member of the basic helix-loop-helix (bHLH)/periodic circadian protein (PER)-AhR nuclear translocator (ARNT)-single minded protein (SIM) superfamily of transcription factors (Rothhammer and Quintana 2019). In contrast, the induction of select genes from P450 families *CYP2*, *CYP3*, and *CYP4* is mediated by transcription factors from the nuclear receptor superfamily. The nuclear receptor known as constitutive androstane receptor (CAR, gene designation *NR1I3*) preferentially induces *CYP2B* genes (Kobayashi et al. 2015), whereas pregnane X receptor (PXR, gene designation *NR1I2*, also known as steroid and xenobiotic receptor and pregnane-activated receptor) preferentially induces *CYP3A* genes and peroxisome proliferator-activated receptor α (PPARα, gene designation *NR1C1*) preferentially induces *CYP4A* genes (Hakkola et al. 2018).

CAR is an orphan nuclear receptor that mediates the widely studied induction of *CYP2B* genes by phenobarbital, other "phenobarbital-like" lipophilic drugs, and other chemicals (Kobayashi et al. 2015). PXR activates *CYP3A* genes in response to diverse chemicals, including certain



Nuclear Receptor Regulation of Hepatic Cytochrome P450 Enzymes, Fig. 1 General mechanism for the direct transcriptional activation of *CYP* genes by xenochemicals that activate their cognate xeno-receptor proteins. In the case of AhR, the receptor's heterodimerization partner is Arnt, whereas in the case of the nuclear receptors CAR, PXR, and PPARα, the heterodimerization partner is RXR. The coactivator and basal transcription factor complexes shown are each comprised of a large number of protein factors

drugs, natural products, and natural and synthetic steroids (Chai et al. 2016). PPARα mediates the induction of fatty acid hydroxylases of the CYP4A family by many acidic chemicals classified as non-genotoxic carcinogens and peroxisome proliferators (Hakkola et al. 2018). These three xenochemical receptors are most highly expressed in the liver, where they may be activated by either by endogenous ligands or foreign chemicals, including many drugs and environmental pollutants. CAR and PXR are also expressed in the intestine, where they may also mediate P450 induction. The discovery of endogenous ligands for CAR (androstanes, which decrease basal receptor activity and serve as inverse agonists), PXR (certain pregnenolone derivatives, bile acids, and other steroids), and PPARα (specific prostaglandins and other fatty acid metabolites) suggests that these three nuclear receptors play an important role in modulating liver gene expression in response to endogenous metabolic or hormonal stimuli, in addition to their established role in modulating liver drug and xenochemical metabolism by induction of cytochromes P450 and other enzymes of foreign compound metabolism.

CYP1 Induction Via AhR

The AhR is localized in the cytosol in the basal state where it exists in a complex containing a dimer of heat shock protein 90 (hsp90), AHR-interacting protein (AIP; also known as the hepatitis B virus X-associated protein, abbreviated as XAP2), the co-chaperone p23 protein, and protein kinase SRC (Rothhammer and Quintana 2019). AhR is activated upon binding a ligand in the cytosol. The ligand-activated AhR translocates to the nucleus where it dissociates from the chaperone and co-chaperone proteins and heterodimerizes with a nuclear protein, AhR nuclear translocator (ARNT). The AhR-ARNT heterodimer binds to DNA enhancer sequences ("dioxin-response elements" or DREs) found upstream of *CYP1* and other AhR target genes and stimulates transcription of target genes, including those involved in biotransformation, cell proliferation, cell differentiation, and control of the immune responses. The overall pathway for AhR activation is conserved in many cell types and across species and accounts for the induction of *CYP1* genes by a large number of aromatic hydrocarbons, including important environmental carcinogens found in auto emissions and cigarette

smoke. Halogenated and polycyclic aromatic hydrocarbons are prototypic AhR ligands, but certain planar compounds, including dietary plant constituents, can also bind to AhR, although with varying affinities. AhR can also regulate gene expression without the direct involvement of DRE DNA-responsive elements, by mechanisms that include (i) interaction of AhR with various coactivators and corepressors, NF-κB, and the retinoblastoma protein; (ii) activation of various protein kinases; (iii) phosphorylation of AhR; and (iv) functioning as an E3 ubiquitin protein ligase to facilitate degradation of target proteins.

Role of CAR in CYP2B Induction and Other Phenobarbital Responses

The orphan nuclear receptor CAR is the key regulated transcription factor that mediates induction of liver *CYP2B* and other genes (Kobayashi et al. 2015). CAR is localized in the cytosol in the basal state (i.e., in the absence of a ligand or an activator), where it is in a complex with co-chaperone proteins Hsp90 and cytoplasmic CAR retention protein (CCRP). CAR is activated by dephosphorylation of Thr-38, which can be induced by direct binding of xenochemicals, such as 1,4-bis[2-(3, 5-dichloropyridyloxy)]benzene (TCPOBOP), a highly specific halogenated agonist of mouse CAR. CAR can also be activated by indirect activators, such as phenobarbital, which stimulates CAR Thr-38 dephosphorylation by disruption of signaling downstream of epidermal growth factor receptor (EGFR, see below). Thr-38 dephosphorylation dissociates CAR homodimers and induces translocation of CAR to the nucleus, where CAR binds to the nuclear receptor retinoid X receptor (RXR). The CAR-RXR complex recruits coactivators such as steroid receptor coactivator 1 (SRC1) and binds to specific DNA response elements to stimulate the transcription of a CAR target gene (e.g., *CYP2B*) (Fig. 1). RXR serves as a common heterodimerization partner for many nuclear receptors, including PXR and PPARα. Further, many activators of CAR can also activate PXR and/or PPARα.

Nuclear translocation of CAR is strongly enhanced in the liver in vivo following administration of phenobarbital. However, unlike classical nuclear receptor agonists, phenobarbital does not bind to the COOH-terminal ligand-binding domain of CAR. Other indirect activators of CAR include the flavonoids galangin, chrysin, and baicalein. Studies in the past decade have provided insights into the nuclear translocation of CAR by phenobarbital (Mackowiak and Wang 2016). The cellular events in the nuclear translocation of CAR by phenobarbital and other indirect inactivators of CAR include (1) binding to EGFR, resulting in inhibition of epidermal growth factor-mediated signaling; (2) dephosphorylation of a protein known as receptor for activated C kinase 1 (RACK1); (3) recruitment of protein phosphatase 2A to the CAR-Hsp90-CCRP complex; and (4) dephosphorylation of CAR.

Mouse *CAR* gene knockout studies demonstrate that CAR is essential, not only for induction of the highly inducible *CYP2B* genes but also for the multiple pleiotropic responses associated with exposure to phenobarbital and phenobarbital-like inducing agents. These include the induction of many genes involved in xenobiotic transport and biotransformation and repression of the expression of certain genes involved in energy metabolism. CAR is also required for various pathophysiological effects of phenobarbital in the liver (e.g., hepatomegaly, enhanced hepatocyte proliferation) and for toxicological or carcinogenic responses that are characteristic of phenobarbital-treated liver, including hepatotoxicity induced by acetaminophen and cocaine, and liver tumor promotion.

CYP3A Induction by PXR: Role in Metabolism of Xenochemicals and Endogenous Lipophilic Substrates

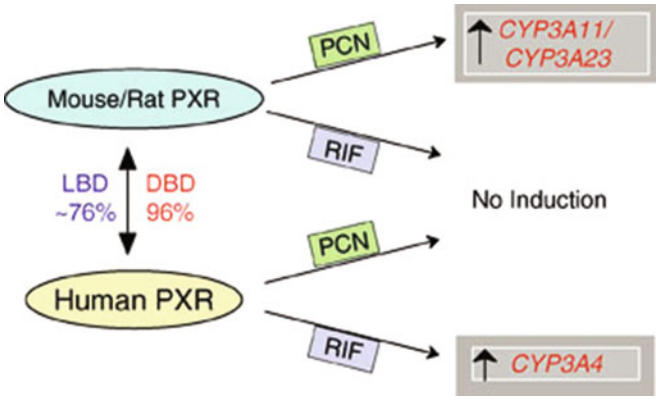
PXR is the major transcription factor that mediates the induction of CYP3A enzymes (Hakkola et al. 2018), most notably CYP3A4, the most abundant P450 enzyme in human liver. CYP3A4 is highly expressed in the liver and intestine, where it metabolizes structurally diverse drugs, environmental chemicals, endogenous steroid hormones, and lipophilic bile acids (Chai et al. 2016). The high level of expression of CYP3A4, coupled with its broad substrate specificity and

widespread inducibility following exposure to diverse steroids, antibiotics, and other pharmacological agents that activate PXR, gives rise to many CYP3A-based drug interactions.

PXR was initially thought to reside exclusively in the nucleus, but subsequent studies identified PXR in the cytosol. In contrast to CAR, PXR has little or no intrinsic basal transcriptional activity in the absence of ligand. Similar to CAR, the cytosolic form of PXR exists in a complex with co-chaperone proteins Hsp90 and CCRP (Mackowiak and Wang 2016). Upon direct binding by an agonist, the ligand-receptor complex dissociates from the co-chaperone proteins and translocates from the cytoplasm to the nucleus. Activated PXR, which is in a heterodimeric complex with RXR and binds various coactivators (e.g., steroid receptor coactivator 1), binds to specific DNA response elements in the promoter or enhancer regions of PXR target genes, enabling PXR to stimulate gene transcription. PXR activity may also be influenced by cellular signaling pathways that control posttranslational modifications, including phosphorylation, ubiquitination, acetylation, and sumoylation. Specific microRNAs (e.g., microRNA-148a-5p and microRNA-18a-5p) have been identified as posttranscriptional determinants in the expression and functionality of PXR.

Major species differences characterize the induction of CYP3A enzymes by drugs, steroids, and other chemicals (Chai et al. 2016). These species differences are a direct result of the species-dependent activation of PXR by individual PXR ligands (Fig. 2). Human PXR but not mouse PXR is activated by rifampicin and other xenochemicals that preferentially induce *CYP3A* genes in human cells and tissues, whereas mouse PXR but not human PXR is activated by the synthetic steroid pregnenolone 16α-carbonitrile (PCN). Mouse *PXR* gene knockout studies establish PXR as the major mediator of *CYP3A* induction by many xenochemicals. Moreover, a human pattern of *CYP3A* inducibility can be achieved when the endogenous mouse *PXR* gene is replaced by its human *PXR* counterpart. Mouse and human PXR exhibit an uncharacteristically high (~25%) divergence of amino acid sequence within the ligand-binding domain, suggesting that these rodent and human PXRs are unusually divergent orthologs whose evolution reflects their adaptation to the unique dietary constituents and distinct endogenous steroid profiles of each species.

PXR may serve as a broadly based "steroid and xenobiotic sensor" whose intrinsic physiologic function is to stimulate synthesis of CYP3A enzymes that catabolize endogenous steroidal



Nuclear Receptor Regulation of Hepatic Cytochrome P450 Enzymes, Fig. 2 Species-specificity of PXR's CYP3A induction response. Shown are the amino acid sequence identities of the COOH terminal-ligand-binding domain (LBD) and the central DNA-binding domain (DBD) of rodent and human PXR. *CYP3A11* and *CYP3A23* are mouse and rat P450 3A genes, respectively, whereas *CYP3A4* is a human P450 3A gene. *PCN* pregnenolone 16α-carbonitrile, *RIF* rifampicin

substrates (Chai et al. 2016). This possibility is supported by the striking responsiveness of PXR to endogenous steroids belonging to several distinct classes (pregnanes, estrogens, and corticoids) and by the catalysis by many CYP3A enzymes of 6β-hydroxylation reactions using diverse steroidal substrates, including androgens, corticoids, progestins, and bile acids. PXR plays a key role in bile acid homeostasis, as shown by the decreased production and increased hepatic uptake and detoxification of cholestatic bile acids, such as lithocholic acid, that is mediated by PXR. Activation of PXR by bile acids in liver leads to (1) decreased expression of *CYP7*, cholesterol 7α-hydroxylase, which catalyzes a key rate-limiting reaction of bile acid biosynthesis; (2) increased expression of the transporter *Oatp2*, which increases hepatic uptake of bile acids from the sinusoidal blood; and (3) induction of CYP3A enzymes that detoxify lithocholic acid by catalyzing its 6-hydroxylation.

PPARα: Xenochemical Induction of CYP4A Enzymes and Role in Rodent Hepatocarcinogenesis

CYP4A enzymes catalyze the oxygenation of biologically important fatty acids, including arachidonic acid and other eicosanoids. *CYP4A* gene transcription can be activated in both the liver and kidney by a range of acidic drugs and other xenochemicals, including hypolipidemic fibrate drugs, phthalate ester plasticizers used in the medical and chemical industries, and other environmental chemicals (Hakkola et al. 2018). These CYP4A inducers are classified as peroxisome proliferator chemicals because they markedly induce liver peroxisomal enzymes, leading to a dramatic increase in both the size and the number of liver cell peroxisomes.

PPARα is the nuclear receptor responsible for *CYP4A* induction, peroxisomal enzyme induction, and hepatic peroxisome proliferation (Hakkola et al. 2018). The tissue distribution of PPARα (liver >kidney >heart >other tissues) mirrors the responsiveness of these tissues to peroxisome proliferator chemicals. *CYP4A* induction in the liver and kidney and hepatic peroxisome proliferation are both abolished in *PPARα* gene knockout mice, demonstrating the essential role of PPARα for these responses in vivo. The general mechanism of PPARα activation is similar to that of other nuclear receptors. PPARα is found in the nucleus in the basal state as a complex with corepressor proteins. Ligand binding leads to dissociation of PPARα from its corepressor proteins, heterodimerization with RXR, recruitment of coactivators, and binding to functional DNA response elements, referred to as peroxisome proliferators response elements (PPREs), in the 5′-flank of *CYP4A* and other target genes, resulting in stimulation of gene transcription. PPARα-RXR complexes bound to PPREs can be synergistically activated by the combination of a PPARα ligand with the RXR ligand 9-*cis*-retinoic acid.

Persistent activation of PPARα can induce the development of hepatocellular carcinoma in susceptible rodent species by a non-genotoxic mechanism, i.e., one that does not involve direct DNA damage by peroxisome proliferator chemicals or their metabolites. This hepatocarcinogenic response is abolished in mice deficient in PPARα, underscoring the central role of PPARα, as opposed to that of two other mammalian PPAR forms (PPARγ and PPARδ), in peroxisome proliferator chemical-induced hepatocarcinogenesis. Other toxic responses, such as kidney and testicular toxicities caused by exposure to certain phthalate di-ester plasticizers, are not abolished in PPARα-deficient mice, raising the possibility that the latter toxicities may be mediated by PPARγ or PPARδ.

Pharmacological Relevance

Importance of Nuclear Receptors for Drug Metabolism and Drug Development

The identification of specific nuclear receptors as molecular targets of P450 inducers impacts drug metabolism and drug development in several important ways:

1. Drug interactions, often associated with interindividual differences in drug metabolism, are a major contributor to idiosyncratic drug responses, which can sometimes be fatal. P450 induction, especially the induction of CYP3A

enzymes via PXR, can contribute significantly to interpatient differences in drug metabolism. High throughput screens for P450 inducers that activate AhR, CAR, PXR, and PPARα have been developed and can readily be applied to characterize the P450 induction potential of drugs currently used in the clinic, as well as investigational drugs and lead compounds under development. These efforts may help to predict, and thereby avoid, drug interactions associated with P450 induction.

2. Interindividual differences in the function and expression of nuclear receptors and their accessory proteins, reflecting either genetic or epigenetic factors, may represent another set of determinants of interindividual differences in pharmacokinetics and possibly pharmacodynamics. Further elucidation of the factors that regulate cellular nuclear receptor levels (e.g., glucocorticoids, which increase expression of PXR in human hepatocytes) and the identification of genetic polymorphisms that impact receptor expression, ligand binding specificity or transcriptional activity are also likely to be important.
3. Receptor proteins involved in the induction of cytochromes P450 and other enzymes of drug metabolism may serve as novel drug targets. Examples of established nuclear receptor drug targets include PPARα, which is a target of hypolipidemic fibrate drugs, and PPARα, which is targeted by anti-type II diabetes drugs of the thiazolidinedione class. CAR and PXR are also therapeutic targets based on the role of CAR activators in the treatment of jaundice and PXR activators in the relief of cholestasis associated with hepatotoxic bile acids, hypercholesterolemia, and inflammatory bowel disease. PXR antagonists might be developed to block CYP3A auto-induction responses, which can substantially shorten the elimination half-life of a drug that simultaneously serves as a CYP3A inducer and a CYP3A substrate, which is a characteristic of several HIV-AIDS protease inhibitors and the anti-cancer drug ifosfamide. The finding that genes encoding liver and intestinal drug transporters are also targets of CAR and PXR (Hakkola et al. 2018) presents additional opportunities but also additional challenges in drug development.

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O

Omega-3 Fatty Acids

► Lipid-Lowering Drugs

$1,25(OH)_2D_3$

► Vitamin D

14-3-3 Proteins

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Synonyms

BMH1; BMH2; Leonardo; RAD24; RAD25

Definition

The 14-3-3 s are a family of <30 kDa proteins that exist as homo- and heterodimers within all eukaryotic cells. 14-3-3 proteins bind target proteins containing phosphothreonine or phosphoserine motifs; however, exceptions do exist. The docking of 14-3-3 s to their target can (i) cause conformational changes in the target protein, (ii) block sequence-specific or structural features on the target protein, or (iii) function as a scaffold allowing other proteins to dock.

Basic Mechanisms

Recognizing Covalent Modifications

Evolution has provided the cell with a repertoire of 20 amino acids to build proteins. The diversity of amino acid side chain properties is enormous, yet many additional functional groups have been selectively chosen to be covalently attached to side chains, and this further increases the unique properties of proteins. These additional groups play a regulatory role allowing the cell to respond to changing cellular conditions and events. Known covalent modifications of proteins now include phosphorylation, methylation, acetylation, ubiquitylation, hydroxylation, uridylylation, and glycosylation, among many others. Intense study in this field has shown the addition of a phosphate moiety to a protein can result in several potential effects including control of enzymatic activity, the shuttling of proteins between cellular compartments, and regulation of proteolytic degradation. More recently the study of signal transduction events has provided a new understanding of the regulation of cellular activities by protein-protein interaction, and many of the covalent modifications mentioned above perform their function by providing specific docking sites for other proteins. Again, the field to protein

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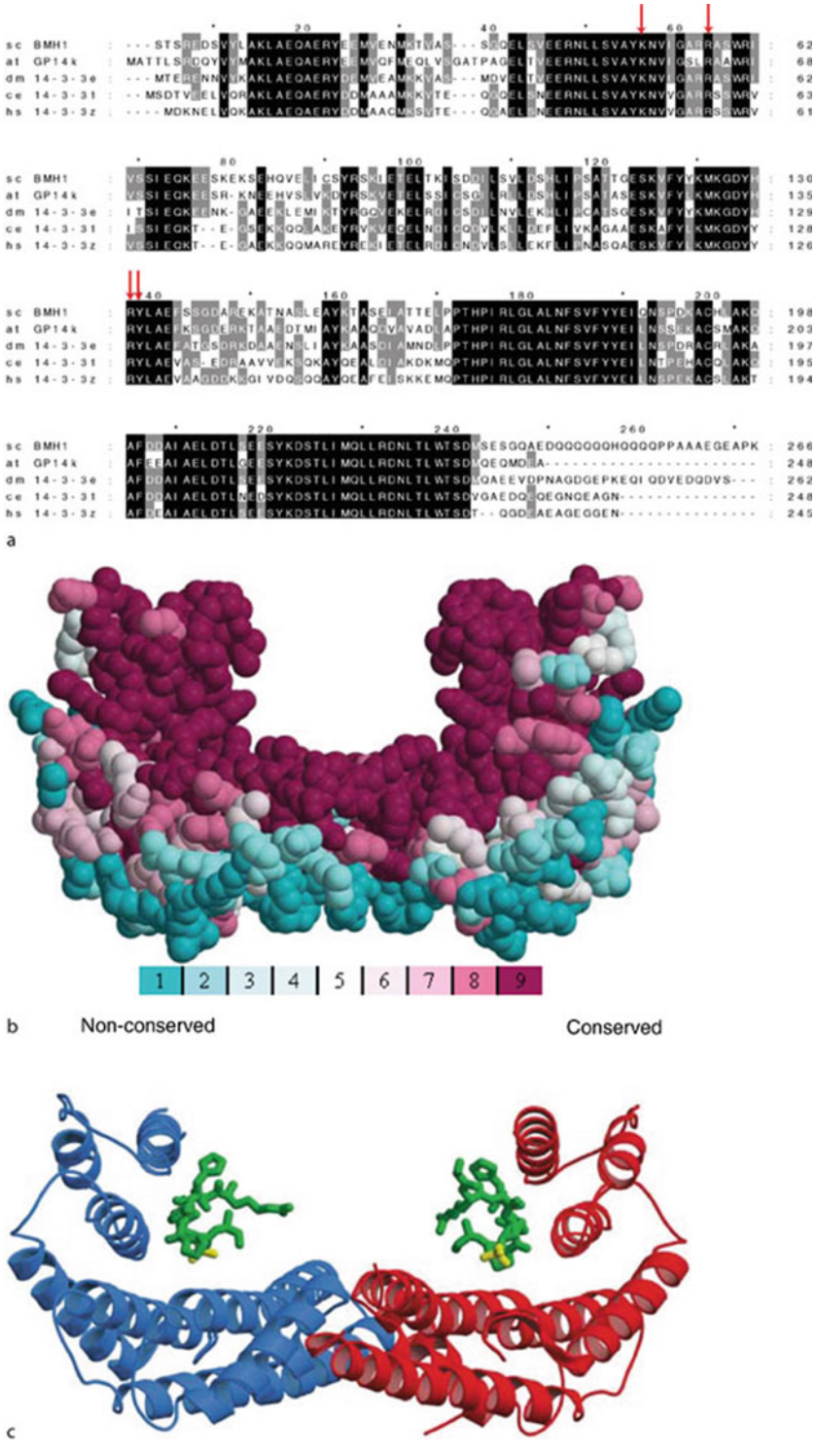
phosphorylation was a forerunner in recognizing and characterizing this property of covalent modifications, and the 14-3-3 proteins were the first protein shown to specifically bind a phosphoserine or phosphothreonine motif. Thus, the generation of specific docking sites for other proteins is an additional, regulatory function for protein phosphorylation. Other phospho-specific binding modules that can be recognized by amino acid sequence within proteins include leucine-rich repeats (LRR), forkhead-associated (FHA), WW, FF, tetratricopeptide repeat (TPR), MH2, WD40, polobox, and BRCA1 carboxyl-terminal (BRCT) domains for serine and threonine phosphorylation and Src homology 2 (SH2) and protein tyrosine binding (PTB) domains for phosphotyrosine. It is possible that additional phospho-specific binding modules exist, although no new ones have been identified for several years now, suggesting the inventory is complete.

The 14-3-3 proteins were initially catalogued as abundant brain proteins and designated 14-3-3 based on their chromatography column elution fraction and position after starch gel electrophoresis (Bridges and Moorhead 2005). Defining the role for these proteins began many years later when they were identified initially as activators of tryptophan and tyrosine hydroxylases, and then as regulators of protein kinase C and the protein kinase Raf-1, and as a direct binding protein for the polyomavirus middle T antigen. This was followed by the key observation that binding of 14-3-3 to its target protein was dependent on the phosphorylation of the target protein. Since this discovery, 14-3-3 s have continuously surfaced as regulators of a multitude of phosphorylation-dependent cellular events (Yaffe 2002; Mackintosh 2004; Bridges and Moorhead 2005; Ballone et al. 2018).

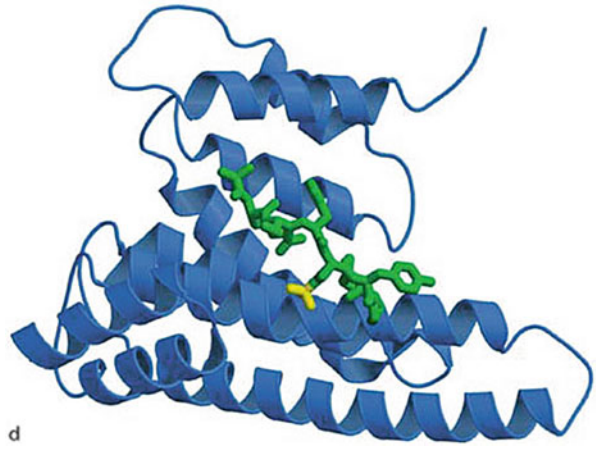
14-3-3 Sequence Conservation, Structure, and Function

Cloning and genome sequencing has shown there to be multiple 14-3-3 genes in all eukaryotic organisms with 12 in *Arabidopsis*, 7 in mammals, and 2 in *Saccharomyces cerevisiae*, *C. elegans*, and *D. melanogaster*. No 14-3-3 or 14-3-3-like genes have been noted any archaeal or bacterial genomes. The *S. cerevisiae* 14-3-3 isoforms are encoded by *BMH1* and *BMH2*, and deletion of either does not cause growth defects, but the double deletion of both is lethal. The 14-3-3 s are highly conserved across species (Fig. 1a) with divergence occurring on the N- and C-termini. The 14-3-3 s have acidic pI values, are small in size (<28–32 kDa), and form homo- and heterodimers. More than 40 crystal structures have been elucidated for the unliganded form and 14-3-3 in complex with a small molecule and/or high-affinity phosphopeptide from a known target protein (Bridges and Moorhead 2005). Dimers form a cup shape with the most highly conserved amino acids lining the inside of the cup (Fig. 1b). It is this conserved inner surface that functions as docking site for the phosphoprotein(s). Each subunit has the ability to bind a phosphopeptide, and as shown in Fig. 1c, phosphopeptides dock in an extended antiparallel orientation. Residues equivalent to Lys ^{49}, Arg ^{56}, Arg ^{127}, and Tyr ^{128} of human 14-3-3ζ are completely conserved in every 14-3-3 known, and it is these residues that are responsible for direct interaction with the phosphate moiety of the bound phosphoprotein (Fig. 1c, d). It is interesting to note that crystallization of the nonsense-mediated decay regulatory protein, SMG7 (suppressor with morphogenetic effects on genitalia 7), revealed structural resemblance of one of its TPR domains to one monomer of a 14-3-3 protein, even with very limited (<10%) sequence homology. Additional work showed that this region, like 14-3-3, binds peptides in a phosphorylation-dependent manner.

When 14-3-3 s were first identified as phosphorylation-dependent binding proteins (note that a selection of non-phosphorylated targets are known), target protein phosphorylation sites were mapped, and it was immediately apparent that 14-3-3 s bound preferentially to specific phosphorylation motifs. The advent of oriented peptide libraries and their application to 14-3-3 binding specificity confirmed this first motif (RSX1, 2pSXP, where R is arginine, X is any amino acid, S is serine, pS is phosphoserine, and



14-3-3 Proteins, Fig. 1 (continued)



14-3-3 Proteins, Fig. 1 Conservation of peptide binding by 14-3-3 protein. (**a**) Sequence alignment of evolutionarily diverse 14-3-3 proteins. Residues that are 100% identical are shaded in black, and residues that are >50% identical are shaded in gray. Proteins from *Saccharomyces cerevisiae* (Bmh1), *Arabidopsis thaliana* (GP14κ), *D. melanogaster* (14-3-3ε), *Caenorhabditis elegans* (14-3-3 isoform 1), and human (14-3-3ζ) are included in the alignment. Residues that directly contact the phosphate group of target proteins are indicated with arrows. (**b**) Structure of 14-3-3ζ shaded according to residue conservation using 14-3-3 isoforms from humans (7 isoforms), *A. thaliana* (12 isoforms), *S. cerevisiae* (2 isoforms), *D. melanogaster* (2 isoforms), and *C. elegans* (2 isoforms). Structures (**b**, **c**, and **d**) based on 14-3-3ζ [PDB 1QJB]. In (**c**) and (**d**), the peptide (ARSHpSYPA) binds in an extended conformation to 14-3-3 (shown as a ribbon diagram in different shades of gray for each monomer) within the conserved groove of each monomer. (Figure from Bridges and Moorhead, *Sci STKE* 2004 RE10. Kindly provided by permission from *Sci STKE*)

P is proline) that is now described as mode I binding. The peptide library method also pulled out another high-affinity binding motif now designated mode II (RXXXpSXP).

In addition to these initially characterized 14-3-3 binding motifs, other studies identified 14-3-3 binding to the C-termini of proteins, and this mode, or mode III, is now recognized as a significant target for 14-3-3 s. This motif was first identified in the plant plasma membrane H^+-ATPase, where the C-terminal sequence QSYpTV-COOH was found to bind 14-3-3 resulting in relief of inhibition of the enzyme by the C-terminus. Binding and relief of inhibition are dependent upon phosphorylation of the second to last residue, in this case a threonine. In a genetic screen of random peptides aimed to identify C-terminal signal motifs that override endoplasmic reticulum (ER) retention of proteins destined for the cell plasma membrane, a group of C-terminal peptides that function through an interaction with 14-3-3 were identified, and this is much like the sequence of the H^+-ATPase C-terminal sequence. The genetic screen suggested preference for a hydrophobic amino acid at the C-terminus and a serine or threonine at −2 from the C-terminus. Further work has defined this binding motif as SWpTX $_{\{Pro\}}$-COOH (where pT is phosphothreonine, X is any amino acid except proline, and COOH is the carboxyl-terminus of the protein). Interestingly, a number of plasma membrane receptor proteins carry this motif.

Another novel 14-3-3 binding motif was uncovered during studies on histones and should be designated mode IV (Macdonald et al. 2005). Histones are among the most conserved proteins known with covalent modification of their tails, which includes lysine acetylation and methylation, arginine methylation, and seine phosphorylation, being responsible for altering access to DNA by charge neutralization and for generating high-affinity binding sites for other proteins. Acetylated lysine and methylated lysine residues are known to dock bromo- and chromodomains, respectively. Histone H3 is phosphorylated at Ser-

10 and 28, which are both preceded by the sequence for Ala-Arg-Lys (ARK). The precise function of this modification was unknown until the N-terminal 20 amino acids were synthesized as unmodified, acetylated (Lys-9 and 14), and acetylated plus Ser-10 phosphorylated peptides and were used for affinity chromatography with HeLa cell nuclear extracts. The only proteins found to bind specifically to the phosphorylated and acetylated (and not acetylated only) versions were 14-3-3 proteins ε, ζ, and γ. The crystal structure of 14-3-3ζ complexed with the acetylated and phosphorylated peptide (AR $_{Ac}$KpSTGG $_{Ac}$K, where $_{Ac}$K is acetylated lysine and pS is phosphoserine) revealed that the basic residues lining the binding pocket as shown in previous structures bind the phosphate moiety. Mode I and II binding motifs have a proline at +2 that allows the bound peptide or protein to change direction and exit the binding cleft. Here positions +2 and +3 have glycine residues that perform the same function. Interestingly, peptides with a glycine at the +2 position were identified as 14-3-3 binding in the original oriented peptide binding studies. The acetyl group at lysine 9 is folded back, and hydrogen bonded to the backbone amide. The exit from the cleft after the two glycines means that acetyl-lysine 14 is not in the binding cleft. Although making no contacts with 14-3-3, acetyl-lysine 14 is thought not to be accessible in any way to proteins that bind acetyl-lysine motifs, through motifs such as bromodomains. This structural study nicely demonstrates how 14-3-3 binding can mask another structural feature altering a property of the protein (see below for 14-3-3 roles).

Modes of Action

Early work on 14-3-3 proteins placed researchers in a peculiar position of trying to define the precise function of 14-3-3 s. This conundrum came from the large number of and apparently diverse types of target proteins. Continued work has brought more and more insights, and it is fairly well accepted that 14-3-3 s play potentially three functions when bound to a target. They are (i) 14-3-3 directed conformational changes, (ii) 14-3-3 blockage of sequence-specific or structural features, and (iii) scaffolding.

14-3-3 directed conformational changes. The 14-3-3 proteins are primarily α-helical and are thus described as rigid in nature. The 14-3-3 structure in the presence of phosphopeptides or the target enzyme serotonin *N*-acetyltransferase supports this notion as no change in 14-3-3 shape is observed in the co-crystals. This has led to the molecular anvil or clamping hypothesis for 14-3-3 function whereby the sturdy 14-3-3, upon binding certain targets, causes them to be reshaped and thus altering their function. This was first described for the enzyme serotonin *N*-acetyltransferase. Serotonin *N*-acetyltransferase is normally catalytically inefficient and after phosphorylation and 14-3-3 binding displays increased Vmax and increased substrate affinity. The binding of 14-3-3 causes a local alteration in enzyme, reshaping the active site. More recently the binding of 14-3-3 to chicken mannitol-1-phosphate dehydrogenase was shown to inactivate the enzyme upon completion of mannitol biosynthesis. This concept can also be extended to long-distance reshaping of proteins as well where it is thought that 14-3-3 binding can expose or hide protein features or motifs distant from the site binding. For instance, data now support the idea that 14-3-3 binding to several nuclear histone deacetylases (HDACs) causes exposure of a nuclear export sequence (NES) and shuttling from the nucleus.

14-3-3 blockage of sequence-specific or structural features. The physical association of 14-3-3 with a target can also mask or occlude sequence-specific motif or structural features. Examples include the masking of nuclear localization or export sequences, and as introduced above, it has been well characterized that the C-terminus of the plant plasma membrane H + -ATPase inhibits the activity of the enzyme. After phosphorylation on a mode III motif, 14-3-3 binding removes the tail of the enzyme, and the ATPase is activated. Another example of 14-3-3 function by sequence masking has been noted for several proteins synthesized in the endoplasmic reticulum (ER) and destined for the plasma membrane. Many proteins synthesized in the ER are retained there by a di-arginine motif that is "recognized" by the coat protein complex I (COPI) retention machinery. Using an affinity-binding method,

14-3-3 s were identified as interacting proteins for the dibasic motif of the C-terminus of the potassium channel α subunit, Kir6.2. 14-3-3 proteins do not recognize the monomeric form of the Kir6.2 tail but do bind the multimeric Kir6.2 complex and compete for binding to the dibasic region with the COPI machinery. This suggests that bound 14-3-3 masks the COPI interaction site and thus causes release of Kir6.2 from the ER.

Scaffolding. Because 14-3-3 s can potentially bind more than one phosphoprotein at once due to the presence of two phosphopeptide-binding sites in a dimer, it has been postulated that they play this role in vivo. Direct observation of this phenomenon is difficult to show, but data from several groups support this idea.

Binding Specificity Among 14-3-3 Isoforms

It is not clear why some organisms have two 14-3-3 isoforms while others have up to 12. Binding 14-3-3 inhibits the plant enzyme nitrate reductase, and there appears to be no selectivity between plant 14-3-3 isoforms; in fact yeast and human isoforms appear to work equally as well in vitro. The best example where selectivity has been demonstrated is human 14-3-3σ. 14-3-3σ preferential homodimerizes with itself, and crystallization revealed a structural basis for this isoform's dimerization properties as well as for its specific selectivity for target binding proteins. Here partner specificity is the result of amino acid differences outside of the phosphopeptide-binding cleft.

14-3-3 Interactome

In 2004, ~350 proteins had been identified in global 14-3-3 interaction studies with the most astounding thing to emerge being the vast repertoire of cellular processes that 14-3-3 plays a role in (Mackintosh 2004). Several early studies purified 14-3-3 as an effector molecule that regulated some assayable property of a protein, or 14-3-3 was simply found as a co-purifying protein with some specific target. One of the best-characterized examples is the purification of 14-3-3 as inhibitor of plant nitrate reductase. In the first global study to find new 14-3-3 interactors, yeast 14-3-3 s (BMH1 and 2) were coupled to a matrix, and a plant extract passed over this affinity column to allow proteins to bind. These were affinity eluted with mode I phosphopeptide and released proteins identified. This work found mostly higher abundance metabolic enzymes as the primary targets. Later studies have employed a similar approach or used specific human 14-3-3-isoforms. Identified targets include proteins involved in many metabolic pathways, protein trafficking, signal transduction, and transcriptional regulation. Prior to 2015, Scansite was used as the standard predictor to identify potential 14-3-3 binding sites based on mapped phosphorylation sites. This tool was developed using a limited number of known 14-3-3 binding sites that all essentially conformed to a mode I motif. By 2015, high-throughput mass spectrometry studies had catalogued more than 2000 phosphoproteins that dock 14-3-3 s. Using this updated dataset, a new 14-3-3 binding predictor was developed for a motif spanning from −6 to +4 around the phospho-binding site ((Madeira et al. 2015) and http://www.compbio.dundee.ac.uk/1433pred). At the time of publication (2015), this new software predicted the number of 14-3-3 binding sites in the human proteome to be 10,881 derived from 5483 proteins, constituting more than 9% of all serine/threonine phosphosites in the human proteome databases.

Pharmacological Intervention

The 14-3-3 s are abundant eukaryotic proteins that have emerged as key regulators of a vast number of cellular functions. Protein-protein interactions are a fundamental component of protein function and essentially all biological process. As a consequence, aberrant protein-protein interactions are often a component of disease states, and modulation of protein-protein association by small molecules (drugs) has emerged as a component of drug discovery programs. As the list of 14-3-3 interacting proteins grows, links with human disease are becoming clearer. Structural studies have defined the interaction surfaces of 14-3-3 s with several targets and have now placed them as potential drug targets. Modulation of 14-3-3-

client binding could be stabilizing or disruption of the interaction with the majority of small molecules binding in or near the interaction site of the two proteins. Given the wide variety and important roles played by 14-3-3 s in biological process, it is no surprise that natural products have evolved to control 14-3-3 partner association. Fusicoccin is a fungal metabolite that promotes the association of 14-3-3 with the plant plasma membrane H + -ATPase. Other complex stabilizers include cotylenin and mizoribine. In addition, high-throughput screening identified pyrrolidone1 as a molecule that stabilizes 14-3-3 H + -ATPase interaction. Several peptide- (R18 and ExoS) and non-peptide-based molecules have also been discovered that disrupt binding of 14-3-3 to clients (Ballone et al. 2018). Just as intriguing is the discovery that the key energy metabolite adenosine monophosphate (5'-AMP) has been shown to stabilize the association of 14-3-3 with the carbohydrate-response element-binding protein (ChREBP) in a phosphorylation-independent manner. The co-crystal structure shows that 5'-AMP occupies the same region as the phosphoserine or phosphothreonine of the phospho-ChREBP.

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Opioid Systems

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Definition

The term "opioids" was traditionally used in a narrow sense for drugs derived from opium that produce a pharmacological effect similar to morphine. After cloning of all opioid receptors and ligand precursor proteins, the term is now referring to a gene family and corresponding drugs acting on opioid receptors. Opioids include all natural and synthetic ligands that interact with the four endogenous opioid receptors (MOP, DOP, KOP, NOP). Endogenous opioid ligands are peptides derived from the precursor proteins proopiomelanocortin (*pomc*), proenkephalin (*penk*), prodynorphin (*pdyn*), and pronociceptin/orphanin FQ (*pnoc*). Traditionally, opioid receptors had been characterized by pharmacological tools which identified only three binding sites, termed mu, delta, and kappa. These are still referred to as "classical opioid receptors" and are promiscuous for their endogenous ligands as well as most of the opium-derived alkaloids. The classical opioid receptors also share the common antagonist naloxone. The nociceptin/orphanin FQ (N/OFQ) system is pharmacologically separate, yet genetically part of the family.

Basic Characteristics

Receptor Subtypes and Opioid Ligands

The three classical opioid receptors mu, delta, and kappa were first defined as promoting the analgesic actions of morphine and its derivatives (Zöllner and Stein 2007). Later, morphine analogues and related synthetic compounds were used to further refine their pharmacological characterization and anatomical distribution. All endogenous ligands of the classical opioid receptors are peptides that share the aminoterminal

sequence Tyr-Gly-Gly-Phe, known as the canonical opioid motif. They are derived from three different precursor proteins (Table 1) and can activate all three classical opioid receptors promiscuously, yet with different potencies. The precursor proteins each harbor multiple active neuropeptides that are released by proteolytic processing. The pro-opiomelanocortin precursor (POMC) is unique in that it contains the opioid peptide β-endorphin together with adrenocorticotropin (ACTH) and the melanocyte-stimulating hormones (MSH) that act on a family of pharmacologically distinct receptors. (NB: The peptides endomorphin 1 and 2 are questionable as endogenous ligands since the human genome contains no equivalent sequences.)

Cloning of the delta opioid receptor in 1992 (Evans et al. 1992; Kieffer et al. 1992) allowed for the swift molecular identification of all three classical opioid receptors and produced an unexpected fourth new family member, originally termed ORL-1 (opioid receptor-like). ORL-1 did not bind and was not activated by any of the known endogenous or synthetic opioid ligands and was thus an orphan GPCR. In 1995, two groups simultaneously identified the endogenous ligand of ORL-1 and termed it nociceptin or orphanin FQ (N/OFQ) (Meunier et al. 1995; Reinscheid et al. 1995). N/OFQ does not bind or activate the three classical opioid receptors and contains distinct structural motifs that ensure and explain its pharmacological selectivity. However, N/OFQ contains the amino terminal sequence Phe-Gly-Gly-Phe and still bears significant homology to the endogenous opioid peptides, especially dynorphin A. It was suggested that the pro-N/OFQ precursor gene may have arisen through gene duplication of the prodynorphin precursor gene.

Opioid Systems, Table 1 Endogenous opioid precursors and ligands

Name	Encoded opioid peptides	Other peptides (active at different receptors)
Proopiomelanocortin	β-Endorphin	ACTH, MSH (α, β, γ)
Proenkephalin	Leu-Enkephalin, Met-Enkephalin	
Prodynorphin	Dynorphin A, Dynorphin B, α-Neoendorphin	
Pronociceptin/ Orphanin FQ	Nociceptin/ Orphanin FQ	Nocistatin (receptor unknown)

Cloning of the human genome ultimately confirmed the existence of only four genuine opioid receptor genes. Receptor subtypes that had been postulated based on pharmacological tools could not be identified at the genetic level and splice variants of the classical opioid receptors did not reproduce the subtype-specific pharmacology. Current hypotheses suggest tissue-specific interaction of certain opioid receptors with other proteins that might be responsible for the distinct pharmacology, although no such protein partners have been identified yet.

Selective agonists and antagonists exist for all four opioid receptors (Table 2). In vivo studies with knockout mice have demonstrated that all main effects of morphine are mediated exclusively by the MOP receptor, including residual analgesic effects of presumably selective compounds such as DPDPE or deltorphin in DOP knockout mice. In general, pharmacological separation exists between the classical opioid receptors (DOP, KOP, and MOP) and the NOP-N/OFQ system. Only at unphysiologically high concentrations some alkaloid opioids produce weak effects at NOP. However, recent medicinal chemistry efforts have discovered bifunctional compounds with dual MOP-NOP activity, such as cebranopadol and AT-121 (Ding et al. 2018).

Physiology

At the cellular level, all opioid receptors interact via $G_{i/o}$-types of G proteins with the same second messenger systems, resulting in inhibition of cyclic AMP accumulation, opening of GIRK potassium channels, inhibition of voltage-sensitive Ca^{2+} channels, and stimulation of common downstream kinases such as MAPK or c-src. Overall, activation of opioid receptors leads to reduced neuronal excitability through hyperpolarization.

At the organism level, all opioids produce some form of analgesia by inhibiting nociceptive

Opioid Systems, Table 2 Opioid receptor subtypes and ligands

Receptor	Endogenous ligands	Selective agonists	Alkaloid/synthetic agonists	Antagonist (∗ selective)
MOP (μ)	β-Endorphin Leu/Met-Enkephalin	DAMGO	Etonitazene Morphine Fentanyl	Naloxone Diprenorphine CTAP∗
DOP (∂)	Leu/Met-Enkephalin β-Endorphin	DPDPE Deltorphin I/II	Diprenorphine Etorphine Morphine	Naltrindole∗ Naltriben∗ Naloxone
KOP (κ)	Dynorphin A Dynorphin B α/β-Neoendorphin	Nalfurafine U-69593	Etorphine Morphine	Diprenorphine nor-Binaltorphimine∗ Naloxone
NOP	Nociceptin/Orphanin FQ	UFP-102 Ro64-6198	Cebranopadol AT-121 (also MOP partial agonist)	UFP-101∗ J-113397

Opioid Systems, Table 3 Physiological effects of opioid systems

Receptor	Analgesia	Sedation	Other agonist effects	Agonist abuse potential
MOP (μ)	Central and peripheral	Profound	Respiratory depression, constipation, Euphoria	Very high
DOP (∂)	Spinal and peripheral	Low	Antidepressant, seizures	Low
KOP (κ)	Visceral and central	None	Dysphoria	None
NOP	Spinal (central: reversal of MOP-mediated analgesia)	Profound	Anxiolysis	None

signaling pathways, either in the brain or periphery (Table 3). MOP carries all central and peripheral effects of morphine that also include therapeutically undesirable side effects such as respiratory depression, constipation, and addiction. DOP-mediated analgesia is mainly produced at the spinal cord level while central DOP activation was shown to produce antidepressant effects, a combination that may have therapeutic potential in the treatment of chronic pain states. DOP full agonists were found devoid of abuse liability but unfortunately produced seizures in animal models, which limited their further clinical development. KOP receptors are expressed both centrally and in sensory fibers innervating many internal organs, so that KOP agonists were found to mediate visceral and central analgesia. However, centrally active KOP agonists also produce significant dysphoria, which has hampered further clinical development. Activation of NOP receptors produces opposing effects on nociception, depending on receptor localization. At the spinal level, NOP agonists produce analgesia, whereas supraspinal administration of NOP agonists reverses effects of endogenous or exogenous opioids, including reversal of stress-induced analgesia. This complex interaction between NOP and MOP signaling has spawned interest into developing bifunctional compounds that may possess a larger therapeutic window with reduced side effect profile. In addition, NOP agonists are potent anxiolytics. Both MOP and NOP agonists also produce significant sedative effects.

Receptor Regulation

After agonist stimulation, all opioid receptors undergo desensitization, followed by internalization and eventual recycling or degradation. Desensitization is intimately linked to agonist-dependent phosphorylation of serine and threonine residues located in the carboxylterminal tail of the receptors. Different G protein-coupled receptor kinases (GRKs) have been identified for each opioid receptor, sometimes depending on

Opioid Systems, Table 4 Opioid receptor regulation

Receptor	Phosphorylation (residues[a])	GRKs	Internalization
MOP (μ)	T372 S377 (primary) T378 T381	GRK2/3 (full agonists) GRK5 (morphine)	High: Fentanyl, DAMGO Low: Morphine, buprenorphine
DOP (∂)	T361 S363 (primary)	GRK2/3	High: SNC80 Low: ADL5859
KOP (κ)	S356 T357 T363 (primary)	GRK2/3	High: U50,488 Low: Nalfurafine
NOP	S346 (primary) S351 S362 S363	GRK2/3	High: Ro64–6198 Low: NNC 63–0532

[a]Amino acid numbering refers to the most common isoforms of human opioid receptor proteins

agonist selection (Table 4). For each opioid receptor, individual "phosphorylation bar codes" were identified that depend on agonist efficacy (Kliewer et al. 2019; Mann et al. 2019; Miess et al. 2018). Phosphorylation always proceeds along a hierarchical pattern and partial agonists usually stimulate only phosphorylation of the primary serine (S) or threonine (T) residue (Table 4).

Recently, phosphorylation-dependent desensitization was demonstrated as a necessary prerequisite for the analgesic effects of MOP opioids and the subsequent development of tolerance, while not affecting reinforcement or respiratory depression and constipation, commonly produced by MOP agonists (Kliewer et al. 2019). Transgenic mice expressing phosphorylation-deficient MOP showed increased analgesic responses to morphine and fentanyl without developing analgesic tolerance after chronic opioid infusions for 1 week. However, nontolerant mice still displayed signs of opioid withdrawal, respiratory depression, and constipation, demonstrating that opioid tolerance and addiction involve separate physiological and molecular mechanisms.

Receptor phosphorylation is a prerequisite for recruitment of beta-arrestins which induce separate intracellular signaling cascades (mostly involving kinases). Arrestin binding to the phosphorylated GPCR is also necessary for receptor internalization. It was recently proposed that signaling through G proteins may be required for the analgesic effects of opioids while arrestin signaling may be involved in the majority of undesirable side effects including respiratory depression and addiction (Manglik et al. 2016). However, phosphorylation-deficient MOP can no longer recruit arrestins, yet the transgenic mice still display the full spectrum of opioid side effects, raising questions about this hypothesis (Kliewer et al. 2019).

Desensitization, phosphorylation, and internalization of other opioid receptors have also been investigated in vitro and in vivo. In general, phosphorylation-deficient opioid receptors no longer desensitize upon prolonged agonist exposure, are unable to recruit arrestins, and thus do not internalize. It remains to be seen whether this novel understanding of opioid receptor regulation can improve the development of future opioid analgesics.

Drugs

Therapeutic Applications and Abuse

Only alkaloid and synthetic MOP agonists are clinically used, primarily for control of severe pain. These include the common analgesics morphine, hydrocodone, oxycodone, codeine, fentanyl, methadone, and buprenorphine. Heroine is a more potent morphine analogue that is commonly abused by opioid addicts but has no clinical application. Prolonged administration of these compounds produces tolerance and strong

reinforcing effects, ultimately leading to addiction that is characterized by severe withdrawal symptoms upon cessation of drug intake. Weak partial agonists such as buprenorphine usually possess lower abuse liability and a reduced risk of fatal overdose, but are still addictive after prolonged administration.

In the last decade, massive over-prescription of opioid analgesics has led to the so-called "opioid crisis," in particular in the United States of America, with an estimated two to three million opioid-addicted Americans and an average of 130 deaths per day by accidental opioid overdose. This public health problem has rejuvenated the search for "safer opioids," especially devoid of the respiratory depressant effects of common opioid analgesics.

It was recently suggested that differential intracellular coupling pathways could separate analgesic effects from undesirable or potentially hazardous effects of MOP agonists. The concept of "biased signaling" posits that MOP agonists preferentially activating the G protein pathway should be mainly antinociceptive, while activation of the intracellular beta-arrestin pathway might be responsible for respiratory depression and constipation. Although it was possible to identify compounds inducing biased signaling at MOP in vitro (Manglik et al. 2016), clinical trials could not confirm the absence of undesirable side effects and failed to show therapeutic benefits compared to morphine. It is thus unclear at this time, whether the concept of biased signaling has any therapeutic potential or if it is an in vitro phenomenon that cannot be easily translated into a complex clinical application.

An alternative approach focuses on bifunctional ligands with agonist activity at both MOP and NOP, since co-administration of MOP and NOP agonists was found to reduce abuse liability while retaining analgesic drug effects. The first compound developed along this line was cebranopadol which is currently in clinical trials for treatment of chronic pain. Another promising candidate might be AT-121 which has partial agonist properties at both MOP and NOP and displayed no respiratory depressant or reinforcing effects in a primate model (Ding et al. 2018).

The diterpenoid (i.e., nonalkaloid) KOP agonist salvinorin A has recently entered the list of abused substances, mainly due to its hallucinogenic effects, although it also produces significant dysphoria and anhedonia. Salvinorin A has nanomolar affinity for KOP, but is also a partial agonist at dopamine D2 receptors, which may explain its atypical pharmacological effects in comparison to pure KOP agonists. Since selective DOP, KOP, and NOP agonists have no psychotropic effects, their abuse liability is considered low and no reports of illicit use have been published.

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Oral Contraceptives

► Contraceptives

Organ Dysfunction

► Sepsis

Ovarian Cancer G Protein-Coupled Receptor 1 (OGR1, GPR68)

► Proton-Sensing GPCRs

Oxygenated Derivative of Cholesterol

► Oxysterols

Oxygenated Sterols

► Oxysterols

Oxysterols

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Synonyms

Cholesterol oxidation products; Oxygenated derivative of cholesterol; Oxygenated sterols

Definition and Introduction

Oxysterols are derived from cholesterol or related molecules and possess one or more additional oxygen-containing functional groups, which can exhibit potent biological activities. They tend to be more water-soluble than cholesterol by virtue of the additional polar functional groups which confer distinct physicochemical properties. Oxysterols are usually found at very low concentrations in biological samples (nM to μM), often at several orders of magnitude lower than cholesterol itself (Brown and Jessup 1999).

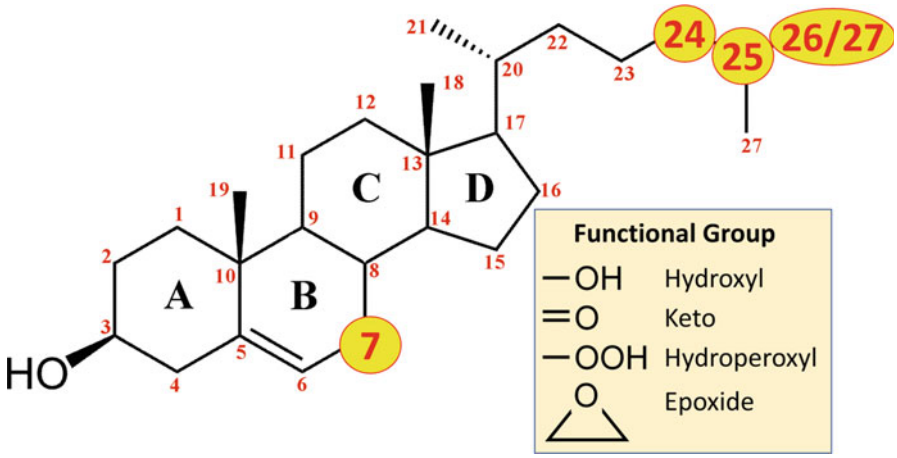
Initial interest in oxysterols stemmed from the idea that rather than cholesterol itself, some oxysterols control feedback regulation of cholesterol synthesis. Subsequent work indicated that oxysterols are likely to play only an auxiliary role in cholesterol homeostasis (Gill et al. 2008). However, work in the past decade has revealed important new roles for oxysterols in diverse fields, including immunity and development (Luu et al. 2016).

Nomenclature

The 27 carbons of cholesterol are numbered according to Fig. 1. In theory, many of these can be oxygenated to form distinct oxysterols. However, in practice the most commonly oxygenated sites are on carbons 7, 24, 25, and 26. Carbon-26 is more frequently referred to as 27 in the biological literature, and this nonsystematic name is used here for convenience. The oxygen-containing functional groups generally comprise hydroxyl, keto (oxo), hydroperoxyl, or epoxide groups. Primary oxysterols can be classified into side-chain (modified at C24, C25, or C27) or ring-modified (C7) oxysterols, while secondary oxysterols have more than one modification. Many of the individual oxysterol structures are provided in a recent review (Griffiths and Wang 2019).

General Principles of Oxysterols

In general, oxysterols can be made by both enzymic and nonenzymic means (**generators**). They can be



Oxysterols, Fig. 1 Numbered carbon structure of cholesterol showing where typical oxysterols are commonly oxygenated as well as the nature of the oxygen-containing functional group. Please note that more than one oxygenation can often occur, typically with oxygenation of the B-ring in addition to the side chain. Oxygenation at carbon-26 has most commonly been referred to as 27-oxygenated sterol, although it should be noted that this is a nonsystematic nomenclature

converted to other oxysterols (by **modifiers**) which can then have activities on various **effector** molecules. These three processes are summarized in the sections below with examples using the major players.

Generators

The oxidized low-density lipoprotein hypothesis of atherosclerosis led to considerable interest in free radical-mediated generation of oxysterols. Subsequently, interest shifted more to enzymic generation and modification of oxysterols. Many of the enzymes that generate oxysterols are cytochrome P450 monooxygenases, and three of these (CYP7A1, CYP7B1, CYP27A1) are pivotal in bile acid synthesis.

Free radical attack on unsaturated lipids can generate peroxyl radicals, resulting in oxysterol species like 7α-/β-hydroperoxycholesterol, which can decompose to 7α-/β-hydroxycholesterol and 7-ketocholesterol. This can occur in our bodies, and during processing of foods we then eat, although the contribution of dietary oxysterols to levels in humans is unclear (Brown and Jessup 1999). Originally, it was considered that many oxysterols that accumulate in disease states were produced by free radical attack. However, the source is now less certain as enzymic routes of generation are now known for many oxysterols which were previously thought to be exclusively nonenzymic in origin (e.g., 7-ketocholesterol).

Cholesterol 7α-hydroxylase (CYP7A1) catalyzes the first step in the classic bile acid synthesis pathway, producing 7α-hydroxycholesterol from cholesterol. CYP7A1 can also act on a precursor of cholesterol, 7-dehydrocholesterol, to make 7-ketocholesterol (Shinkyo et al. 2011). Consistent with this, patients who accumulate 7-dehydrocholesterol (due to a cholesterol synthesis defect, namely, Smith-Lemli-Opitz syndrome) also display elevated levels of 7-ketocholesterol.

Sterol 7α-hydroxylase (CYP7B1) is also a critical enzyme in bile acid synthesis, performing 7α-hydroxylation on side-chain oxygenated sterols like 27-hydroxycholesterol (Stiles et al. 2009).

Cholesterol 24-hydroxylase (CYP46A1) converts cholesterol into the more polar 24*S*-hydroxycholesterol (also known as cerebrosterol), providing the principal means by which excess cholesterol can be eliminated from the brain across the blood-brain barrier (Bjorkhem et al. 2019). In humans, CYP46A1 expression is restricted to the brain, whereas rodents have appreciable hepatic expression as well, highlighting that caution should be used when trying to extrapolate findings from mouse or rat models to humans.

Cholesterol 25-hydroxylase (CH25H) is not a cytochrome P450 but a microsomal di-iron-containing enzyme that converts cholesterol to 25-hydroxycholesterol. Its expression is dramatically induced in immune cells during inflammation (e.g., following treatment of macrophages with lipopolysaccharides) (Cyster et al. 2014).

Cholesterol 27-hydroxylase (CYP27A1) is a mitochondrial cytochrome P450, catalyzing the first step in the alternative bile acid synthesis pathway to produce 27-hydroxycholesterol from cholesterol (Bjorkhem et al. 2019). It can further oxidize the same carbon to ultimately produce cholestenoic acid. It is believed to be a significant mechanism by which cells (including macrophages) can eliminate excess cholesterol. Accordingly, patients deficient in this enzyme (cerebrotendinous xanthomatosis) have an abundance of cholesterol-filled macrophages (foam cells), despite having normal circulating cholesterol levels.

24*S*,25-Epoxycholesterol is made in a shunt in the cholesterol synthesis pathway but using the same enzymes (Gill et al. 2008). The key enzymes are squalene monooxygenase (or epoxidase) which introduces an epoxide group onto the isoprenoid squalene and the next enzyme, lanosterol synthase (or oxidosqualene cyclase), which then uses elegant molecular origami to fold the epoxide-bearing hydrocarbon into the four-fused ring structure characteristic of steroids. Partial inhibition of lanosterol synthase allows accumulation of mono-oxidosqualene which can be acted on again by squalene monooxygenase to introduce a second epoxide which ultimately becomes 24*S*,25-epoxycholesterol. This oxysterol can also be generated by the action of CYP46A1 on an immediate precursor of cholesterol, where CYP46A1 introduces the epoxide across the C24–C25 double bond of desmosterol. 24*S*,25-Epoxycholesterol plays a critical role in fine-tuning cell cholesterol homeostasis (Gill et al. 2008).

Modifiers

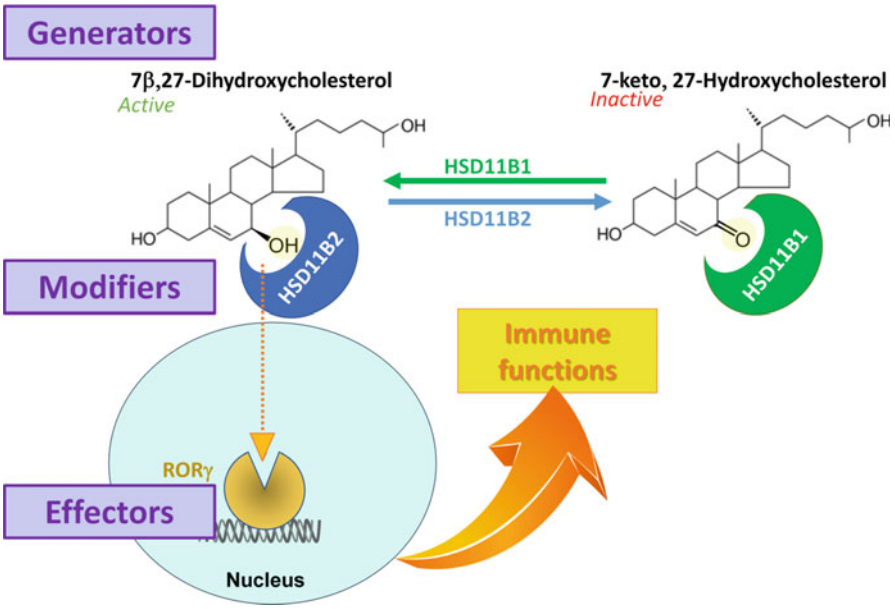
Oxysterols can be modified to other oxysterols which may alter their activities. Notable modifiers include members of the short-chain dehydrogenase/reductase superfamily such as the microsomally located enzymes HSD3B7, HSD11B1, and HSD11B2.

Hydroxy-Δ5-steroid dehydrogenase, 3β- and steroid delta-isomerase 7 (HSD3B7) is another enzyme involved in bile acid synthesis, oxidizing the 3β-hydroxyl to a keto group, as well as isomerizing the C5–C6 double bond to C4–C5. These modifications can deactivate the 7α,25-hydroxycholesterol ligand for the G-protein-coupled receptor, EBI2 (Cyster et al. 2014).

Hydroxysteroid 11β-dehydrogenase 1 (HSD11B1) functions mainly as an oxidoreductase and is best known for converting inactive cortisone to the active stress hormone cortisol in glucocorticoid target tissues. Cortisol is a ligand for both the glucocorticoid and mineralocorticoid receptors. In the context of oxysterols, HSD11B1 exhibits similar reactivity, reducing 7-ketocholesterol to 7-β-hydroxycholesterol (Mitic et al. 2013).

Hydroxysteroid 11β-dehydrogenase 2 (HSD11B2) functions as a dehydrogenase, deactivating cortisol to cortisone. HSD11B2 is highly expressed in mineralocorticoid target tissues, where it prevents cortisol acting as a ligand for the mineralocorticoid receptor. An analogous situation may exist with particular oxysterols. For instance, 7β,27-dihydroxycholesterol can activate the nuclear receptor RORγ (Fig. 2), but oxidation by HSD11B2 would be expected to produce an inactive 7-keto-derivative (Beck et al. 2019).

Esterification refers to the attachment of a fatty acid to the 3β-hydroxyl group of the sterol by an ester linkage. Like cholesterol, most oxysterols can be esterified in cells by either SOAT1 or SOAT2 (sterol *O*-acyl transferase 1/sterol *O*-acyl transferase 2, also known as ACAT1/ACAT2), or in the plasma by LCAT (lecithin cholesterol acyl transferase), where they can be transported mostly by lipoproteins. Esterification is another way that oxysterols are likely to be inactivated, as it is difficult to envisage an oxysterol ester binding to an effector, like a nuclear receptor, with the same affinity as an unesterified oxysterol ligand. It should be noted that most oxysterols found in the circulation and in diseased tissue are predominantly esterified (Brown and Jessup 1999), suggesting limited



Oxysterols, Fig. 2 **The origin and action of oxysterols generally involve three processes:** (1) **generation** of oxysterols from enzymic and nonenzymic reactions on cholesterol and related molecules; (2) **enzyme modifiers** convert specific oxysterols to active or inactive derivatives; (3) **effectors** transduce the biological effects of the oxysterols, often involving inducing particular transcriptional programs. The example given has been derived from Beck et al. (2019)

biological activity. Whether these esters can serve as stores of oxysterols, and how well they can then be liberated by sterol esterases, remains to be determined.

Other processes. As with cholesterol, oxysterols can be eliminated from cells by transporters like the ATP-binding cassette proteins ABCA1 and ABCG1. In humans, side-chain oxysterols like 24*S*-hydroxycholesterol can undergo reactions normally associated with hepatic metabolism of bile acids, steroid hormones, and drugs, such as sulfation and glucuronidation, which may facilitate their excretion from the body. Free radical-derived oxysterols, including 7-β-hydroxycholesterol and 7-ketocholesterol, are metabolized to unusual bile acids via novel branches of the alternative pathway (Griffiths et al. 2019).

Effectors

Oxysterols can have potent biological effects by binding to various effectors and eliciting a variety of transcriptional programs. These include nuclear receptors (LXRα/β, RORγ) which are ligand-activated transcription factors and G-protein-coupled receptors (GPCRs like EBI2 and Smoothened) which also transduce specific transcriptional programs, often in concert with small molecule activators.

Insig is like a brake for the activation of the SREBPs which are master transcriptional controllers of lipid metabolism. Binding of oxysterols to Insig (or cholesterol to Scap) prevents the proteolytic processing of SREBPs into active transcription factors and hence results in effective downregulation of lipogenic transcriptional programs. The oxysterols that can bind most avidly to Insig are those with oxygenated side chains like 25-hydroxycholesterol (Gill et al. 2008).

Liver X receptor α/β (LXRα/β) is a nuclear receptor where the endogenous ligands are oxysterols, with 24*S*-hydroxycholesterol, 27-hydroxycholesterol, and 24*S*,25-epoxycholesterol being particularly potent. Activation of LXRα/β favors cholesterol elimination from cells (for instance, through upregulation of *ABCA1* and *ABCG1*) and

from the body more generally by stimulating reverse sterol transport. LXRα/β activation also tends to dampen the immune response, providing one of the first glimpses into the importance of oxysterols in immunity (Spann and Glass 2013).

RAR-related orphan receptor gamma (RORγ) is another nuclear receptor and has been studied most with respect to the immune system. Notably in the thymus, RORγ promotes differentiation of thymocytes into a subclass of mature, pro-inflammatory T helper cells defined by their production of interleukin-17 (Th17 cells). A number of oxysterols can bind to RORγ, but 7α/β,27-dihydroxycholesterol is particularly potent. As shown in Fig. 2, oxidation by HSD11B2 of the 7β-hydroxy oxysterol to the 7-keto version will likely bar its ability to bind to and activate RORγ (Beck et al. 2019).

Epstein-Barr virus-induced G-protein-coupled receptor 2 (EBI2) is crucial for B-cell trafficking within lymphoid tissues and thus the humoral immune response in general, including antibody-mediated immunity. 7α,25-Dihydroxycholesterol is an especially potent ligand, binding to EBI2 at subnanomolar concentrations (Cyster et al. 2014).

Smoothened transduces the signal in the Hedgehog pathway of vertebrate development. Hedgehog binds to a multipass membrane receptor called Patched which then leaves the primary cilia where Smoothened is located. Various oxysterols can bind to Smoothened to activate it, upregulating a developmental transcriptional program. 24*S*,25-Epoxycholesterol is enriched in cilia from sea urchins and potently activates mammalian Hedgehog signalling by binding to Smoothened (Qi et al. 2019).

Oxysterols, Disease, and Concluding Remarks

Oxysterols have long been implicated in various diseases, including atherosclerosis, some cancers, and neurodegenerative diseases. Blood levels of certain oxysterols may be useful disease risk markers in some cases; however, it should be noted that accurately measuring oxysterol levels can be particularly challenging (Luu et al. 2016). In cell culture, oxysterols exhibit many properties that would be consistent with them contributing to pathogenesis. However, direct evidence of a particular oxysterol having a causal role in disease has been harder to attain. Specifically manipulating endogenous levels of a particular oxysterol is challenging, since the generation and metabolism of oxysterols share some of the same machinery as used in cholesterol, bile acid, steroid hormone, vitamin D, and xenobiotic metabolism. Oxysterol research has advanced significantly in recent years and continues to be a fruitful area of research.

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Oxytocin

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Synonyms

8-leucine-vasotocin; Pitocin (trade name); Syntocinon

Definition

Oxytocin is primarily involved in parturition and milk ejection, stimulating smooth muscle contraction in the uterus and mammary glands in female mammals. It is also known to contract smooth muscle in the male reproductive tract. Oxytocin is a peptide hormone secreted by the neurohypophysis and the hormonal targets for oxytocin in females are the myoepithelial cells that surround the alveolar channels in the mammary gland and the uterus and in males in the reproductive organs such as the seminal vesicles and epididymis. Oxytocin is also a neuropeptide, released into the brain and regulating sexual, maternal, social, and feeding behaviors. Several conditions are associated with altered oxytocin activity or effects. The main clinical disorder directly associated with altered circulating (neurohypophysial) oxytocin is preterm labor. Other conditions include a range of defects in social (e.g., autism spectrum disorders) and sexual behavior, which are primarily controlled by oxytocin released into the brain.

Basic Characteristics

Oxytocin is a small peptide hormone comprising nine amino acids arranged in cyclic form, which is maintained by a single disulfide bridge between cysteines 1 and 7. Together with the modified C-terminal amino acid, this confers resistance to rapid degradation in the blood stream, as well as providing a unique three-dimensional structure that complements the hormone-binding pocket of its receptor. In all mammals, oxytocin is very closely associated with the structurally related hormone vasopressin, both of which are derived by gene duplication from a common invertebrate ancestral hormone. Oxytocin is expressed as part of a polyprotein comprising more than one functional unit. Besides oxytocin, the gene also encodes a larger cysteine-rich polypeptide, neurophysin 1. Oxytocin mRNA encodes a pre-pro-polyprotein, which because of its N-terminal signal peptide is posttranslationally translocated into the lumen of the endoplasmic reticulum, where the signal is removed. The pro-polyprotein is then transferred to the Golgi apparatus and finally to acidic secretory vesicles. During this process, specific proteolytic enzymes cleave the oxytocin from neurophysin and catalyze C-terminal transamidation of the oxytocin, which remains associated with its neurophysin until released from the cell (de Bree 2000).

The principal sites of processing of the pre-prohormone into its final products are the cell bodies of the large magnocellular neuroendocrine neurones of the hypothalamus within the brain. These neurones produce either oxytocin or

vasopressin and are arranged symmetrically close to the third ventricle in two nuclei, the supraoptic nucleus and the paraventricular nucleus. The few thousand neurons located in both nuclei are characterized by long axons that project downwards out of the hypothalamus along the pituitary stalk ending in the posterior lobe of the pituitary gland (also called the neurohypophysis). From there the peptides are secreted into the blood after appropriate stimuli (Brown et al. 2013). Oxytocin secretion from the pituitary gland is triggered by the stimulation of the nipples, resulting in the milk-ejection reflex, and by the distension of cervix and vagina during labor. Oxytocin release in rodents and some other mammalian species is also stimulated by plasma hyperosmolality (high sodium) and hypovolemia, suggesting a regulatory role in natriuresis and blood volume for this hormone. Secretion also occurs during sexual arousal and ejaculation in many mammals, including in man. Oxytocin is also released during stress and (in some species) in response to food intake. Oxytocin secretion is suppressed by alcohol and opioids (Leng et al. 2012).

In addition, secretory granules containing oxytocin are released from the cell bodies and especially dendrites of the magnocellular neurons within the hypothalamus (Ludwig and Leng 2006). In the paraventricular nucleus, there are, as well as magnocellular neurons, smaller (parvocellular) oxytocin neurons that project not to the neurohypophysis but to sites in the caudal brainstem that are involved in the control of energy balance and to sites in the spinal cord involved in the control of erectile function and copulation (Argiolas and Melis 2013) and in the regulation of pain (Boll et al. 2018). Some scattered isolated neurones in the lateral and anterior part of the hypothalamus are between the paraventricular and supraoptic nucleus and some are located in the dorsomedial hypothalamic nucleus (Grinevich et al. 2016).

The anatomical distribution of oxytocin neurones and fibers is relatively consistent among mammals, but there is considerable species variation in oxytocin receptor expression and this is associated with differences in reproductive behavior. For example, in rodents oxytocin receptors are concentrated in brain regions involved olfactory processing, but in primates they are concentrated in regions in visual processing. Oxytocin (and vasopressin) are an integral part of the mammalian emotional circuitry and are associated with the emergence of social bonding, parental care, stress regulation, social communication, and emotional reactivity (Donaldson and Young 2008). Finally, several other organs, such as the gastrointestinal tract, heart, ovary, amnion, chorion, decidua, testis, epididymis, and prostate, have been reported to synthesize oxytocin (albeit at extremely low levels), suggesting a possible paracrine role for this hormone in these tissues.

Oxytocin Receptor Activation

Oxytocin elicits its physiological and pharmacological responses through the oxytocin receptor, a cell surface receptor belonging to the rhodopsin-like (type A) group of G-protein coupled receptors. So far, only one type of oxytocin receptor is known (but three different subtypes of vasopressin receptors). Due to its high sequence and structural homology with vasopressin, oxytocin has the ability to bind for all three types of vasopressin receptors, namely, V_{1a}, V_{1b}, and V_2 (Manning et al. 2012).

The oxytocin receptor is encoded by a single gene that is highly homologous across species, comprising three exons and in the human located on chromosome 3p25. In spite of the importance of the oxytocin receptor for the induction of labor and mammary function, little is known about the transcriptional regulation of the oxytocin gene. It is regulated by steroids such as estrogens, although probably in a nonclassical manner whereby activated estrogen receptors act indirectly via other transcription factors within the upstream promoter region of the gene. Other specific transcription factors such as MafF are also involved, acting within an intron of the receptor gene (Gimpl and Fahrenholz 2001). Moreover, DNA methylation within different regions of the gene appears to correlate with cell specificity of expression. There is evidence of physiological

redundancy in some functions; oxytocin and oxytocin receptor knockout mice appear to undergo parturition normally (Ratajczak and Muglia 2008).

Posttranslational modifications, localization in special plasma membrane compartments (lipid rafts or caveolae), and interactions with downstream signal transduction components modify oxytocin receptor signaling. Importantly, the oxytocin receptor responds to activation by oxytocin by rapid desensitization and internalization, as is typical for most hormones that are secreted in a pulsatile fashion. Up-regulation of the oxytocin receptor system in a cell requires either a delay in the desensitization process or simply an increase in the expression of the oxytocin receptor gene. At full-term pregnancy, the levels of oxytocin receptor gene transcripts appear to be increased almost 100-fold in the uterus.

The oxytocin receptor interacts with heterotrimeric G protein complexes (Gα, Gβ, and Gγ) and can be linked to multiple signaling pathways depending on the specific G protein complex involved (e.g., Gαq, Gαo, Gαi). Upon ligand binding to the complex, the β and γ subunits dissociate from the Gα-protein. Several different α-protein subforms can contribute to the composition of the complex. Depending on the type of Gα protein, the functional outcome of receptor activation can be stimulatory or inhibitory.

The main signaling pathway is the Gq/PLC/Ins3 pathway (Gimpl and Fahrenholz 2001; Arrowsmith and Wray 2014). Oxytocin receptor activation stimulates phospholipase C (PLC) resulting in the generation of inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG). Whereas IP3 mobilizes Ca^{2+} from intracellular stores, DAG activates protein kinase C (PKC), leading to phosphorylation of a number of other downstream target proteins. In addition, Ca^{2+} influx through voltage-operated Ca^{2+} channels contributes to the full response after myometrial oxytocin receptor activation. The increase in cytosolic calcium concentration is the main trigger for smooth muscle contraction but also plays a role in neuronal oxytocin responses such as transmitter release (Busnelli and Chini 2018; Jurek and Neumann 2018). MAPK (ERK5) and the RhoA/Rho kinase pathways are also activated, contributing to increased prostaglandin production and direct contractile effect on myometrial cells. OTR activation also leads to activation of ERK1/2 in myometrial and endothelial cells (proliferation), an effect that involves the release from $G_{q/11}$ and transactivation of the EGFR. In decidua activation of oxytocin receptor induces PGF2α release, which enhances uterine contractions, cervical ripening, and luteolysis. This effect is G_i- and $G_{q/11}$-dependent and involves Ca^{2+}-mediated translocation of PLA_2 from the cytoplasm to cell membranes, in addition to the activation of PLA_2 by MAPK. When only a minor fraction of OTRs is located in lipid rafts (caveolae), they inhibit cell proliferation (via G_i, PLC, c-Src, PI3K, EGFR transactivation and persistent ERK1/2 activation), but when targeted to lipid rafts they have a strong mitogenic effect (via $G_{q/11,\ b/c}$ release, EGFR transactivation and transient ERK1/2 activation). Oxytocin receptor-activated ERK signaling is also involved in regulating selective behaviors in the brain, including social and maternal behaviors. In the cardiovascular system, oxytocin receptor is associated with the ANP-cGMP and NO-cGMP pathways, which reduce the force and rate of contraction and increase vasodilatation.

O

Oxytocin Effects

Parturition

Oxytocin is one of the most potent uterotonic factors, a property that is used today in the induction of birth. Shortly before the end of pregnancy (term), estrogen concentrations rise and there is functional progesterone withdrawal. Oxytocin receptors are up-regulated in the myometrial smooth muscle layers of the uterus and become sensitive to the increasing pulses of oxytocin released from the posterior pituitary. Labor and birth are a result of a positive feedback loop (Ferguson reflex) resulting in expulsion of the uterine contents. Part of this involves the induction of local prostaglandin-F2α expression in the myometrium, which, like oxytocin, causes smooth muscle contractility by mobilizing intracellular Ca^{2+} stores. Factors promoting

myometrial quiescence, such as progesterone and the hormone relaxin in most species (though not in humans), decrease. Uterine contractility, along with fetal movements, elicits the Ferguson reflex, whereby neuronal afferents from the cervix via the vagal and hypogastric nerves result in further activation of the magnocellular neurons in the hypothalamus.

In rats and other species, the magnocellular oxytocin neurones give rise to intense synchronized bursts of electrical activity, which cause the release of increasingly large secreted pulses of oxytocin. Activation of the magnocellular neurons also results in dendritic oxytocin release from the cells within the hypothalamus. Dendritic oxytocin release induces functional neuronal plasticity to further orchestrate the synchronization within the magnocellular nuclei. Oxytocin release in a pulsatile manner is important since continuous and sustained release of oxytocin leads to oxytocin receptor desensitization and a slowing of the birth process. Oxytocin is involved not only in the expulsion of the fetus at birth, but also subsequently of the placenta, and then critically ensures the contraction of the empty uterus and to separate the placenta from the womb. Therapeutically, oxytocin may be given to support this process, after giving birth if not before.

Lactation

Oxytocin is not involved in mammary gland development, nor in milk synthesis and secretion, but transgenic mouse studies have shown that oxytocin is essential for milk let-down in the mammary glands. Oxytocin is released from the posterior pituitary in response to suckling at the nipples, causing contraction of the smooth muscle cells surrounding the mammary alveoli and release of milk. As in the uterus, oxytocin receptors are increasingly expressed in the myoepithelial cells of the developing alveoli towards the end of pregnancy, and their responsiveness to oxytocin also increases. Studies in rats indicate that there are short bursts of electrical activity every 5–15 min in the magnocellular neurons of the hypothalamus, leading to pulses of oxytocin release from the posterior pituitary and release of milk to the suckling pups (Rossoni et al. 2008). This appears to be a feature common to all mammals; imaging in humans has shown a similar intermittent milk-release in response to continuous suckling. Oxytocin receptors in the mammary gland become desensitized if exposed to continuous oxytocin delivery, and require relatively high concentrations of oxytocin within a pulse.

Copulation

The oxytocin-dependent induction of smooth muscle contractility in the reproductive system is not limited to female mammals. In male mammals, pulses of oxytocin can be measured accompanying the contraction of smooth muscle cells in the epididymis and vas deferens, associated with ejaculation. Oxytocin produced locally within the testes is responsible for the slow peristaltic contractility of the seminiferous tubules associated with sperm release from the seminiferous epithelium. Oxytocin also promotes penile erection, through actions at the hypothalamus and spinal cord; the spinal effects are mediated by parvocellular oxytocin cells of the paraventricular nucleus, but the hypothalamic actions may reflect effects of dendritic release from magnocellular neurons (Argiolas and Melis 2013).

Hypothalamo-Pituitary-Gonadal Axis

Oxytocin not only has direct effects on receptors within the reproductive organs themselves, but also influences the hypothalamo-pituitary-gonadal axis. Activation of oxytocin receptors on the gonadotropin-producing cells of the anterior pituitary causes activation of phosphoinositide-3-kinase and intracellular Ca^{2+} release, which together interact with the GnRH-induced signaling activity in the same cells. Whether this oxytocin, like GnRH, arrives through the portal system from the hypothalamus or comes from the posterior pituitary is not known, but it does suggest that the hormones of the HPG axis can be modulated at times of high oxytocin expression, for example, at the end of pregnancy or during lactation.

Natriuresis

The magnocellular oxytocin neurons are, like vasopressin neurones, osmoreceptors; they

respond to increased osmotic pressure by releasing oxytocin, but oxytocin is released in a more sustained fashion than during uterine contraction and lactation, the circulating levels increasing without evident pulsatility (Leng and Russell 2019). In animal models, it has been shown that oxytocin can directly stimulate the kidney to increase Na^+ excretion (natriuresis), presumably via oxytocin receptors, though possibly also via vasopressin V1 receptors, with which oxytocin can interact weakly. However, much of this effect is due to the stimulation of cardiac atrionatriuretic peptide secretion, which in turn influences the kidney. The natriuretic response is species-specific, with humans and sheep exhibiting little effect compared to rodents.

Behavioral Effects of Oxytocin

Since oxytocin is largely unable to cross the blood–brain barrier, the various behavioral effects of oxytocin must be due to centrally released hormone, including that from the dendrites of the magnocellular neurones (Ludwig and Leng 2006). Moreover, such effects do not correlate with levels of oxytocin measured in blood, and these do not reflect the levels in cerebrospinal fluid. Central oxytocin is responsible for a range of maternal and affiliative behaviors: for example, in sheep oxytocin induces the bonding of a newborn lamb with its mother, while in the female prairie vole, it promotes monogamous pair-bonding. It is generally described as "anti-anxiolytic," encouraging more exploratory and olfactory behavior in laboratory animals. It is also linked to sexual responsiveness and behavior in female and male rats, respectively (Neumann and Landgraf 2012).

Cholecystokinin (CCK) can stimulate hypothalamic oxytocin secretion via CCK receptors on vagal efferents from the stomach. Since CCK is produced by the duodenum following a meal, these vagal actions evidently mediate a satiety reflex. It has been shown experimentally in rats that injection of oxytocin into the brain can suppress appetite, and there have been numerous claims of similar effects in humans (Leng and Sabatier 2017), though possibilities for therapeutic approaches using oxytocin in certain mental disorders are very limited, due to the difficulty of targeting the brain by a peptide that cannot cross the blood-brain barrier. Although it has been proposed that oxytocin applied as a nasal spray might circumvent this difficulty by reaching the brain directly across the nasal mucosa, the findings from such studies are not without substantial controversy (Leng and Ludwig 2016).

Drugs

Agonists

Oxytocin is a potent uterotonic, used in obstetrics as an intravenous infusion to induce labor in women with sufficiently ripened cervix, to augment hypotonic uterine contractions during labor, in the prevention and treatment of postpartum hemorrhage, and in the medical treatment of miscarriage (Molitch and Schimmer 2018).

The most common adverse effects following parenteral application of oxytocin are arrhythmias, headache, nausea, and vomiting. There are two caveats to intravenous application of oxytocin: rapid infusions can cause peripheral vasodilation, manifested in patients as hypotension followed by the reflex tachycardia due to the subsequent activation of baroreceptor reflex, whereas excessive doses of intravenously administered oxytocin may lead to uterine hyperstimulation and consequent complications for both mother and fetus. These complications include uterine rupture, impaired fetal oxygenation as a result of decreased uterine perfusion and cervical and/or fetal trauma during forced propulsion of fetus through the incompletely ripened cervix.

In recent years, a growing number of studies have speculated that oxytocin delivered nasally could be used as a possible treatment option for human conditions characterized by social deficits, such as autism spectrum disorder (Yamasue and Domes 2018). Further research directed towards unraveling the pharmacokinetic profile of intranasally applied oxytocin is yet to show if this approach may have any benefit in any psychiatric disorders.

Carbetocin is an oxytocin-derived analogue with a longer half-life than oxytocin itself. It is

used to prevent postpartum hemorrhage, especially in prevention of uterine atony after caesarean section. Unlike oxytocin, carbetocin can be applied parenterally in a single dose. It has also been shown to possess better tolerability and safety profile than oxytocin. Heat-stable formulation of carbetocin, which does not require cold-chain transport and storage, may be used to reduce the maternal mortality due to postpartum hemorrhage in less privileged parts of the world.

Antagonists

Discovered more than three decades ago, atosiban still remains the only oxytocin receptor antagonist that has found application in clinical medicine. For its ability to inhibit uterine contractions when given as an intravenous infusion, atosiban is used as a tocolytic in the acute treatment of preterm labor (Akerlund 2006). However, its lack of selectivity (atosiban shows even higher affinity for vasopressin V1a receptors than for oxytocin receptors) and its peptide nature make it unsuitable for prolonged maintenance treatment as a result of the limited oral bioavailability. A considerable effort has been made to identify novel, orally active, nonpeptide selective OTR antagonists.

Retosiban is an orally active, selective oxytocin receptor antagonist with tocolytic properties that shows high affinity for human oxytocin receptor (Ki = 0.65 nM). It is an antagonist at least 15-fold more potent on the oxytocin receptor than atosiban, with a better selectivity profile. Results from phase 2 clinical studies indicated that retosiban has a favorable safety and tolerability profile, but the phase 3 clinical studies have recently been terminated.

Epelsiban and ciglosiban are selective orally available oxytocin antagonists, suggested to be effective in the treatment of premature ejaculation.

The ability of oxytocin receptor antagonists to reduce the frequency of uterine contractions in nonpregnant women has been used in clinical studies as a potential means of increasing implantation and pregnancy rates prior to embryo transfer, in patients undergoing subfertility treatment through in vitro fertilization. However, randomized clinical trials have yet to provide reasonable grounds for using oxytocin receptor antagonists in assisted reproduction.

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P

P1 Receptors

► Adenosine Receptors

P450 Monooxygenase System

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Synonyms

Cytochrome P450 (CYP); Cytochrome P450 isozymes; Cytochrome P450 monooxygenases; Mixed-function oxidases

Definition

Cytochrome P450 (CYP) monooxygenases, also called mixed-function oxidases, are versatile hemoprotein enzymes that catalyze the cleavage of molecular oxygen to incorporate one oxygen atom into a substrate molecule and one atom into water (Ortiz de Montellano 2015). The general stoichiometry of the reaction is as follows (S-H, substrate):

$$NADPH + H^+ + O_2 + S\text{-}H \rightarrow NADP^+ + H_2O + S - OH$$

The numerous biotransformations catalyzed by cytochrome P450 enzymes include aromatic and aliphatic hydroxylations, epoxidations of olefinic and aromatic structures, oxidations and oxidative dealkylations of heteroatoms, and some reductive reactions. Cytochromes P450 of higher animals may be classified into two broad categories depending on whether their substrates are primarily endogenous or xenobiotic substances. Thus, CYP enzymes of families CYP1, CYP2, and CYP3 catalyze the phase I metabolism of most drugs and other xenobiotics, often a prerequisite for a phase II conjugation reaction and subsequent elimination from the body. Sometimes the product of CYP-catalyzed reactions can be pharmacologically active (in this case the substrate is termed a prodrug), or it may be carcinogenic or toxic. The CYP enzymes of families CYP4 to CYP51 primarily participate in various physiological pathways of fatty acids, eicosanoids, steroid hormones, bile acids, prostaglandines, and other endogenous compounds.

Basic Characteristics

Cytochrome P450 Electron Transport Systems

Cytochrome P450 monooxygenase function depends on electron transport chains, in which electrons are transferred from cellular pyridine nucleotides (NADPH/NADH) to the P450 heme moiety. In vertebrates, there are two principal types of P450 electron transfer chains. One is

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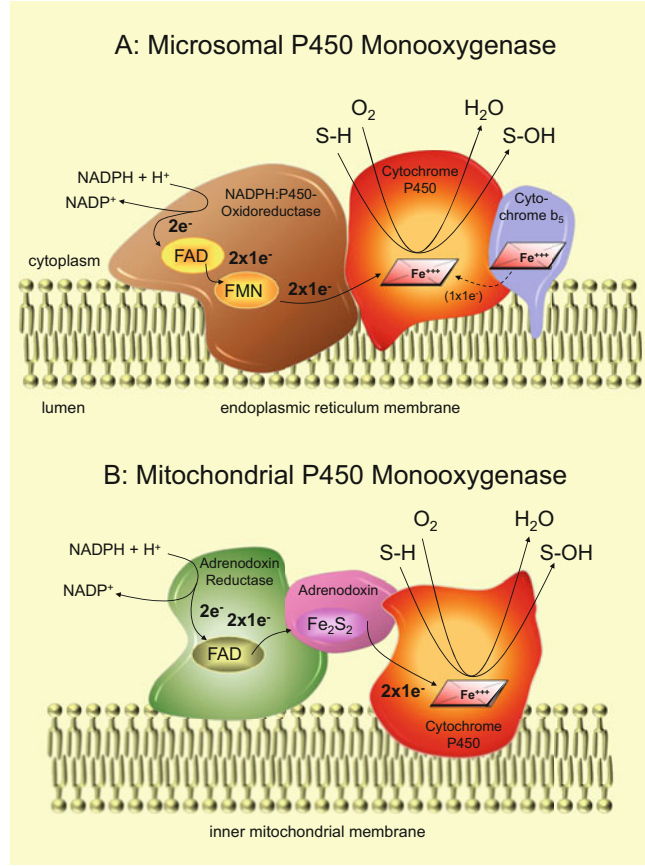


P450 Monooxygenase System, Fig. 1 P450 monooxygenase system. (**a**) Components of microsomal P450 monooxygenases. (**b**) Components of mitochondrial P450 monooxygenases

found in the endoplasmic reticulum (ER), the other in the mitochondrial inner membrane. The electron-donating protein of the ER system (Fig. 1a) is called NADPH-cytochrome P450 oxidoreductase (CPR). It consists of two domains with two different prosthetic flavin groups, FAD (flavin adenine dinucleotide) and FMN (flavin mononucleotide), which transfer two electrons acquired from NADPH directly, but only one at a time, to the P450 heme iron. CPR is bound to the ER membrane by its N-terminus with the bulk of the protein on the cytosolic side. In humans, CPR is encoded by a single gene (*POR*) on chromosome 7, which encodes a protein of 677 amino acids. The crystal structure of CPR has been determined. In certain reactions the second electron can be transferred via cytochrome b_5, a smaller heme containing protein (MW ~ 15 kDa), which usually accepts electrons from NADH via the flavoprotein NADH-cytochrome b_5-reductase.

In the mitochondria (Fig. 1b), the electron acceptor protein is also a flavoprotein termed NADPH-adrenodoxin reductase (MW ~ 50 kDa). It is present in the adrenal cortex and donates electrons not directly to P450 but to the smaller redox protein adrenodoxin (MW ~ 12.5 kDa). The two iron-sulfur clusters of this protein serve as electron shuttle between the flavoprotein and mitochondrial P450s.

Cytochrome P450 Structure

The name cytochrome P450 (P for pigment) derives from the unusual spectrum compared to other hemoproteins. Carbon monoxide strongly binds to the reduced (ferrous) heme iron inducing a strong absorption at 450 nm that was first discovered in 1958 and that can be used to quantitate cytochrome P450 via its reduced CO difference spectrum. The structural difference between cytochrome P450 and other hemoproteins is the

unusual fifth ligand to the heme iron, which is a histidine in other hemoproteins but a conserved cysteine thiolate in all P450s, located close to their C-termini. X-ray crystal structures available for bacterial and several human microsomal P450s have confirmed the role of the conserved thiolate ligand and provided deep insights into substrate binding and structural peculiarities (Guengerich et al. 2016). The many sequences available and the recent structural data indicate significant structural similarities between all P450s, which consist of about 500 amino acids and most likely evolved from a single common ancestor gene. Microsomal P450s are bound to the membrane by a single hydrophobic N-terminal transmembrane anchor in such an orientation that the heme moiety is colocalized with the CPR flavodomain at the cytoplasmic side of the ER. Mitochondrial P450s are synthesized as slightly larger precursors with an N-terminal signal sequence that is being cleaved off during translocation of the protein to the inner mitochondrial membrane.

Cytochrome P450 Gene Superfamily

Cytochromes P450 are the products of a gene superfamily with currently more than 50,000 known forms (isozymes) from all types of living organisms. Cytochrome P450 proteins are classified into families and subfamilies based on their sequence similarities. Any two forms with more than 40% identity at the amino acid level belong to the same family (indicated by an Arabic numeral), whereas they belong to the same subfamily (indicated by a capital letter) if they share more than 55% of identical amino acids. Individual isozymes of the same subfamily are distinguished by an additional Arabic number. Humans have 57 individual functional *CYP* genes that belong to 18 families and 43 subfamilies and that are located over all autosomal chromosomes. A particular aspect of mammalian cytochrome P450 systems is the high number of pseudogenes, which harbor mutations that prevent the expression of functional proteins. Humans have more than 25 *CYP* pseudogenes that make molecular genetic analysis complex due to their high sequence homology. Other mammals share the same 18 *CYP* families with humans, but the number of individual functional isozymes in each family or subfamily can be very different. Based on their role in metabolism, the 18 *CYP* families can be broadly classified into those catalyzing the metabolism of xenobiotics (families *CYP1*, *CYP2*, and *CYP3*) and those that are responsible for biotransformations of endogenous substances (families *CYP4* to *CYP51*; Table 1). Most *CYP* genes of the first category are polymorphic in the population, i.e., they harbor common genetic variants that may lead to polymorphic drug oxidation (Zanger and Schwab 2013). In contrast, mutations in CYPs catalyzing physiological reactions are usually rare as they may cause inherited metabolic disorders.

Human Drug Metabolizing Cytochromes P450

Typical characteristics of the drug-metabolizing P450s are their broad and overlapping substrate specificities and highly variable expression and function, both in terms of inter- and intraindividual variation, with tremendous influence on the pharmacokinetics of most drugs in clinical use. The in vivo activity of a specific P450 enzyme can principally be estimated by measuring metabolite concentrations of an ingested selective probe drug in urine, blood, or breath of patients. This is necessary because there are no reliable surrogate markers to estimate liver expression and activity. Three basic mechanisms are responsible for variability in the activity of drug-metabolizing enzymes: (i) genetic variation including common polymorphisms and rare mutations, as well as epigenetic variation; (ii) gene regulatory mechanisms that lead to enzyme induction or downregulation; and (iii) direct inhibition of enzyme activity. The importance of each type of variation differs substantially between different CYP enzymes (Fig. 2).

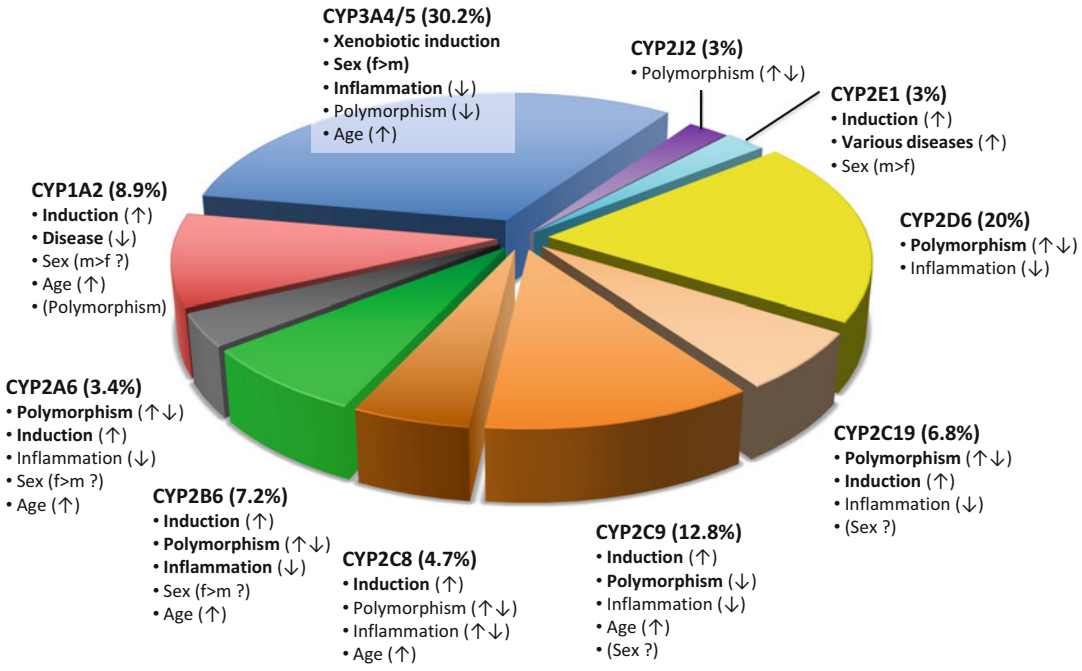
Genetic Variation in Drug-Metabolizing P450s

A genetic polymorphism is a difference in DNA sequence that has a frequency of at least 1% in a population. Many drug-metabolizing enzyme genes are highly polymorphic with consequences for expression and function of the gene product,

P450 Monooxygenase System, Table 1 P450 monooxygenase system. Overview of the cytochrome P450 superfamily in humans

Family	Subfamily, genes (P indicates pseudogenes, not all are shown)							Typical substrates/functions
CYP 1	A1	A2	A8P					PAHs, PCBs, estrogens, aromatic amines
	B1							PAHs, PCBs, retinoids
CYP 2	A6	A7	A13	A18P				Nicotine, coumarin, nitrosamines
	B6	B7P1						Cyclophosphamide, bupropion, efavirenz
	C8	C9	C18	C19	C23P			Taxol (C8), NSAIDS, warfarin (C9), omeprazole (C19)
	D6	D7P	D8P					Antidepressants, opioids, beta-blockers
	E1							Ethanol, halothane, acetone
	F1	F1P						Naphthalene, styrene
	G1P	G2P						No function
	J2							Arachidonic acid, ebastine hydroxylation
	R1							Vitamin D3 25-hydroxylase
	S1							All-trans retinoic acid, naphthalene
	T2P	T3P						No function
	U1							Arachidonic acid omega-hydroxylation
	W1							Bioactivation of procarcinogens
CYP 3	A4	A5	A5P3	A7	A43			Cyclosporin, antidepressants, testosterone
CYP 4	A11	A22						Medium- and long-chain fatty acids, arachidonic acid ω-hydroxylation
	B1							Inactive in humans
	F2	F3A	F3B	F8	F11	F12	F22	Medium- and long-chain fatty acids; arachidonic acid, leukotrienes, prostaglandines
	V2							ω-3 polyunsaturated fatty acids, medium-chain fatty acids, arachidonic acid
	X1							Unknown function
	Z1	Z2P						Medium-chain fatty acids, arachidonic acid (?)
CYP 5	A1							Thromboxane A2 synthase
CYP 7	A1							Cholesterol 7alpha-hydroxylase (→ bile acids)
	B1							Oxysterol 7alpha-hydroxylase
CYP 8	A1							Prostacyclin synthase
	B1							Steroid 12alpha-hydroxylase (→ cholic acid)
CYP 11	A1							Cholesterol side chain cleavage
	B1	B2						Steroid 11-β or 18 hydroxylase (→ cortisol, aldosteron)
CYP 17	A1							Steroid 17-apha hydroxylase/17–20 lyase
CYP 19	A1							Steroid aromatase (→ estrogens)
CYP 20	A1							Unknown function
CYP 21	A1P	A2						Steroid 21-hydroxylase
CYP 24	A1							Vitamin D degradation (24-hydroxylase)
CYP 26	A1							All-trans-retinoic acid hydroxylase
	B1							Retinoic acid hydroxylase
	C1							Retinoic acid hydroxylase
CYP 27	A1							27-hydroxylation in bile acid biosynthesis
	B1							Vitamin D3 1alpha-hydroxylase (kidney)
	C1							Unknown function
CYP 39	A1							24-Hydroxycholesterol 7-hydroxylase
CYP 46	A1	A4P						Cholesterol 24-hydroxylase
CYP 51	A1	P1	P2	P3				Lanosterol 14alpha-demethylase (cholesterol biosynthesis)

PAHs polycyclic hydrocarbons, *PCBs* polychlorinated biphenyls



P450 Monooxygenase System, Fig. 2 Major human drug-metabolizing P450s. Contribution (in %) of individual cytochrome P450 isoforms to the metabolism of 248 commonly used drugs. Only major pathways were considered. The legends indicate variability factors with direction of influence indicated (↑, increased activity; ↓, decreased activity; ↑↓, increased or decreased activity). Factors of controversial significance are shown in parentheses. (Reproduced with permission from Zanger and Schwab (2013))

such that they affect the disposition of drugs and xenobiotics (pharmacogenetics). The best studied example of a P450 genetic polymorphism is that of *CYP2D6* (Zanger and Schwab 2013). It results in four phenotypes in the population, termed ultrarapid (UM), extensive or normal (EM), intermediate (IM), and poor metabolizer (PM) phenotype. The PM phenotype is inherited as an autosomal recessive trait and affects about 5–10% of Caucasians who are unable to metabolize a range of drugs which are substrates for CYP2D6. For drugs with narrow therapeutic window, these individuals carry an increased risk to develop adverse drug reactions when given normal drug doses. Further examples for polymorphisms in *P450* genes are mentioned below. The Pharmacogene Variation Consortium (PharmVar) at www.PharmVar.org maintains a central repository for pharmacogene variation to facilitate CYP allele (haplotype) designation (Gaedigk et al. 2018).

Gene Regulatory Mechanisms in Drug-Metabolizing P450s

A variety of regulatory mechanisms are acting on different *P450* genes to regulate their constitutive expression and response to environmental stimuli. Members of the *CYP1* family and some other drug-metabolizing enzymes including some UDP-glucuronosyltransferases are collectively induced by polycyclic aromatic hydrocarbons (PAH) that serve as ligands to a specialized receptor called the aryl hydrocarbon receptor (AhR), which translocates to the nucleus following binding of ligand and another protein component called ARNT (AhR nuclear translocator). In the nucleus the complex binds to the DNA and activates transcription. Another regulatory mechanism is responsible for the 40–50-fold induction of CYP2B enzymes of humans and rodents following the administration of phenobarbital and other barbiturates. The orphan nuclear receptor CAR (constitutively activated receptor) plays a

central role in mediating the effect of phenobarbital that leads to the induction of human CYP2B6. PXR, another orphan nuclear receptor, binds a different range of ligands including the antibiotic rifampin and leads to the induction of a different profile of genes, in particular CYP3A4. Both CAR and PXR, as well as several other ligand-activated nuclear receptors, form heterodimeric complexes with the retinoic X-receptor, RXR, that subsequently translocate to the nucleus, bind to DNA elements, and activate transcription. Nuclear receptor-independent mechanisms to induce P450s are also known, e.g., the induction of the ethanol-oxidizing CYP2E1 enzyme by ethanol. The general feature of these regulatory mechanisms is that substrates induce their own metabolism.

Direct Inhibition of P450

Direct inhibition of P450 enzymatic activity is the most common reason for drug-drug interactions. P450 inhibitors can be either of the competitive- or of the mechanism-based type. Potent competitive inhibitors are often, but not always, substrates with high affinity for the enzyme. Mechanism-based or suicide enzyme inhibition occurs when a substrate is activated to a reactive intermediate that subsequently binds either to the P450 polypeptide or to the heme moiety, thereby inactivating it irreversibly. Examples for both types of inhibitors are known for almost every drug-metabolizing P450. Substances interfering with P450 enzyme activity may also originate from food, e.g., grapefruit juice contains CYP3A4 inhibitors that have significant effects on in vivo drug concentrations lasting several days (Flockhart 2007).

Drugs

Human Drug-Metabolizing CYPs

CYP1A1: Typical substrates are polycyclic aromatic hydrocarbons (PAHs) like benzo(a)pyrene or methylcholanthrene. The carcinogenicity of these substances depends on their metabolic activation by CYP1A1. Some of the PAHs that induce CYP1 enzymes via the Ah receptor are found in cigarette smoke and charred food. CYP1A1 is expressed at very low levels in the liver of uninduced individuals but is found in extrahepatic tissues including the placenta, lung, and lymphocytes. **CYP1A2** has a broader substrate specificity than CYP1A1, including many aromatic and heterocyclic amines, and is more abundantly expressed in the human liver. Typical substrates are caffeine, phenacetin, clozapine, estrogens, and others. Caffeine N3-demethylation can be used as a selective 1A2 marker activity, whereas 7-ethoxyresorufin O-demethylation or phenacetin O-demethylation reflects both CYP1A isozymes. Genetic polymorphisms have been found in all human *CYP1* genes, and their associations with various forms of cancer were intensely studied. The *CYP1A1/2* genes are located on chromosome 15.

CYP1B1 (chromosome 5) has been linked to primary congenital glaucoma. CYP1B1 is not regularly expressed in the liver but is often found in various kinds of tumors. It metabolizes retinoids and many aromatic amines and PAHs to potentially carcinogenic products.

The CYP2 family is the largest CYP family in humans, and it comprises about 16 functional genes and 11 pseudogenes.

CYP2A6 is the principal enzyme for nicotine metabolism. A selective probe drug for CYP2A6 is coumarin. Clinically important drug substrates are rare, but examples are tegafur, efavirenz, and letrozole. Genetic polymorphisms affect mainly expression levels. It has been suggested that smokers with genetically determined low CYP2A6 expression need to consume less nicotine to achieve the same satisfying blood levels of the drug.

CYP2B6 is the only functional isozyme of the 2B subfamily in humans, as *CYP2B7* is a pseudogene. Both genes are located within a large *CYP2* gene cluster on chromosome 19. CYP2B6 is highly variable owing to its inducibility by barbiturates and other drugs, but its expression and function are also affected by frequent genetic polymorphisms. Clinically important substrates are the cytostatic cyclophosphamide, the antidepressant bupropion, and the antiretroviral drugs used in HIV treatment, efavirenz and nevirapine. S-Mephenytoin *N*-demethylation and bupropion hydroxylation are selective marker activities that can be used both in vitro and in vivo.

The **CYP2C** subfamily comprises the four genes *CYP2C8*, *2C9*, *2C18*, and *2C19*, which are together localized on chromosome 10. The members of this subfamily show surprisingly large variation in substrate specificity and regulation. **CYP2C8** catalyzes taxol 6alpha-hydroxylation as a selective marker activity; other substrates include pioglitazone and amodiaquine. **CYP2C9** is very abundantly expressed in the human liver and has a broad substrate specificity accepting many weakly acidic substances like the hypoglycemic agent tolbutamide, the anticoagulant warfarin, the anticonvulsant phenytoin, and several NSAIDs (nonsteroidal anti-inflammatory drugs). **CYP2C19** substrates are S-mephenytoin, the 4-hydroxylation of which provides a very specific marker activity, the proton pump inhibitor omeprazole, the antimalarial proguanil, and diazepam. All *CYP2C* genes are genetically polymorphic. Clinically relevant are the two major variant alleles of CYP2C9, *2C9∗2* and *2C9∗3*, which are associated with decreased enzyme activity, as well as the genetic polymorphism of *CYP2C19*, also known as the S-mephenytoin polymorphism, which affects about 3–5% of Caucasians and up to 20% of Asian populations. **CYP2C18** appears to be expressed in the liver only as mRNA but not as a protein.

CYP2D6 is a very important enzyme for drug oxidation, although its expression in the liver is rather low. Substrates include antiarrhythmics (e.g., propafenone), antidepressants (e.g., amitriptyline, venlafaxine), antipsychotics (e.g., thioridazine), beta-blockers (e.g., metoprolol), opioids (e.g., codeine), and more. It was the first P450 for which a classical pharmacogenetic polymorphism became known. More than 100 functionally distinct alleles which are associated with either complete lack of function (null-alleles) or with decreased or increased enzyme activity are known (www.pharmvar.org). The individual inherited allele combination (genotype) determines whether an individual will have the ultrarapid metabolizer (UM), extensive (EM), intermediate (IM) or poor metabolizer (PM) phenotype. About 5–10% of Caucasians carry two null-alleles and are consequently PMs; about 10–15% are IMs carrying alleles with reduced function, and about 1–5% are UMs due to an allele with a duplicated functional gene. In other ethnic populations, these percentages can be very different. Thus, in Asians the PM phenotype has a frequency of only 0.5–1%, whereas in certain Arabian and Eastern African populations, the frequency of the UM phenotype can be as high as 30%. The phenotype can be determined either by using one of several available specific probe drugs, e.g., dextromethorphan or metoprolol, or it can be predicted by genetic diagnosis.

CYP2E1 metabolizes small molecules including ethanol, halogenated hydrocarbons like halothane, as well as small aromatic and heterocyclic compounds, many of which also act as inducers. For example, it is known that CYP2E1 is induced in alcoholics. Only few drug substrates of CYP2E1 are known, but the enzyme activates many xenobiotic metabolites to toxic intermediates. Chlorzoxazone 6-hydroxylation has been proposed as a marker activity. Polymorphisms in the *CYP2E1* gene seem to be of low significance, although some are believed to be associated with increased cancer risk.

CYP2F1 appears to be expressed preferentially in lung where it bioactivates the selective pneumotoxins 3-methylindole and naphthalene.

CYP2J2 is abundant in cardiovascular tissue and active in the metabolism of arachidonic acid to eicosanoids that possess potent anti-inflammatory, vasodilatory, and fibrinolytic properties. Polymorphic alleles with reduced function are known.

CYP2R1 is the principal hepatic enzyme for the hydroxylation of vitamin D to 25-hydroxyvitamin D. Inactivating mutations in CYP2R1 can lead to a certain form of vitamin D-deficiency rickets.

CYP2S1 appears to be expressed preferentially in respiratory tissues and the intestine. It is induced by dioxin and is able to activate and deactivate certain carcinogens.

CYP2U1 metabolizes arachidonic acid and has been linked to hereditary spastic paraplegia.

CYP2W1 appears to be expressed in the fetal colon and in colon tumors, but its functions are still unknown.

The **CYP3A** subfamily, in particular CYP3A4, plays a very important role due to its broad

substrate selectivity and because it is abundantly expressed not only in the liver but also in intestinal enterocytes. CYP3A4 makes significant contributions to the metabolism of more than half of all clinically used drugs including large molecules like the immunosuppressant cyclosporin A, macrolide antibiotics like erythromycin, or anticancer drugs like taxol and smaller molecules like benzodiazepines, HMGCoA reductase inhibitors, anesthetics, and many more. CYP3A4 expression levels are increased following exposure to a number of drugs that bind to the nuclear receptor PXR (pregnane X-receptor) which increases the rate of *CYP3A4* gene transcription. Furthermore, there are sex-related differences in CYP3A4 expression with women having significantly higher levels, which translate into higher in vivo clearance of several drug substrates. Although an allele (*CYP3A4*∗22) has been linked to lower activity, genetic polymorphism has not been found to be of major importance. In contrast, the expression of the two subfamily members, CYP3A5 and CYP3A7, is confined to a smaller fraction of the population who carry particular alleles of these genes. CYP3A7 is more abundantly expressed in the fetal liver than in adult liver. CYP3A43 was found to be expressed at very low levels.

CYPs in Physiological Pathways

The **CYP4** family comprises a large number of subfamilies and isozymes. The major substrates for CYP4A enzymes are fatty acids (ω-hydroxylation) and eicosanoids. Non-fatty acid substrates may be metabolized by specific CYP4A forms. For example, CYP4F2 is clinically relevant for warfarin dose adjustment. Expression of CYP4 enzymes is regulated by peroxisome proliferators like clofibrate (peroxisome oxidize fatty acids), drugs that bind to another nuclear receptor termed PPAR (peroxisome proliferator-activated receptor).

CYP5 synthesizes thromboxane A2, a fatty acid in the arachidonic acid cascade that causes platelet aggregation. Aspirin prevents platelet aggregation because it blocks the cyclooxygenases COX1 and COX2 which catalyze the initial step of the biotransformation of arachidonic acid to thromboxane and prostaglandins.

CYP7A1 catalyzes the 7α-hydroxylation of cholesterol, the first and rate-limiting step of bile acid synthesis. This is also the principal way to eliminate cholesterol. **CYP7B1** is primarily expressed in the brain and catalyzes the synthesis of various neurosteroids and also the 7α-hydroxylation of oxysterols.

CYP8A1 is the complementary enzyme to CYP5 in that it synthesizes prostacyclin in the arachidonic acid cascade. **CYP8B1** catalyzes the steroid 12alpha-hydroxylation in the cholic acid biosynthesis.

CYP11A1 is known as the mitochondrial side-chain cleavage enzyme that converts cholesterol to pregnenolone, the first step in steroid hormone biosynthesis. Steroid hormone levels are under tight endocrine control via the P450 enzymes involved in their biosynthesis, which are transcriptionally regulated by ACTH (adrenocorticotropic hormone) via intracellular cAMP. Genetic defects in CYP11A1 lead to a lack of glucocorticoids, feminization, and hypertension. **CYP11B1** is the mitochondrial 11 beta-hydroxylase that synthesizes cortisol and corticosterone. Genetic defects in this gene lead to congenital adrenal hyperplasia. **CYP11B2**, aldosterone synthase, hydroxylates corticosterone at C-18. The genetic deficiency of CYP11B2 is the cause of congenital hypoaldosteronism.

CYP17 is the 17alpha-hydroxylase and 17–20 lyase, two different reactions of the same substrate catalyzed by one enzyme and required for production of testosterone and estrogen, respectively. Defects in this enzyme affect development at puberty.

CYP19 is known as aromatase that synthesizes estrogen by converting ring A of the steroid nucleus into an aromatic ring. The lack of this enzyme causes a lack of estrogen and failure of women to develop at puberty. Because estrogens are involved in breast cancer development, CYP19 is an important target to develop specific anti-breast cancer agents (aromatase inhibitors) that inhibit the enzyme.

CYP21 catalyzes steroid C21 hydroxylation required for cortisol biosynthesis. Genetic defects in this gene cause congenital adrenal hyperplasia.

CYP24 is a 25-hydroxyvitamin D3–24-hydroxylase that degrades vitamin D metabolites.

CYP26 consists of three enzymes each representing a separate subfamily, all involved in retinoic acid hydroxylation. **CYP26A1** is an all-trans-retinoic acid hydroxylase which degrades retinoic acid, an important signaling molecule for vertebrate development. It acts through retinoic acid receptors.

CYP27A1 is a mitochondrial enzyme that catalyzes the side-chain oxidation (27-hydroxylation) in bile acid biosynthesis and the 25-hydroxylation of vitamin D_3. Because bile acid synthesis is the only elimination pathway for cholesterol, mutations in the *CYP27A1* gene lead to abnormal deposition of cholesterol and cholestanol in various tissues. This sterol storage disorder is known as cerebrotendinous xanthomatosis. **CYP27B1** is the 1alpha-hydroxylase of vitamin D3 that converts it to the active vitamin form. The function of **CYP27C1** is not yet known.

CYP46 is a brain enzyme and hydroxylates cholesterol at the 24-position, a reaction that appears to play a role for brain cholesterol homeostasis.

CYP51 catalyzes lanosterol 14alpha-demethylation required in the biosynthesis of cholesterol in a mammalian liver, but it is also expressed in the sperm where it synthesizes meiosis-activating sterols. This enzyme is evolutionarily highly conserved in plants, fungi and animals, and bacteria and may be the ancestor of all eukaryotic P450s. CYP51 enzymes are important targets for cholesterol-lowering drugs, antifungal agents like ketoconazole, and herbicides.

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Pain Medication

▶ Analgesics

Parkinson's Disease

▶ Dopamine System
▶ Monoamine Oxidases and Their Inhibitors

Pathogenesis

▶ Rheumatoid Arthritis

PCSK9 Inhibition

▶ Lipid-Lowering Drugs

P

Peptides and Peptidomimetics as Foundations for Drug Discovery

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Synonyms

Drug discovery; Gram-negative bacteria; Multidrug-resistant; Peptidomimetics; Polymyxins; Somatostatin; Substance P

Definition

Peptides and Peptidomimetics

Peptides (derived from Greek language peptós "digested") are short chains of amino acids, typically comprising 2–50 amino acids, that are linked by peptide bonds. Peptides are naturally occurring biological molecules that are ubiquitous in all living organisms and display a large diversity of structures and biological effects. They are known to regulate most physiological processes in the body, acting as hormones, neurotransmitters, and antimicrobial agents. Peptides also mediate various essential biological functions, such as signal transduction, heart rate regulation, mating, chemotaxis, food intake, pain perception, growth development, and immunity to achieve homeostasis and maintain health. Like proteins, peptides are formed from transcription of a sequence of the genetic code, DNA; however, peptides are distinguished from proteins on the basis of size, being significantly shorter (oligopeptides (two to ten amino acids) and polypeptides (10–50 amino acids)), although the cutoff number of amino acids can be arbitrary (Lau and Dunn 2018).

Peptidomimetics are modified peptides whose essential elements (pharmacophore) mimic a natural peptide, designed to maintain the biological function of the natural peptide, while simultaneously removing their liabilities, such as their rapid metabolism, lack of receptor selectivity, and poor bioavailability. Peptidomimetics arise from the combination of chemical synthesis and employment of techniques from various other sciences offering preservation of the advantages of linear peptides, including high potency and low toxicity, and removing their disadvantages (Cunningham et al. 2017; Rubin et al. 2018; Rubin and Qvit 2018).

Peptides and peptidomimetics as drugs show unique characteristics and can be very effective. They are highly bioactive and very specific, demonstrate low toxicity, and in many cases are developed from natural endogenous scaffolds with known biological activity, thereby making them particularly attractive therapeutic agents. Over the years, peptides and peptidomimetics have been evolved as promising therapeutic agents in the treatment of different diseases (Rubin and Qvit 2016; Qvit et al. 2017; Lau and Dunn 2018) such as parasitic diseases (Qvit and Kornfeld 2016; Qvit et al. 2016c), cancer (Marqus et al. 2017), diabetes (Flatt and Conlon 2018), and cardiovascular diseases (Qvit et al. 2016a; Qvit et al. 2016b).

The Role of Peptides and Peptidomimetics in Drug Discovery

Over the past six decades, many scientific and technological tools have been improved remarkably in many various fields, contributing to innovation and progression in basic research, drug discovery, and drug development. Despite the various developments that have improved efficiency, reliability, and reproducibility, and in a much more cost-effective way, the research and discovery (R&D) productivity of the pharmaceutical industry has been declining. With every billion United States (US) dollars allocated to drug R&D, the number of approved drugs has halved approximately every 9 years since 1950, raising the estimated average cost per new approved drug to $1395 million (DiMasi et al. 2016). Scannell et al. coined this phenomenon as Eroom's Law (the reverse spelling of Moore – as in Moore's Law, which describes the doubling every 2 years from the 1970s to 2010 of the number of transistors which can be placed at a reasonable cost onto an integrated circuit) (Scannell et al. 2012).

Peptide therapeutics have played a notable role in medical practice since the isolation and commercialization of insulin, a 51-amino acid peptide, which was initially purified in 1921, reaching the first diabetic child for treatment with bovine insulin a year later and becoming the first commercially available peptide drug in 1923. Before insulin was discovered, there was no treatment for people with diabetes, other than to put patients on very strict diets that could buy patients a few extra years. Therefore, it is not surprising that insulin was considered a "miracle drug." The pioneering and extensive work on insulin's sequence, structure, and molecular pharmacology leads to several important accompaniments, including the introducing of the first recombinant drug, human insulin, in 1982. Since then there is a

constant increase in the number of peptides entering clinical studies, from 1.2 per year (1970s) to over 16.8 per year (2000s) (Kaspar and Reichert 2013). Importantly, peptides demonstrate superior success rates in transitioning from phase 1 to phase 2 trials (83%) compared to small molecules and biological drugs (63% and 77%, respectively) and in transitioning from phase 3 to regulatory review (68%, compared to 61% of small molecules and 63% of biological drugs). Overall, it is not surprising that the number of available therapeutic peptides and peptidomimetics is increasing, and currently, there are 60 approved peptide and peptidomimetic drugs in the market, about 155 peptide drugs are in clinical trials, and over 500 are in preclinical development (Fosgerau and Hoffmann 2015). In addition, four peptides reached global sales of over $1 billion in 2010, including glatiramer acetate ($4.0 billion), leuprolide acetate ($3.0 billion), octreotide acetate ($1.3 billion), and goserelin acetate ($1.1 billion). In the next section we will discuss the basic mechanisms and pharmacological intervention of three representative peptides: a hormone, somatostatin; a neurotransmitter, substance P; and antimicrobial peptides from the polymyxin family, polymyxin B and polymyxin E.

Somatostatin

Somatostatin ((SST) aka growth hormone-inhibiting hormone (GHIH)) is an endogenous cyclic peptide hormone that regulates the endocrine system. It was discovered in 1973, by the research group of Dr. Roger Guillemin (Brazeau et al. 1973), and in 1977 Dr. Guillemin shared the Nobel Prize in Medicine with Dr. Andrew V. Schally, for their discoveries concerning the peptide hormone production of the brain. It is initially secreted as a 116-amino acid precursor, pre-prosomatostatin, which undergoes endoproteolytic cleavage to prosomatostatin (92 amino acids). Prosomatostatin is further processed into two active forms produced by alternative cleavage, somatostatin-14 (SST-14) and somatostatin-28 (SST-28), which is an extended variant of SST-14 sequence, with the extension appended to the N-terminus. SST-14 is the predominant form in the brain, and SST-28 is mainly produced by intestinal enteroendocrine cells.

Basic Mechanisms

Somatostatin is a hormone produced by many tissues in the body, principally in the nervous and digestive systems, and it exhibits a large variety of actions in different organ systems throughout the body through binding to somatostatin-specific receptors that belong to the family of G protein-coupled receptors (GPCRs). Somatostatin receptors are members of the rhodopsin family of GPCRs and comprise five receptor subtypes (SSTR1-SSTR5), which are found in a variety of tissues, including the gastrointestinal (GI) tract and brain. SST-14 and SST-28 bind to SSTR1-SSTR4 with similarly high affinities (Kd values in the low nanomolar range); however SST-28 demonstrates about tenfold higher affinity for SSTR5 than to the other receptors (Kumar et al. 1997). Somatostatin release is stimulated by several nutrients, such as amino acids and glucose, as well as by stimulation of various hormones. It is secreted by the delta cells of the islets of Langerhans within the pancreas producing inhibitory effects on pancreatic endocrine and exocrine secretion. Somatostatin is also expressed throughout the gastrointestinal tract, reducing gastric secretions and the emission of gastrointestinal hormones. In addition, somatostatin is also generated by neurons in the anterior periventricular nucleus and arcuate nucleus within the hypothalamus, where it inhibits the release of growth hormone and thyroid-stimulating hormones from the anterior pituitary.

Pharmacological Intervention

Somatostatin's short circulating half-life ($t_{1/2}$ 1–3 min) limits its therapeutic potential. The development of ligands selective for each receptor subtype is important when producing new therapies to minimize interaction with other receptor subtypes and therefore reduce unwanted side effects. A common therapeutic use of somatostatin receptor agonists is their anti-secretory effect on neuroendocrine tumors.

Lanreotide (trade name: Somatuline) is a synthetic long-acting analogue of somatostatin with a significantly longer half-life (22 days), used to treat neuroendocrine tumors that secrete excessive amounts of growth hormone, other active hormones, or neuropeptides (Caplin et al. 2014). The Food and Drug Administration (FDA) approved lanreotide in 2007.

Octreotide (trade name: Sandostatin) is an octapeptide synthetic analogue of somatostatin that mimics natural somatostatin pharmacologically. It is a peptide analogue of somatostatin with longer half-life (1.7–1.9 h) compared to somatostatin, with pharmacological activity mimicking those of the natural hormone somatostatin; however it is a more potent inhibitor of growth hormone, glucagon, and insulin (Lamberts and Hofland 2019). It was first synthesized in 1979 by the chemist Wilfried Bauer and approved by the FDA in October 1988.

Pasireotide (aka SOM230, trade name: Signifor) is a cyclic hexapeptide synthetic analogue of somatostatin that resembles the native hormone in its ability to suppress levels and activity of growth hormone, insulin, glucagon, and many other gastrointestinal peptides. It is a long-acting molecule (half-life 12 h) that was approved as a drug for Cushing's disease by the European Medicines Agency (EMA) in October 2009 and by the FDA in December 2012. Pasireotide LAR (long-acting release) was approved by the EMA in November 2014 and by the FDA in December 2014 for treatment of acromegaly (Colao et al. 2014).

Substance P

Substance P is a undecapeptide of the tachykinin ((TK), aka neurokinin (NK)) family of neuropeptides that are evolutionarily the oldest neurotransmitters and are derived from the tachykinin-1 (Tac1) (aka preprotachykinin-A (PPTA)) gene. It was first identified in extracts of equine brain and intestinal tissue in 1931 (Euler and Gaddum 1931). Substance P is widely expressed throughout the animal kingdom, and it is abundant in the peripheral and the central nervous system (CNS), being the most abundant neuropeptide in the brain. The TK peptides are synthesized as large precursor proteins, which undergo posttranslational proteolytic processing by alternative mRNA splicing to generate the mature peptide products. TKs have an important role in health and disease (*e.g.*, cancer, inflammation, pain, and cardiovascular diseases) (Nässel et al. 2019).

Basic Mechanisms

Substance P biological activity is mediated by specific TK receptors that belong to the class of (rhodopsin-like) GPCRs. There are three main classes of neurokinin receptors: GPCR neurokinin-1 receptor (NK1R), NK2R, and NK3R, and substance P has the highest affinity to NK1R (affinity 0.05–0.5 nM), followed by NK2R and NK3R (Mantyh 2002). The various TKs have different binding affinities to the neurokinin receptors, yet all TK, sharing a common C-terminal amino acid sequence which essentially dictates their biological activity (Zieglgänsberger 2019). Substance P interaction with the neurokinin-1 receptor results in major downstream cellular actions, which include changes in fluxes of calcium (Ca^{2+}) and ERK, as well as phosphorylation of p21-activated kinase and nuclear factor kappa B (NF-κB) activation (Spitsin et al. 2018). There are two isoforms of NK1R, the full-length (NK1R-F, 407 amino acid residues) and truncated NK1R (NK1R-T, 311 amino acid residues), mediating the actions of substance P. NK1R-F is the predominant isoform in the central and peripheral nervous systems, while NK1R-T disrupts a vital receptor signaling motif resulting in decreased ligand binding affinity and thus lowering activity (Douglas and Leeman 2011). There are functional differences between NK1R-F and NK1R-T; herein we will focus on NK1R-F. Substance P activation of NK1R-F leads to the activation of several major signal transduction pathways, such as activation of phospholipase C (PLC) that causes phosphatidylinositol 4,5-bisphosphate cleavage to form diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) (Mizuta et al. 2008). NK1R-F activation also leads to calcium (Ca^{2+}) mobilization (release) and activation of protein kinase C (PKC) (Morris et al. 2006). NK1R-F signaling stimulates numerous downstream

mechanisms leading to diverse and sometimes cell type-specific effects that include inflammation, proliferation, anti-apoptosis, cellular growth, neuronal excitation, and migration, to mention a few (Martinez and Philipp 2016; Mashaghi et al. 2016).

Pharmacological Intervention

Substance P's role in pain, inflammation, cancer, depressive disorder, immune function, gut function, hematopoiesis, sensory processing, and hormone regulation has led to extensive research into the pharmacology and molecular biology of this signaling system as a therapeutic target. Structure-activity relationship (SAR) studies employing substance P fragments (Regoli et al. 1984), alanine-substituted (Couture et al. 1979), and D-amino acid-substituted analogs (Wang et al. 1993) have indicated that changes in the NH_2-terminal region have only minor effects on the peptide potency. Since substance P rapidly degrades both *in vivo* and *in vitro*, with a half-life of less than 2 min and 12 min, respectively, academic and industrial researchers are in search for metabolically stable analogues. Many potent and selective peptidomimetics and non-peptide TK receptor antagonists have been developed and proven effective in preclinical studies: herein we will describe some of these analogues.

Aprepitant (trade name: Emend) is a selective, high-affinity NK1R antagonist that is able to alleviate the emetic effects of substance P, which was initially approved as a drug in 2003. Oral aprepitant is effective in the prevention of acute and delayed chemotherapy-induced nausea and vomiting (CINV) associated with initial and repeat courses of highly emetogenic cancer chemotherapy when combined with a corticosteroid (*e.g.*, dexamethasone) and a serotonin 5-HT3 receptor antagonist (*e.g.*, ondansetron). Fosaprepitant for intravenous (IV) administration (Emend (US) and Ivemend (EU)) is a prodrug of aprepitant that is a well-tolerated substitute for oral aprepitant, with half-life of 9–13 h.

Netupitant/palonosetron (trade names: NEPA, Akynzeo) is a combination of netupitant, a small molecule antiemetic substance P analogue with high affinity to the NK1R antagonist, and palonosetron, a serotonin-3 ($5\text{-}HT_3$) receptor antagonist with a strong binding affinity for this receptor used as an antiemetic and antinauseant agent. It is an oral fixed-dose combination of netupitant/palonosetron for the prevention of acute and delayed CINV, with a half-life of 96 h, that was initially approved as a drug in 2014. Fosnetupitant is a prodrug form of netupitant approved as an alternative treatment option for CINV patients, with half-life of 80 h, that was initially approved as a drug in 2018.

Rolapitant (trade names: Varubi (US) and Varuby (EU)) is an oral potent and long-acting (169–183 h) NK1R highly selective antagonist. It is indicated in combination with other antiemetics for the prevention of delayed CINV, initially approved as a drug in 2015 (Goldberg et al. 2017).

Polymyxins

Polymyxins are an old class of non-ribosomal naturally occurring cyclic lipopeptides produced by the widely distributed Gram-positive spore-forming soil bacterium *Paenibacillus polymyxa*, which demonstrate narrow antibacterial spectrum against Gram-negative infections. Initially the polymyxin family was identified in the 1940s by three research groups concurrently (Ainsworth et al. 1947; Benedict and Langlykke 1947; Stansly et al. 1947). Members of the polymyxin family demonstrated unique characteristic by its specificity for Gram-negative bacteria (GNB), which distinguished it from previously reported antibiotics (Velkov et al. 2019).

At the beginning five different polymyxin molecules were known (polymyxins A, B, C, D, and E) differing in their amino acid sequences and fatty acid side chains. Later, several other members of the family were discovered, including polymyxin M, polymyxin S, and polymyxin T. Interestingly, polymyxins M and T are also active against Gram-positive bacteria. In 1963, the chemical structures for polymyxin B1, polymyxin B2, and colistin A (polymyxin E1) were determined, followed by that of colistin B (polymyxin E2) (Hayashi and Suzuki 1965). Polymyxin B and polymyxin E share an almost

identical primary amino acid sequence with the major difference present at position six, where polymyxin B contains D-phenylalanine, while colistin contains D-leucine (Velkov et al. 2010).

Polymyxin B is a cationic lipopeptide that binds the lipid-A portion of endotoxin, which was discovered and isolated from *Bacillus polymyxa* in the 1940s. It is a mixture of at least four closely related compounds, polymyxin B1 to B4; however polymyxin B1 and polymyxin B2 are the two major components (Rigatto et al. 2019). Polymyxin E (aka colistin) is a cyclic polypeptide antibiotic from *Bacillus colistinus* that was first described in 1950. At least 30 different components have been detected in colistin, which differ only in the fatty acid side chain components (Li et al. 2001).

Basic Mechanisms

Polymyxin B and colistin share the same mechanism of action; both have the diaminobutyric acid residues, which are positively charged, targeting the negatively charged bacterial cell membrane. They disrupt the bacterial cell membrane through interaction with anionic lipopolysaccharide and phospholipid molecules in the outer membrane of GNB, a detergent-like mechanism. The initial association of both peptides with the bacterial cell membrane occurs through electrostatic interactions between the cationic polypeptide and anionic lipopolysaccharides. They displace divalent cations, such as calcium (Ca^{2+}) and magnesium (Mg^{2+}), from the phosphate groups of membrane lipids, which stabilize the lipopolysaccharide membrane leading to derangement and an increase in the permeability of the cell envelope. The result of this process causes leakage of the cell contents and ultimately cell death (Gai et al. 2019).

Pharmacological Intervention

Polymyxin B and colistin became available clinically in the late 1950s and early 1960s. However, their use waned in the 1970s with the development of less toxic agents, such as extended-spectrum penicillins, due to their potential nephrotoxicity and neurotoxicity except for treatment of patients with cystic fibrosis. Currently they are used as a last therapeutic option for multidrug-resistant (MDR) Gram-negative organisms, caused by "superbugs" such as *Pseudomonas aeruginosa*.

Antibiotic resistance (AMR) causes higher medical costs, longer hospital stays, and increased mortality. It is estimated that a cumulative US $100 trillion of economic output is at risk due to AMR by 2050; moreso, about ten million people per year will die of AMR infections. Both polymyxin B and colistin are clinically available polymyxins that reemerged in clinical practice to treat infections caused by MDR GNB. The two clinically available polymyxins have very different pharmacological properties, as colistin is mainly administered intravenously to patients in an inactive prodrug form, colistimethate sodium (CMS) (Rigatto et al. 2019). Surprisingly, in several recent studies, polymyxin B and colistin demonstrated acceptable effectiveness and considerably less toxicity than that reported in old studies (Nation and Li 2017).

The most relevant clinical use of polymyxin B is for the treatment of infections caused by extensively MDR using polymyxin B sulfate (trade name: Polimixina B), which is the form available for IV administration, with half-life of 11.5 h. Colistin is available in two forms. First is colistin sulfate (trade name: Cortisporin-TC) for the treatment of acute or chronic infections due to sensitive strains of certain Gram-negative bacilli, with half-life of 5 h. In addition, it may be used for the treatment of lung infections due to MDR GNB in patients with cystic fibrosis. Colistin second form is colistimethate sodium ((CMS); trade name: Coly-Mycin M) which is an inactive prodrug of colistin less potent and less toxic than colistin sulfate, and it is used in parenteral formulations and can be administered intravenously, intramuscularly, or by nebulization, with half-life of 2–3 h.

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Peptidomimetics

▶ Peptides and Peptidomimetics as Foundations for Drug Discovery

Pharmacokinetic and/or Pharmacodynamic Consequence of Multiple Drug Therapy

▶ Drug Interactions

Pharmacokinetic and/or Pharmacodynamic Interactions

▶ Drug Interactions

Pharmacology

▶ Ghrelin, Physiological Roles and Clinical Relevance of

Phosphatidylinositide Kinases

▶ Phospholipid Kinases

Phosphatidylinositol Kinases

▶ Phospholipid Kinases

Phosphoinositol Kinases

▶ Phospholipid Kinases

Phospholipid Kinases

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Synonyms

Phosphatidylinositide kinases; Phosphatidylinositol kinases; Phosphoinositol kinases

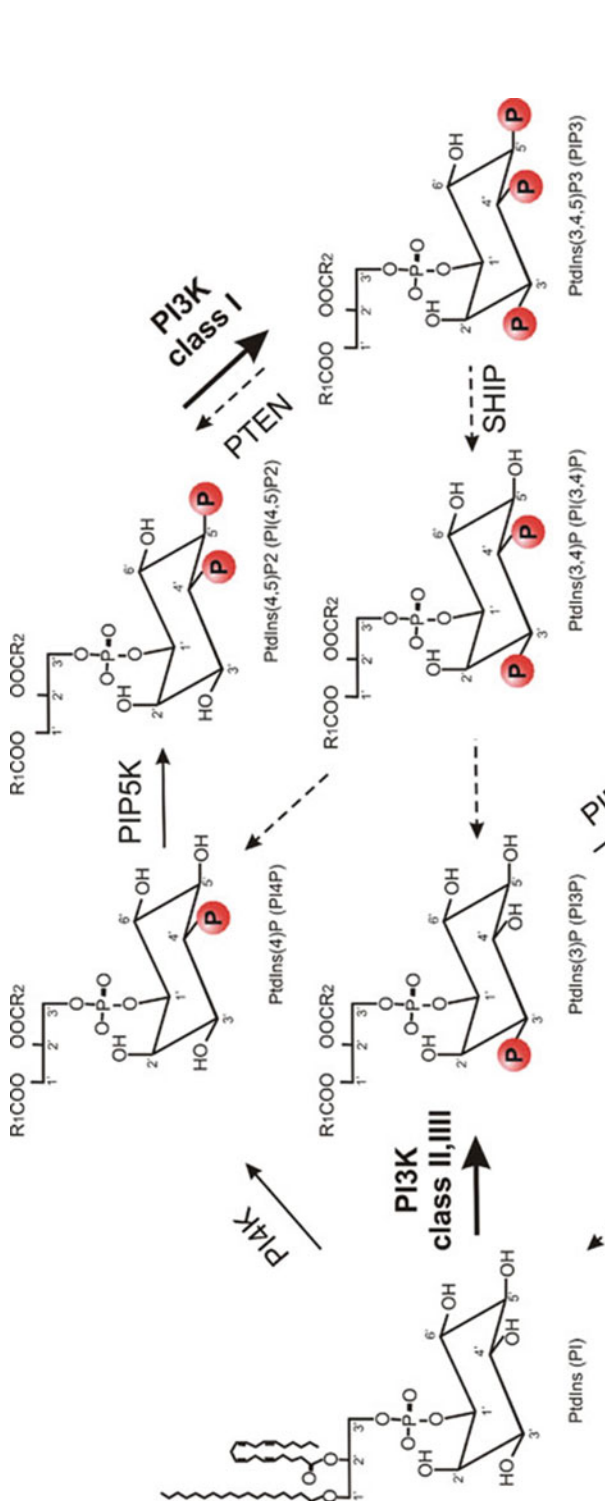
Definition

Phospholipid kinases comprise a family of enzymes that phosphorylate phosphatidylinositol (PtdIns) and phosphatidylinositides at positions 3′, 4′, and 5′ but not at positions 2′ and 6′ of the inositol ring (Fig. 1). PtdIns and its derivates are referred to as phosphoinositides or PIs. PIs represent approximately 1% of all membrane lipids such as those of the plasma, nuclear, and endomembranes. In unstimulated cells, more than 90% of all phosphoinositides correspond to unphosphorylated PtdIns, whereas the remaining 10% consist of roughly equal amounts of phosphatidylinositol 4-phosphate (PtdIns(4)P) and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) (Loo et al. 2015). The remaining PIs, i.e., phosphatidylinositol 5-phosphate (PtdIns (5)P) and phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P_2), are present in small quantities in cells (Shisheva 2008). Initially, it was assumed that these phosphoinositides function exclusively as precursors for signaling molecules. For instance, PtdIns(4,5)P_2 serves as a substrate for phospholipase C and phosphoinositide 3-kinases (PI3Ks). In addition, PtdIns(4,5)P_2 interacts with intracellular proteins in a regulatory manner, thereby affecting their endocytosis, trafficking, localization, and activity (Shisheva 2008). In resting cells, less than 0.25% of the PIs are 3′-phosphorylated. Stimulation of cells with ligands that activate, for instance, receptor tyrosine kinases or G protein-coupled receptors (GPCRs) results in the rapid and transient phosphorylation of PtdIns(4,5)P_2 to phosphatidylinositol 3,4,5-triphosphate (PtdIns (3,4,5)P_3). This 3′-phosphorylated phosphoinositide behaves as a typical second messenger. Hence, the responsible phosphoinositide 3-kinases are considered as important regulatory molecules of the cell and therefore targeted for pharmacological intervention.

Basic Characteristics

Cellular PI concentrations are under tight control by phospholipid kinases and phosphatases. Phospholipid kinases preferentially phosphorylate distinct positions of the inositol ring and hence are subdivided into phosphoinositide 3-kinases (PI3Ks), phosphoinositide 4-kinases (PI4Ks), and phosphoinositide 5-kinases (PI5Ks) that phosphorylate PIs on positions 3′, 4′, and 5′, respectively. In a canonical pathway, PtdIns(4,5)P_2 is generated from PtdIns(4)P by the enzymatic activity of phosphatidylinositol 4-phosphate 5-kinase (PIP5K) (Fig. 1). Maintenance of the plasma membrane pool of PtdIns(4)P and PtdIns (4,5)P_2 relevant for function and regulation of GPCRs and ion channels is preferentially controlled by the IIIα isoform of phosphatidylinositol 4-kinases (PI4Ks) and by the type 1 phosphatidylinositol 4-phosphate 5-kinases (PIP5Ks) (Loo et al. 2015). Two classes of PI4Ks are known that can be distinguished by their sensitivity toward Wortmannin. Interestingly, inducible PI4K IIIα knockout mice are lethal. A lethal phenotype was also reported for constitutive PIP5K and for global PIKfyve knockout mice. The latter produce phosphatidylinositol 5-phosphate (PtdIns (5)P) and phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P_2) from PtdIns(3)P that have been implicated in regulation of endosomal trafficking and cellular signaling. The vital functions of these PI-forming enzymes question them as suitable drug targets for therapeutic intervention despite specific inhibitors are available and a rapidly growing number of mutations in PI-forming enzymes are correlated with human diseases.

3′-phosphorylated PIs function as important intracellular mediators or modulators of signaling processes and act as second messengers following stimulation of cells by a broad range of extracellular stimuli. They are involved in cell survival pathways, the regulation of gene expression, proliferation, cell metabolism, membrane trafficking, and cytoskeletal rearrangements involved in cell motility (Loo et al. 2015; Bilanges et al. 2019). Correspondingly, the enzymes producing 3′-phosphorylated PIs,, i.e. phosphoinositide 3-kinases (PI3K), have been linked to major human diseases and conditions such as cancer, nonmalignant overgrowth syndromes, immune dysfunction and inflammatory processes, diabetes, or aging. This has lead not only to the development of highly specific pharmacological tools to interfere with



Phospholipid Kinases, Fig. 1 Major phosphoinositide pathways in mammalian cells. All phosphoinositides are found in the cytosolic half of the lipid bilayer of the plasma or intracellular compartment membranes (left part). The different kinases acting on phosphoinositides in mammalian cells are shown in solid lines and the phosphoinositide 3-kinases in bold. The phosphoinositide counterpart pathways catalyzed by known phosphatases are represented by dashed lines. The best known phosphatases are PTEN (phosphatase and tensin homolog deleted on chromosome 10) and SHIP (SH_2 domain-containing inositol 5-phosphatase). For further information, see Rudge and Wakelam (2014)

Phospholipid Kinases, Table 1 Approved PI3K inhibitors for treatment in patients

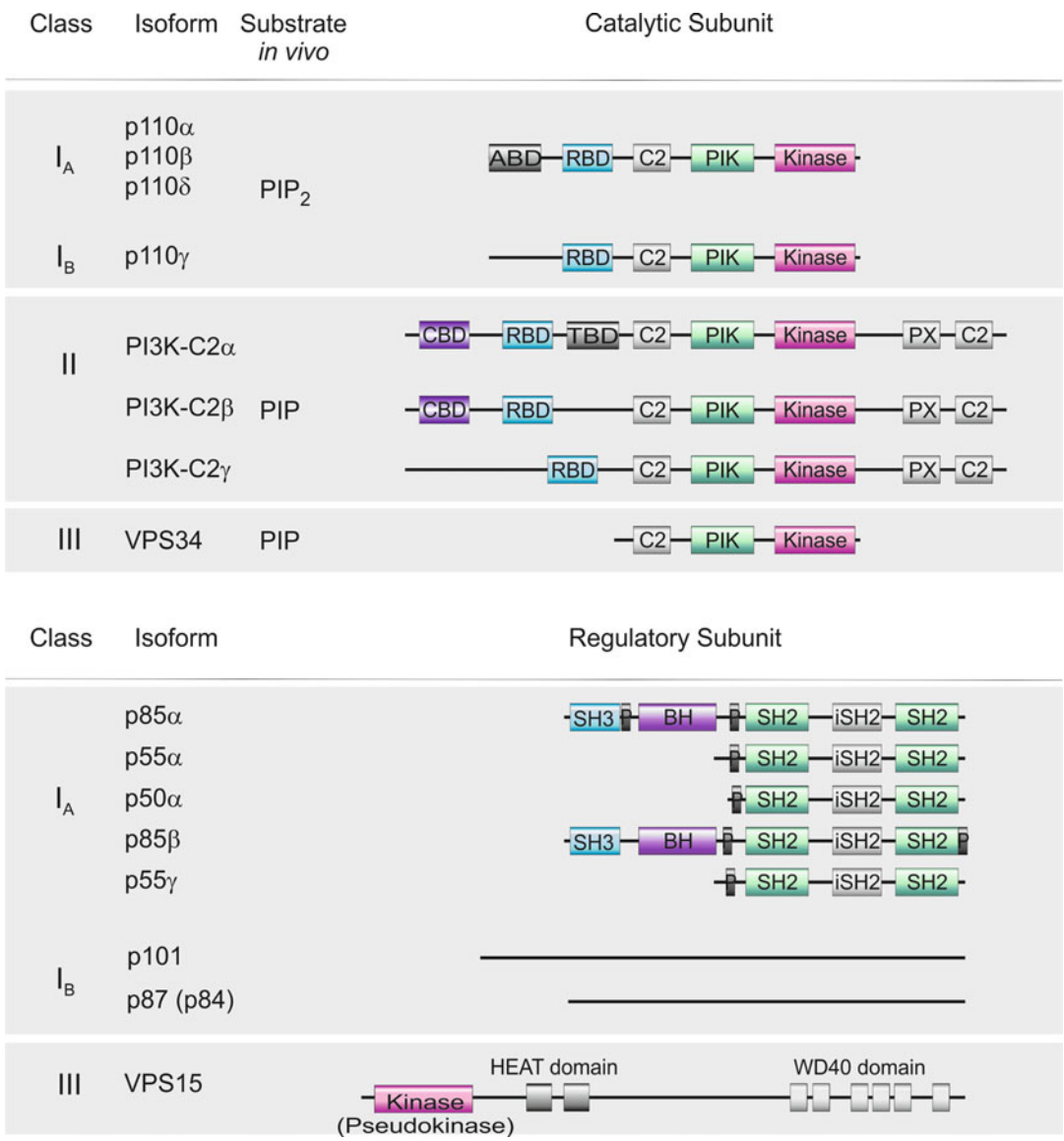
INN name	Drug name	Properties	Indication	Date of approval
Alpelisib	Piqray	PI3Kα inhibitor	Breast cancer in combination with endocrine therapy	2019
Copanlisib	Aliqopa	PI3K inhibitor (predominantly PI3Kα and PI3Kβ isoforms)	Relapsed follicular lymphoma	2017
Duvelisib	Copiktra	PI3Kδ and PI3Kγ inhibitors	Relapsed or refractory chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma (SLL), follicular lymphoma	2018
Idelalisib	Zydelig	PI3Kδ inhibitor	Chronic lymphocytic leukemia (CLL), refractory follicular lymphoma	2014

PtdIns 3-dependent pathways but also to the application of PI3K-inhibiting drugs for the therapy of patients (Table 1).

Classification of PI3Ks

Based on substrate specificity and homology, mammalian phosphoinositide 3-kinases (PI3K) are divided into three classes (Fig. 2). Class I PI3Ks are heterodimeric enzymes composed of a 110 kDa catalytic subunit (with the isoforms p110 α, β, δ, or γ) that associates with a regulatory subunit. Although the class I PI3Ks are capable of phosphorylating PtdIns, PtdIns(4)P, and PtdIns (4,5)P_2 in vitro, it appears that they only use PtdIns(4,5)P_2 as a substrate in vivo (Bilanges et al. 2019). Receptor-induced formation of PtdIns(3,4,5)P_3 from PtdIns(4,5)P_2 seems restricted to the enzymatic activity of class I kinases, whereas the metabolites of PtdIns(3,4,5)P_3, PtdIns(3,4)P_2, and PtdIns(3)P are also produced by class II or class III PI3Ks.

Class I_A PI3K catalytic subunits are relatively unstable and form a complex with either one of five different regulatory subunits (p85α, p55α, p50α, p85β, or p55γ), which contain two src-homology 2 (SH2) domains (Fig. 2). These SH2 domains specifically recognize phosphorylated tyrosine residues that are present on activated growth factor receptors or on growth factor receptor substrates. This interaction results in a translocation of the cytosolic heterodimeric enzyme to the inner leaflet of the plasma membrane, thus bringing the catalytic subunit p110 close to its lipid substrate PtdIns(4,5)P_2. In addition, the interaction with the tyrosine-phosphorylated receptor induces a conformational change of p85, which disinhibits the enzymatic activity of the p110 catalytic subunit. The p85 subunits contain characteristic protein domains which allow multiple protein–protein interactions. For instance, the inter-SH2-region (iSH2) of p85 mediates the interaction with the catalytic p110 subunit. Interestingly, p85 seems to exist independently from p110 and may possess PI3K-activity independent roles such as cell migration and cytokinesis (Bilanges et al. 2019). All class I PI3Ks are also controlled by monomeric GTPases via a Ras-binding domain. Whereas p110α, p110γ, and p110δ bind monomeric Ras proteins, the p110β isoform preferentially interacts with Rac1 and Cdc42. In addition, p110β binds Rab5 by a discrete binding site within its helical domain which is required for autophagy (Bresnick and Backer 2019). Though the impact of the regulation by monomeric GTPases is currently not fully understood, it is assumed to contribute to subcellular localization and the signaling specificity of the different class I PI3Ks. PIK3β is somehow unique that it is not only controlled by growth factor-activated tyrosine kinases but also under the control of GPCRs via direct interaction with Gβγ dimers released from receptor-activated heterotrimeric G proteins. Accordingly, it is thought to act as a coincidence detector integrating signals



Phospholipid Kinases, Fig. 2 Classification of phosphoinositide 3-kinases. Shown are schematic representations of the catalytic (upper panel) and regulatory (lower panel) subunits of mammalian PI3Ks. Isoforms are subgrouped into three classes. Class I_A comprises the three catalytic members p110α, p110 β, and p110δ that are encoded by the *PIK3CA*, *PIKC3B*, and *PIKC3D* genes, respectively, and five regulatory p85 subunits encoded by three genes, i.e., *PIK3R1*, *PIK3R2*, and *PIK3R3*. The only catalytic class I_B subunit, p110γ, is encoded by the PI3KCG gene and associates with one of two regulatory subunits, p87 (also known as p84 and encoded by the P*IK3R6* gene) or p101 (encoded by the *PIK3R5* gene). Three enzymes belong to the class II termed PI3K-C2α, PI3K-C2β, and PI3K-C2γ that are encoded by the *PIK3C2A*, *PIK3C2B*, and *PIK3C2G*, respectively. The only class III PI3K catalytic isoform VPS34 (encoded by the *PIK3C3* gene) complexes with the regulatory VPS15 regulatory subunit (encoded by the *PIK3R4* gene). ABD indicates the binding site for the regulatory subunits. BH represents a BCR homology domain. The C2 domain is a membrane-binding domain that was first identified as a calcium-binding domain in protein kinase C (PKC), iSH2: inter-SH2 domain. The PIK (phosphoinositide kinase) domain is shared by all the lipid kinases. CBD (clathrin-binding domain), P (proline-rich region), RBD (Ras-binding domain), SH2 and SH3 (src-homology domains 2 and 3), and TBD (TACC3-binding domain) represent protein–protein interaction domains, PX: Phox homology domain

from different receptor classes. Little is known in molecular terms about the apparent preference of a given receptor for a class I_A-specific catalytic subunit. Class I_A PI3Ks are mainly involved in the regulation of cell growth, proliferation, metabolism, and immunity (Bilanges et al. 2019; Bresnick and Backer 2019). An increase of the class I_A PI3K activity has been found in a number of different human cancers, overgrowth, or immunodeficiency syndromes which lead to the development of PI3K-inhibiting drugs (Bilanges et al. 2019).

The class I_B PI3Kγ signals downstream of GPCRs and Ras (Nürnberg and Beer-Hammer 2019). It is a heterodimeric enzyme that occurs in two different variants. The only class I_B catalytic p110γ subunit is associated with one of two regulatory subunits, p101 or p87 (also known as p84). p101 and p87 are structurally distinct from class I_A PI3K adaptor proteins with an overall sequence similarity of 24%. Their structures have not been resolved so far. Similar to class I_A PI3Ks, unstimulated class I_B PI3Kγ is predominantly localized in the cytosol. Upon GPCR activation, Gβγ dimers and Ras directly bind PI3Kγ and induce a recruitment to the plasma membrane and a subsequent activation of PI3Kγ. The p87-containing PI3Kγ variant is less sensitive towards Gβγ than the p101-containing PI3Kγ variant. The physiological significance of this difference is not understood. In addition to its predominant expression in hematopoietic cells where it controls immune functions, PI3Kγ is also found in a broad range of other organs and tissues including cardiac, vascular, endocrine, and neuronal tissues. PI3Kγ dysfunction is also implicated in malignancy.

Two other classes of PI3Ks have been less intensively investigated. Class II PI3Ks comprise three members, PI3K-C2α, PI3K-C2β, and PI3K-C2γ (Margaria et al. 2019). The proteins are large monomeric enzymes of 170–210 kDa molecular weight that produce PtdIns(3)P and PtdIns(3,4)P_2 in vivo. They share overlapping functions but exhibit also distinct isoform-specific roles on membrane dynamics and trafficking most likely due to different subcellular expression profiles. In contrast to class I PI3Ks being mainly cytosolic, class II PI3Ks are predominantly found in membrane fraction of cells including plasma and various intracellular membranes. They respond to a wide array of stimuli, such as chemokines, insulin, and growth factors through mechanisms that are rather indirectly controlling cell signaling. A pivotal role was proposed for PI3K-C2α's enzymatic activity regulating primary cilium formation, vesicle trafficking, and cell migration. In contrast, PI3K-C2β governs selectively endosomal trafficking. Little is known about PI3K-C2γ that is predominantly expressed in the digestive system such as the liver, the pancreas, and the small intestine and in the exocrine tissue of the breast and the prostate. It has specific effects on cell metabolism through regulating endosomes. In addition, mutations of *PIK3C2G* have been linked to malignant tumors. Based on their pathophysiological roles, class II PI3Ks are considered as potential targets for anticancer therapies.

The vacuolar protein-sorting protein (VPS34) represents the only catalytic class III PI3K subunit which produces PtdIns(3)P (Bilanges et al. 2019). This PI is recognized by effector proteins harboring PtdIns(3)P-recognizing domains such as the Fab-1, YGL023, Vps27, and EEA1 (FYVE) domain, the phox homology (PX) domain, or the β-propellers that bind polyphosphoinositides (PROPPINS) domain. VPS34 was initially identified in *Saccharomyces cerevisiae*. The human orthologue, hVPS34, is 100 kDa of size and complexes with the VPS15 (also known as p150) adapter protein that has regulatory functions and is responsible for the membrane association of this PI3K. Of note, the N-terminal kinase domain of VPS15 includes a kinase-like structure that appears to be a pseudokinase. This region is important for the function of VPS34 due to its capacity to form multi-protein complexes (Ohashi et al. 2019). Two tetrameric class III PI3K-containing complexes have been defined that are designated complex I and complex II. In complex I, VPS34/VPS15 assembles with the accessory proteins VPS30 (also known as beclin 1, a Bcl-2-interacting protein) and ATG14L. It is involved in the regulation of autophagy, a conserved lysosomal degradation pathway. Complex II is composed of the UVRAG (UV irradiation resistance-

associated gene) protein instead of ATG14L and VPS34, VPS15, and VPS30. This complex plays roles in membrane trafficking including endocytic sorting. Interestingly, somatic mutations in the catalytic, regulatory, or accessory proteins of the complexes are linked to various human cancers such as esophageal, colorectal, bladder, and various forms of melanoma. VPS15 mutations have also been correlated with ciliopathy and neurodevelopmental disorders in humans. Because of its role in autophagy, type III PI3K is considered to be a potential therapeutical target for the treatment of malignancies (Shintani and Klionsky 2004).

Protein Kinase Activity of PI3K

In all known catalytic subunits of PI3K, the C-terminal kinase domains show considerable similarity with classical serine/threonine kinases (Nürnberg and Beer-Hammer 2019). Indeed, PI3Ks can function as dual-specific enzymes and exhibit a protein kinase activity in addition to their lipid kinase activity. This activity may have autoregulatory functions since for some enzymes their PI3K lipid kinase activity is modulated by autophosphorylation of the catalytic subunit p110 and/or by transphosphorylation of the associated regulatory p85. Accordingly, it became apparent that both enzymatic qualities, i.e., the lipid and the protein kinase activities, are required for certain physiological processes. For instance, the endocytosis of β-adrenergic receptors requires both lipid and protein kinase activities of PI3Kγ. Clear evidence for protein kinase-based independent downstream signaling pathways of PI3Ks is missing. Only very few protein substrates for PI3K have been described, and their physiological relevance remains to be determined.

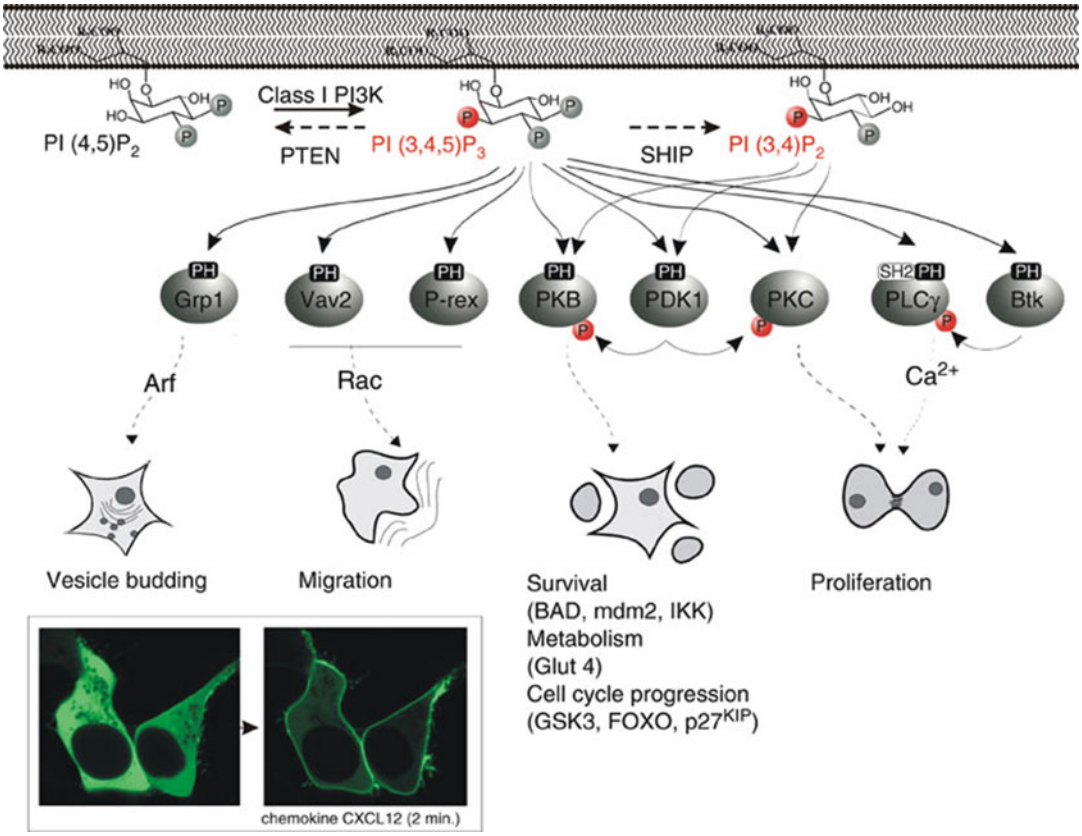
Nonenzymatic Roles of PI3K

Emerging evidence suggest that PI3Ks have also non-catalytic functions (Bilanges et al. 2019). They stabilize binding partners and orchestrate signaling complexes. Their roles as highly selective molecular scaffolds were detected when differential results became evident from genetically engineered mice lacking the kinase and from mice expressing a catalytically inactive point mutant of the same enzyme. These differences were not due to a redundancy of functions of related PI3K isoforms that may have jumped into the role of the deleted protein. Examples include nonenzymatic roles for PI3Kβ during glucose-stimulated insulin secretion or receptor-induced endocytosis and for PI3Kγ to ensure cyclic adenosine monophosphate (cAMP) homeostasis in the cardiovascular system (Bresnick and Backer 2019; Nürnberg and Beer-Hammer 2019). The class II PI3K-C2α stabilizes the mitotic spindle during metaphase in a non-enzymatic fashion (Margaria et al. 2019). In contrast to their lipid kinase functions that are controlled by upstream regulators, a demonstration of dynamic modulation of their scaffolding functions awaits further studies.

Downstream Effectors of PI3Ks

The importance of PI3Ks arises from the fact that numerous important signaling molecules need to be localized to the plasma membrane to fulfill their functions. Binding to 3′-phosphorylated phosphoinositides constitutes a major mechanism of membrane translocation for the signaling molecules. The prototypical phosphoinositide-binding domain is the pleckstrin homology-domain (PH-domain), which binds to the second messengers PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 with different affinities (Fig. 3). A PH-domain protein known to play a pivotal role in PI3K signaling is protein kinase B (PKB), also called *Akt* (Bilanges et al. 2019). Plasma membrane-recruited and plasma membrane-activated PKB triggers multiple signaling cascades implicated in an array of normal and pathophysiological cell processes, such as inflammation, metabolism, and tumorigenesis (Fig. 3).

Numerous other PH-domain-containing proteins are implicated in important physiological processes, such as the *g*uanosine nucleotide *e*xchange *f*actors (GEFs) Vav and P-Rex. GEFs



Phospholipid Kinases, Fig. 3 Role of PH-domain-containing effectors of class I PI3Ks in various cellular processes. Bottom left panel: visualization of the translocation of GFP-fused Grp1 PH-domain in living cells. Human embryonic kidney cells (HEK293) were stimulated with the chemokine CXCL12. The GFP-fused Grp1 PH-domain protein translocates from the cytosol to the plasma membrane, reflecting an increased PI3Kγ-mediated PtdIns $(3,4,5)P_3$ production. HEK293 were transfected with the chemotactic GPCR (CXCR4), PI3Kγ, and GFP-fused Grp1 PH-domain

exchange GDP for GTP and lead to an activation of small GTPases. Vav and P-Rex regulate the activity of the small GTPase Rho/Rac and are involved in cytoskeletal remodeling and cell motility. Grp1, a GEF for the small GTPase Arf, is necessary for vesicle budding (Fig. 3).

PtdIns(3)P generated by class II or III PI3Ks interacts with the FYVE zinc finger domains (acronym of FYVE: *F*ab1p, *Y*OTB, *V*ac1p, and *E*EA1) as well as PX domains (a domain originally identified as a common motif in the p40phox and p47phox of the neutrophil NADPH oxidase complex). Other phosphoinositide-binding domains have been identified. Among them are FERM domains (band four-point one, ezrin, radixin, moesin) that link cytoskeleton to the plasma membrane or ENTH domains (*e*psin *N*-*t*erminal *h*omology) that play a role in endocytosis.

Drugs

As described above, PI3K isoforms are critical for many signaling pathways in a highly selective manner that control growth and survival of the cells and metabolic and immunological functions of the organism. Consequently, PI3Ks are regarded as attractive drug target for cancer therapy and for the treatment of other human diseases. Whereas initial classical PI3K inhibitors such as Wortmannin and LY294002 show very little

selectivity for the different PI3K isoforms and display considerable off-target effects, nowadays a significant number of highly potent inhibitors have been developed. Major challenges for successful drug development of PI3K inhibitors are insufficient efficacy and serious unwanted drug effects (De Santis et al. 2019). The underlying reasons are manifold and often caused by mechanistically different developments of resistance and off-target toxicity. Pan-inhibitors, dual-specific inhibitors, or selective inhibitors are applied as part of a combination therapy, trying to increase therapeutic efficiency. Conversely, new classes of isoform-selective inhibitors are being designed to reduce off-target effects. Up to date, regulatory authorities have approved four PI3K inhibitors for treating patients suffering from various malignant tumors (Table 1). More are expected to come to the clinic in the future since a series of novel compounds are tested in clinical trials.

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Phosphoprotein Phosphatase

▶ Protein Phosphatases

Phthisis

▶ Tuberculosis

Physiology and Treatment of Hyperuricemia and Gout

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Synonyms

MSU; sUA; URAT1

Definition

Hyperuricemia is a condition characterized by abnormally elevated levels of serum urate (sUA), while gout, the most common form of inflammatory arthritis, arises from the subsequent deposition of urate crystals when concentrations become saturated (Dalbeth et al. 2019).

Hyperuricemia is commonly defined as a serum urate concentration >6.8 mg/dL, based on the *in vivo* solubility of urate above which crystal deposition may occur leading to gout. It should be noted that alternative definitions of hyperuricemia are sometimes applied, which needs to be considered during comparative data analysis (Stamp and Dalbeth 2017).

Gout has been defined as "a progressive metabolic disease characterized by symptomatic hyperuricemia and deposition of monosodium urate (MSU) crystals in joints and soft tissues due to an imbalance in uric acid uptake, synthesis or excretion" (Bardin and Richette 2014). Not all individuals with hyperuricemia go on to develop gout, and therefore gout represents a subset of individuals with symptomatic hyperuricemia.

Basic Mechanisms

Uric Acid

Uric acid is a weak diprotic acid with an aqueous pK_{a1} of 5.4 and pK_{a2} of 9.8. At physiological pH, uric acid is predominantly found as the deprotonated urate anion. The solubility of uric acid at normal physiological pH is around 6.8 mg/dL. The reference ranges for sUA are 3.5–7.2 mg/dL (210–430μmol/L) and 2.6–6.0 mg/dL (155–360μmol/L) in males and premenopausal females, respectively (Desideri et al. 2014). Notably, the upper limit of the normal male reference range includes concentrations that exceed the concentration at which uric acid precipitates. The normal range for serum urate concentrations in humans, and some other primates, is significantly above the typical mammalian range of 0.5–2.0 mg/dL (30–120μmol/L). Potential benefits of high sUA levels remain to be elucidated; however, hyperuricemia, in conjunction with genetic and/or environmental factors, can lead to significant health problems associated with urate crystal deposition.

Uric Acid Generation

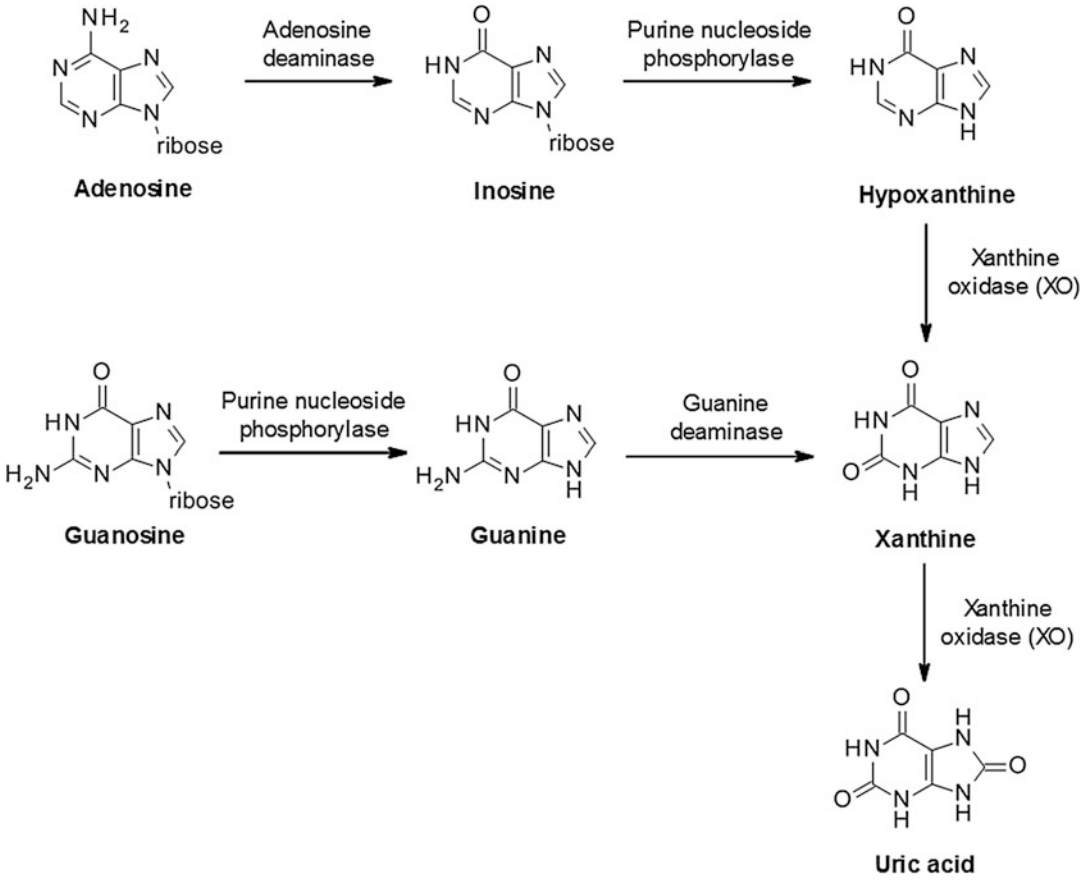
Uric acid is produced during the metabolism of both endogenous (daily synthesis rates of ~300–400 mg) and exogenous (dietary contribution, ~300 mg) purines within a total pool size of 1200 mg in healthy males (600 mg in females) on a purine-free diet. Uric acid biosynthesis is catalyzed by xanthine oxidase (XO/xanthine oxidoreductase/XOR), coded by the xanthine dehydrogenase gene *XDH* (Pacher et al. 2006). The enzyme is normally present as an inactive NAD-dependent cytosolic dehydrogenase precursor, which is processed by oxidation or proteolytic modification to form active enzyme. XO is widely distributed throughout various organs including the liver, gut, lung, kidney, heart, and brain as well as the plasma and is involved in two stages of uric acid generation: conversion of hypoxanthine to xanthine and subsequently xanthine to uric acid (Fig. 1).

Urate Crystal Formation

The initial clinical sign of an acute gout attack is severe disabling pain, usually involving a single joint, which typically spontaneously resolves over a period of a few days to weeks without intervention. Treatment with anti-inflammatory drugs such as colchicine, NSAIDs (nonsteroidal anti-inflammatory drugs), and corticoids will generally improve symptoms more rapidly. Upon resolution of an acute attack, the patient will enter a symptom-free interval; however flares can recur with increased frequency and duration if the underlying pathology is not addressed. If sUA (serum uric acid) values remain high, MSU (monosodium urate) crystal deposits can grow and expand to other sites leading to further inflammation and associated tissue/joint injury. A subset of individuals will transition to chronic tophaceous gout, characterized by nodular urate crystal deposits, recurrent flares, and concurrent arthritis (Bardin and Richette 2014).

While sustained hyperuricemia is a prerequisite of crystal formation, it is not possible to accurately predict which individuals will go on to develop gout, even for those with very high sUA. There is no global consensus on approaches for asymptomatic hyperuricemia nor in response to acute gout attacks. Different strategies are advocated by national or international guidelines, which range from reactive approaches to active sUA management in the absence of evoked symptoms.

The process of crystal deposition leading to gout is reversible by means of reducing sUA levels below its saturation point. The rate of crystal reduction will be modulated by both the total crystal load and reduction in sUA levels. Urate-lowering therapy (ULT) initiation generally leads to increased flare rate and associated pain,



Physiology and Treatment of Hyperuricemia and Gout, Fig. 1 Biosynthesis of uric acid from purines. Purine mononucleotides are catabolized to produce uric acid although the underlying pathway can vary in different tissues and cells

potentially as a direct consequence of urate crystal dissolution leading to the removal of a protein deposit protecting the underlying surface from attack by inflammatory cells. Treatment with anti-inflammatories alone may modulate the acute inflammatory response to crystals, but it is unlikely to alter crystal deposition and ongoing joint damage.

Uric acid urolithiasis refers to the development of a stone or calculus composed of significant amounts of urate in the renal pelvis, ureter, or bladder and is reported to account for 5.0–16.5% of all kidney stones. Uric acid solubility is modulated as a function of its weakly acidic pK_a, and hence, individuals with more acidic urine (pH 5.5) are more likely to have increased uric acid stone formation than individuals with a normal pH range (pH 6.0–6.5). Urinary alkalinization should therefore reduce stone growth/recurrence and promote stone dissolution.

Uric Acid Clearance

In most mammalian species, uric acid is further metabolized by the enzyme uricase to the more soluble allantoin which is subsequently excreted in the urine. However, humans and some higher-order primates lack a functional uricase enzyme, and therefore uric acid is the final breakdown product of the pathway. This discrepancy in uric acid handling between species represents a challenge in the preclinical evaluation of urate-lowering drugs during drug discovery. Urate elimination from humans occurs via two main routes; two-thirds is excreted in urine with normal

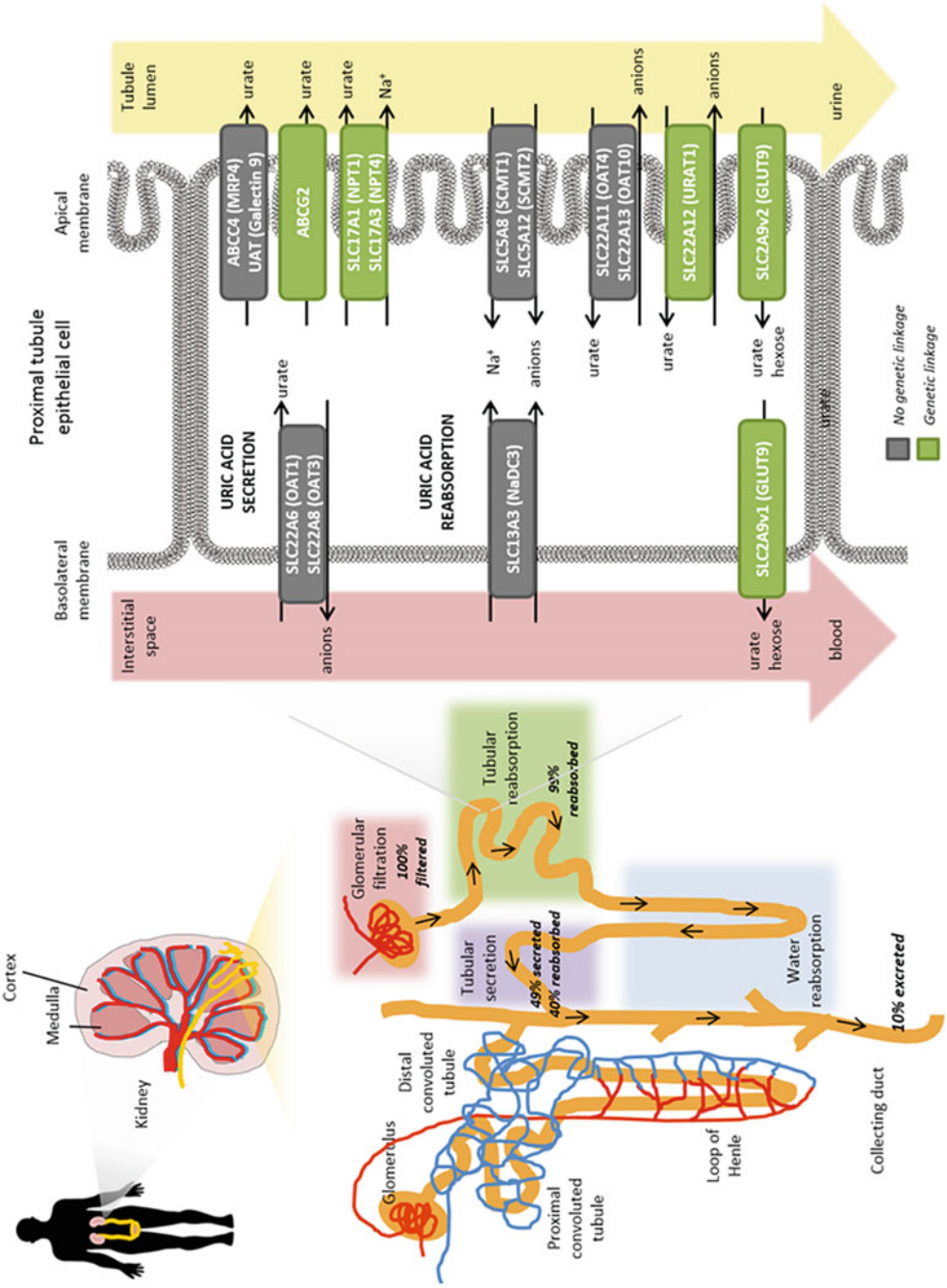
uricosuria levels of 620 ± 75 mg/day in adult, while the remainder is thought to be excreted via the gastrointestinal tract (Hyndman et al. 2016). Hyperuricemia may also be associated with hyperuricosuria (defined as urinary excretion of urate >800 mg/day in men and >750 mg/day in women). Urate elimination can also be quantified as clearance (normal males, 8.7 ± 2.5 mL/min) or as fractional excretion of urate (FEUA) which indicates the net urate excretion by the kidney (normal males, 7.25 ± 2.98%). Healthy subjects have an average FEUA in the range of 6–8%, whereas gout patients generally have average FEUA of 3–5%. These observations are consistent with the notion that decreased renal excretion or low FEUA represents a major contributor to hyperuricemia as opposed to increased generation of uric acid.

Despite the high fraction of renally excreted uric acid, the process is more complex than simple glomerular filtration, with approximately 91–95% of filtered urate being reabsorbed in the proximal tubule. Reabsorption is a key factor underpinning the comparatively high levels of circulating urate and is primarily mediated by transporters that exchange intracellular anions for urate. Reabsorption and secretion of urate predominates in the S1 and S2 regions of the proximal tubule although it is not clear whether the secretion happens concomitantly with reabsorption and/or if there is post-reabsorptive secretion within the tubule. Ultimately, around 3–10% of the filtered urate emerges in the urine. Several transporters playing a role in reabsorption and secretion have been identified, and some of these are genetically linked to hyperuricemia and gout (Köttgen et al. 2013; Hyndman et al. 2016) (Fig. 1).

URAT1 Is the Predominant Urate Reabsorption Transporter

URAT1 (*SLC22A12*) is the dominant apical (luminal) urate exchanger in the human proximal tubule. URAT1 is a 12-transmembrane domain protein, predominantly localized to the apical brush border membrane of proximal tubule epithelial cells in the kidneys. URAT1 has been reported to transport urate with a K_m of 371 ± 28μM as well as other organic anions such as orotate, salicylate, lactate, and nicotinate (Enomoto et al. 2002). URAT1-mediated urate transport is a tertiary active process dependent on sodium gradients initially established via basolateral Na^+K^+ATPases which actuate a number of apical Na^+-coupled organic anion transporters, in turn providing the driving force for urate reabsorption. Loss-of-function mutations of URAT1 are associated with FEUA of 40–100% and extremely low serum urate levels (average levels of 0.93 mg/dL). Additional urate transport pathways are inferred given that FEUA is <100% even in patients with complete URAT1 loss-of-function mutations. URAT1 is now a well-established drug target with a number of primary and secondary uricosurics (drugs capable of increasing FEUA) such as benzbromarone, probenecid, and lesinurad, known to derive at least part of their efficacy through this mechanism albeit, in earlier cases, this was not understood when they were developed. Interestingly, compounds such as pyrazinamide have been shown to trans-stimulate URAT1 activity to impact on vectorial transport of urate.

GLUT9 (*SLC2A9*) variants are strongly associated with both hyperuricemia and gout. GLUT9 appears to function predominantly as a facilitative urate uniporter with at least some additional capacity for hexose transport. GLUT9-mediated urate transport is voltage dependent, but independent of sodium, chloride, and other ions. GLUT9 is distinct from other members of the glucose transporter (SLC2) family due to its substrate specificity although it shares common structural features such as 12 transmembrane helices, cytoplasmic termini, and an N-linked glycosylation site. The short isoform (GLUT9a) appears to be expressed at both apical and basolateral membranes in proximal tubule epithelium cells (and indeed may contribute to the import of urate from the peritubular interstitium and thus facilitate renal urate secretion). The long isoform (GLUT9b) is predominantly expressed on the basolateral membrane and is the only known basolateral efflux transporter for urate. Interestingly, a positive relationship has been described between glycosuria and uricosuria suggesting that there could be interference between the tubular reabsorption of glucose and the tubular capacity to reabsorb urate.



Physiology and Treatment of Hyperuricemia and Gout, Fig. 2 (continued)

OAT4 (*SLC22A11*) and OAT10 (*SLC22A13*) have a range of organic anion substrates and are expressed on the apical membrane of proximal tubule epithelium cells together with URAT1 (*SLC22A12*). Both transporters have been demonstrated to exhibit low levels of urate transport capabilities.

Urate excretion transporters include ABCG2, and NPT1 (*SLC17A1*) and NPT4 (*SLC17A4*) are apical efflux transporters that are genetically associated with hyperuricemia and gout risk. OAT1 (*SLC22A6*) and OAT3 (*SLC22A8*) mediate the basolateral entry of urate into renal proximal tubule cells via the outwardly directed gradient for dicarboxylates, which in turn is generated by Na^+-dependent uptake. Thus, urate exchange is significantly trans-stimulated by dicarboxylates (Fig. 2).

Pharmacological Intervention

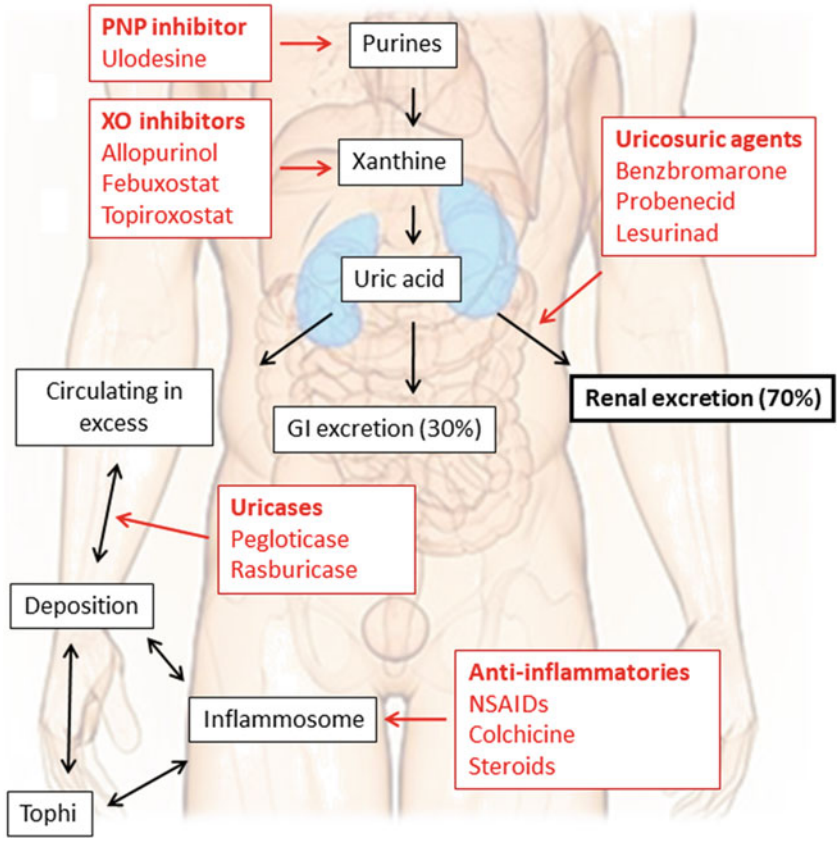
There are several approved ULT drugs which fall into three main classes: reduction of uric acid synthesis (xanthine oxidase inhibitors), increasing uric acid excretion (uricosurics, e.g., URAT1 inhibitors), and enabling systemic metabolic hydrolysis of uric acid (urolytics, e.g., recombinant uricases) (Figs. 3 and 4).

Xanthine Oxidase Inhibitors (XOi)

These reduce endogenous production of uric acid and thus lower sUA levels (Pacher et al. 2006). Inhibitors fall into two main classes: the classical purine analogs (including allopurinol) and more recently developed non-purine analog compounds such as febuxostat and topiroxostat.

Allopurinol was the first ULT to reach the market and remains the first-line therapy for hyperuricemia and gout. Allopurinol is a relatively weak competitive XO inhibitor and is rapidly metabolized to the more potent oxypurinol, an isostere of xanthine, which is then renally cleared. Allopurinol has been reported to possess additional pharmacology, such as decreasing blood pressure and creatinine levels, which supports the notion that XO inhibition can have effects independent of urate lowering. However, studies have suggested that less than 50% of patients taking the drug achieve a sUA level <6 mg/dL at an allopurinol dose of 300 mg/day although it is recommended to up-titrate allopurinol to 800/900 mg per day (depending on geography) until the target of sUA <6 mg/dL is achieved. Allopurinol has been associated with

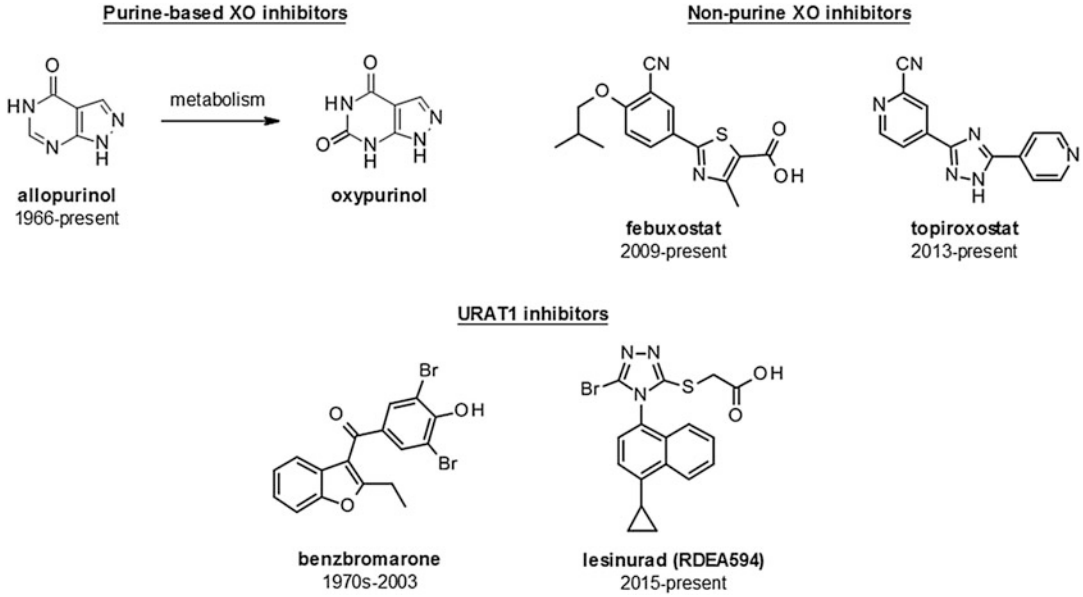
Physiology and Treatment of Hyperuricemia and Gout, Fig. 2 Role of transporters in the renal proximal tubule on urate handling. Within an individual nephron in the kidney (yellow), filtration of water and solutes occurs in the glomerular capsule from the afferent arteriole into the renal tubule (pink shading). Tubular reabsorption (green shading) is predominantly mediated by the proximal convoluted tubule, whereas tubular secretion extracts uric acid (and other substances) from peritubular capillaries (purple shading) and secretes them into the tubular fluid for urinary excretion. Urate transporters in renal proximal tubule epithelial cells actively mediate the secretion and reabsorption of urate. The balance between these processes determines the net excretion levels from the kidney. The anion transporters SLC22A6 (OAT1) and SLC22A8 (OAT3) localized on the basolateral membranes transport urate from the interstitial space in the blood depending on the gradients for exchanged anions but have not been shown to exhibit a genetic linkage with hyperuricemia or gout risk (gray box). On the apical membrane, ABCG2, SLC17A1 (NPT1), SLC17A3 (NPT4), ABCC4 (MRP4), and UAT (galectin 9) have all been shown to contribute to the secretory transport of urate into the tubule lumen and leading to urinary excretion; a number of these have been genetically associated with hyperuricemia and gout risk (green boxes). Exchange gradients upstream of urate anion exchange are enabled through the actions of SLC13A3 (NaDC3), SLC5A8 (SCMT1), and SLC5A12 (SCMT2). In renal reabsorption, the apical urate-anion exchanger SLC22A12 (URAT1) has been shown to play a predominant role in urate homeostasis, and indeed several variants have been identified to be associated with gout and hyperuricemia risk (green box). Additional contributions to urate reabsorption are mediated by SLC22A11 (OAT4) and SLC22A11 (OAT10) (gray boxes, not genetically associated with gout/hyperuricemia risk) and the short isoform of SLC2A9v2 (GLUT9, green box) on the apical membrane. The long isoform of SLC2A9v1 (GLUT9, green box) is the only known transporter to mediate basolateral efflux of urate back into circulation, which is in accordance with its genetic association for gout and hyperuricemia risk in addition to rare mutations associated with hypouricemia



Physiology and Treatment of Hyperuricemia and Gout, Fig. 3 Multiple points for rational pharmacological intervention (red boxes/text) to address hyperuricemia and gout. Biosynthesis of uric acid via purine catabolism to xanthine to uric acid can be targeted via purine nucleoside phosphorylase (PNP) or xanthine oxidase (XO) inhibition. Uric acid is predominantly cleared renally, with a significant proportion of the excreted uric acid being reabsorbed. It has been reported that underexcretion of uric acid contributes notably to hyperuricemia and gout; and this is the mechanism targeted by uricosuric agents. Uricases are synthetic recombinant enzymes that metabolize circulating uric acid. All aforementioned approaches are disease-modifying in contrast to symptomatic management approaches such as anti-inflammatories

several adverse effects including gastrointestinal effects, rash, Stevens-Johnson's syndrome, and allopurinol hypersensitivity syndrome in 2–8% of patients. Complications can also arise when patients have renal impairments.

Febuxostat is a selective, non-purine, inhibitor of XO approved by the FDA in 2009 for management of patients with hyperuricemia in patients with gout but not for asymptomatic hyperuricemia. Febuxostat has a reported IC_{50} of 1.8 nM, which is significantly more potent than allopurinol (IC_{50} 7.8μM), and consequently has been shown to be more efficacious at doses of 80 or 120 mg for achieving target urate levels of <6 mg/dL vs. allopurinol at 100–300 mg daily. Interestingly, gout flares on commencement of treatment have been reported to be more frequent with febuxostat than with allopurinol, likely due to the more rapid and pronounced sUA reduction. Febuxostat clearance is predominantly via hepatic metabolism which suggests the potential at least for broader prescribing with respect to impaired renal function though this has not been fully assessed in the clinic. In addition, febuxostat has been reported to have fewer drug-drug interactions than allopurinol and is better tolerated in patients with allopurinol hypersensitivity syndrome.



Physiology and Treatment of Hyperuricemia and Gout, Fig. 4 Structures of launched (marketed and withdrawn) drugs for the management of hyperuricemia. Purine-based (allopurinol and oxypurinol) and non-purine-based (febuxostat and topiroxostat) XO inhibitors are shown with dates of approval for clinical use. URAT1 inhibitors are represented by benzbromarone (withdrawn in 2003) and lesinurad (RDEA594).

Topiroxostat (FYX-051) is a structurally distinct, non-purine, selective XO inhibitor which was approved for use only in Japan in 2013. Topiroxostat has been shown to inhibit xanthine oxidase via formation of a hydroxylated 2-pyridine metabolite that forms a covalent linkage to the molybdenum via oxygen and also interacts with amino acid residues in the solvent channel.

Uricosurics

The underlying cause of hyperuricemia in a notable subset of patients appears to be due to renal underexcretion of uric acid (Ichida et al. 2012). This suggests the application of uricosuric agents to increase renal urate excretion as a rational approach for the treatment of hyperuricemia. However, by enhancing the renal clearance of uric acid, uricosurics may increase the risk of renal adverse events (e.g., nephrolithiasis). Uricosurics are currently recommended as a second-line therapy when target sUA levels are not reached, particularly as add-on therapies in combination with XO inhibitors. Combination therapy with uricosuric agents and XOi have been suggested to provide enhanced urate lowering and potentially more rapid resolution of tophi.

Several drugs, including probenecid, sulfinpyrazone, fenofibrate, and losartan, were serendipitously discovered to have uricosuric properties although the underlying pharmacology was not initially understood. Probenecid was originally introduced to prolong the action of antibiotics by reducing their renal clearance and functions as a nonselective inhibitor of organic anion transporters. URAT1 has now been demonstrated to be one of the molecular targets of probenecid. However, its lack of selectivity and subsequent potential for drug-drug interactions limit its clinical use as a uricosuric.

Benzbromarone, the first drug specifically developed for its uricosuric properties, was discovered and developed without any understanding of its pharmacology at a molecular level (Lee et al. 2008). Benzbromarone, first marketed in 1976, has subsequently been shown to be a potent URAT1 inhibitor (IC_{50} 22 nM). Benzbromarone is effective as a single-agent ULT although, like allopurinol, it requires dose titration from 50 to

200 mg to achieve maximum efficacy. Unlike allopurinol, benzbromarone could be used in patients with renal impairment though caution was advocated by clinicians where there was history of renal calculi. Benzbromarone was not approved in the USA and was withdrawn from the market in many other countries in 2003 following reported incidents of idiosyncratic hepatotoxicity.

Lesinurad is the first novel uricosuric to reach the market since benzbromarone. The primary mechanism of action of lesinurad derives from inhibition of URAT1, although it is also reported to be an inhibitor of OAT4 which could also potentially contribute to its efficacy. 200 mg dose of lesinurad received FDA approval at the end of 2015 for use in combination with a xanthine oxidase inhibitor for the treatment of hyperuricemia associated with gout in patients who have not achieved target serum uric acid levels with a xanthine oxidase inhibitor alone (Pérez-Ruiz et al. 2019). Further optimization of the RDEA594 (lesinurad) template culminated in the development of RDEA3170 (verinurad) which reportedly has a URAT1 IC_{50} of 25 nM (compared to 3.4 uM IC_{50} of lesinurad against URAT1) and is currently in clinical trials. Additional uricosurics in development have been reported including arhalofenate, FYU-981, and URC-102 (Benn et al. 2018). Another interesting molecule for which development has been terminated is PF-06743649 which was a dual URAT1/XO inhibitor. Ulodesine (BCX4208) is also noteworthy as it represents a novel mode of action, outside of XO inhibitors, uricosuric and uricolytic agents. This is a potent purine nucleoside phosphorylase (PNP) inhibitor acting upstream of xanthine oxidase inhibitors.

Urate Hydrolysis by Uricases

Humans, and some primate species, differ from other mammals by the lack of a functional uricase enzyme. Therapies based on the intravenous administration of a functional recombinant uricase enzyme have been developed which enable an immediate and significant reduction of sUA levels (Benn et al. 2018). Pegloticase, a PEGylated recombinant uricase, was approved in 2010 for limited-patient use in the treatment of chronic refractory gout in patients with tophaceous deformities and complications or where XOi and uricosurics are contraindicated. However, likely due to the very rapid urate reduction caused by administration of pegloticase, its application has been associated with significant prevalence of acute flares. Additionally, while pegloticase has been used effectively in the clinic, it is also prone to immunogenic infusion reactions with a significant portion of the patients receiving pegloticase developing antibodies within the first few months of treatment. A non-PEGylated uricase, rasburicase, has also been approved for specifically treating hyperuricemia associated with tumor lysis syndrome as its quick mode of action and short half-life relative to pegloticase (8 h vs. 12 days) aligns well with this particular application. However, rasburicase also causes high rates of immunogenic infusion reactions, methemoglobinemia, hemolysis, and anaphylaxis in patients which limit its more general use.

Conclusions

High levels of circulating uric acid have been associated with the increased risk for developing gout and kidney stones. Gout is the most common form of inflammatory arthritis with well-studied epidemiology. The natural history of gout can be summarized as asymptomatic hyperuricemia, acute gouty arthritis, intercritical period, and chronic tophaceous gout, with diagnosis based on both laboratory and radiological features. Gout is an eminently curable condition with approaches that include management of the inflammatory pain associated with flares, urate-lowering therapies that address the underlying cause, as well as other approaches such as improved patient education. If the treatment paradigm shifts further toward active management of hyperuricemia, we anticipate that patients will benefit from more facile methods for determining uric acid concentrations in biofluids together with increased availability of mobile applications for disease management.

The role of genetic predisposition is becoming more evident, and new insights into the pathophysiology of hyperuricemia and gouty arthritis (both acute and chronic) allow for an even better understanding of the disease. The identification of the URAT1 transporter and its role in urate reuptake from the proximal tubule has shed light on the pharmacology of a number of clinical uricosuric compounds and enabled the development of a new generation of specifically targeted molecules. This has been exemplified by development of lesinurad and verinurad in addition to other agents whereby drug discovery efforts have leveraged the measurement of URAT1-mediated transport as a functional primary pharmacology assay (Storer et al. 2016; Benn et al. 2018). Other transporters genetically linked with urate homeostasis such as GLUT9 may provide further targets for novel drug development in the future. In this context it is worth mentioning that determining requisite potency levels for advancing a compound can be hampered by the lack of a valid preclinical model. In addition, direct clinical PK/PD comparison can be challenging, due to complicating factors such as active metabolites, active renal excretion (which may imply that ultrafiltrate concentrations in the proximal tubule significantly differ from unbound plasma), and organic anion transporter polypharmacology. Nonetheless, there is genuine potential for further drug development to lead to better management in patients with hyperuricemia, gout, and associated conditions in the coming years.

Cross-References

- ABC Transporters
- Antigout Drugs

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Physiology of Blood Pressure

- Blood Pressure Control

PIAS Proteins

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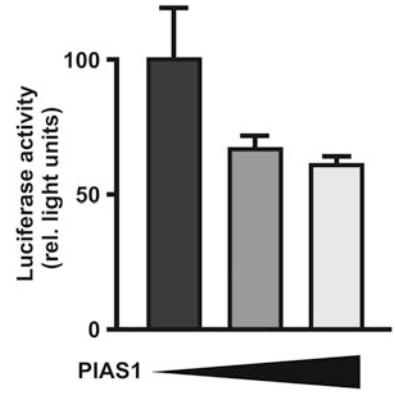
Definition

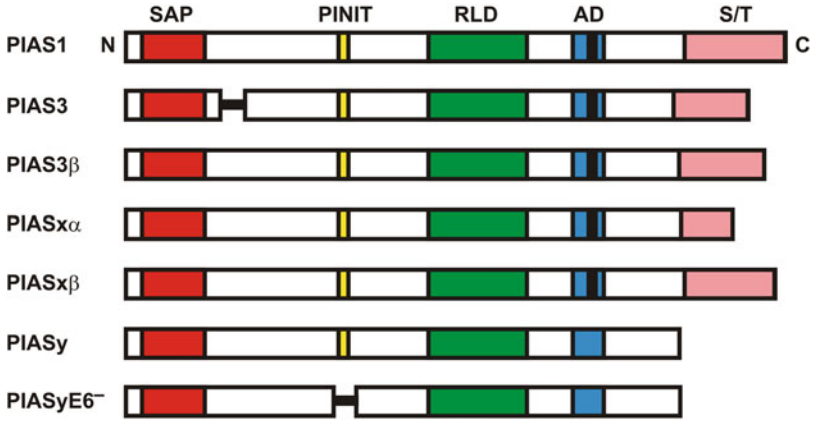
PIAS (*p*rotein *i*nhibitors of *a*ctivated *S*TATs) proteins were first discovered in yeast two-hybrid screens as interacting molecules with STAT transcription factors. The mammalian family consists of the founding member PIAS3, which was described as a repressor of STAT3, and three additional members, PIAS1, PIAS2 (also known as PIASx with α and β splice variants, termed ARIP3 [androgen receptor-interacting protein 3] and Miz1 [Msx-interacting zinc finger]), and PIAS4 (also known as PIASy), respectively. Despite their name, PIAS proteins do not selectively inhibit STAT-mediated cytokine signaling (Fig. 1), but function as transcriptional co-regulators in diverse pathways either by activating or repressing gene expression (Palvimo 2007). PIAS proteins have been shown to interact with a broad range of transcription factors unrelated to STATs, such as p53, c-Jun, SMAD, β-catenin/lymphoid enhancer factor 1, NF-κB p65, nuclear hormone receptors, and other proteins. PIAS proteins have been identified as SUMO E3 ligases, which catalyze the covalent addition of small ubiquitin-like modifier (SUMO) to different target proteins acting in these pathways. Some reports demonstrated that the effect of PIAS proteins on substrate function can also be exerted by mechanisms independent of their SUMO ligase activity (Rytinki et al. 2009). PIAS proteins are thought to act as adapter proteins, thereby regulating transcription through several mechanisms, which include inhibition of DNA-binding activity of transcription factors, recruitment of transcriptional corepressors, sequestration of transcription factors in distinct subcellular or subnuclear compartments, and epigenetic modification. Given their function in both SUMOylation-dependent and SUMOylation-independent processes, PIAS proteins are involved in a broad spectrum of biological processes, including embryonic development, innate and adaptive immune responses, hematopoiesis as well as spatial learning and long-term memory.



PIAS Proteins, Fig. 1 Reporter gene assay using an interferon-γ-driven artificial promoter demonstrating the inhibitory transcriptional activity of increasing concentrations of PIAS on STAT1 signal transduction by an unknown mechanism

Domain Structure and Function

With the exception of a variable carboxy-terminal region, all mammalian PIAS proteins share a high degree of sequence homology and a common domain structure (Fig. 2). The overall sequence identity at the amino acid level is more than 40%. PIAS1 with a length of 651 amino acid residues is the largest member, whereas PIASy lacking the C-terminal serine-/threonine-rich domain is the smallest. In the amino-terminus of all PIAS proteins, a SAP domain is located which contains an LXXLL amino acid motif (where X denotes any amino acid residue). The acronym refers to three defining members of the class of SAP-containing proteins, namely, scaffold attachment factor (SAF), apoptotic chromatin condensation inducer in the nucleus (ACINUS), and PIAS, and has been



PIAS Proteins, Fig. 2 Domain structure of PIAS proteins. For abbreviations see text

suggested to confer binding to chromatin structures. The SAP domain of PIAS1 and PIASy has been shown to bind synthetic (A+T)-rich DNA sequences *in vitro*, suggesting that it targets PIAS proteins to the nuclear matrix. The PINIT (Pro-Ile-Asn-Ile-Thr) motif is present in all PIAS proteins except the splice variant PIASyE6-, which lacks exon 6. Mutation in the PINIT motif results in disrupted nuclear localization of PIAS3, suggesting a role in nuclear retention (Heppler and Frank 2017). Another conserved domain is the RING-finger-like domain (RLD), which resembles the zinc-binding RING fingers found in a subclass of ubiquitin E3 ligases, except for a different spacing of the zinc-coordinating residues. And indeed, the RLD domain has been shown to confer E3 ligase activity for SUMO modification. The carboxy-terminus of PIAS proteins contains a highly acidic domain (AD) with a putative SUMO-interaction motif (SIM, except for PIASy, in which the SIM is missing), which modulates their enzymatic activity, as well as a serine-/threonine-rich domain (S/T rich).

Characteristics of PIAS Proteins

PIAS proteins have been described as adapter molecules that function as E3-like ligases in enhancing the interaction between the SUMO-conjugating enzyme Ubc9 and its respective substrates (Rabellino et al. 2017). PIAS proteins facilitate the formation of an isopeptide bond between the C-terminus of SUMO and the ε-amino group of a lysine residue in the target protein. SUMOylation has been suggested to regulate a variety of cellular processes, such as modulation of transcriptional activity, targeting of proteins to subnuclear structures, and protein stability. PIAS might repress or stimulate gene expression, depending on the target gene and the transcriptional cofactors involved. The molecular mechanisms of PIAS-mediated gene regulation include inhibition of DNA-binding of transcription factors as well as the recruitment of co-regulators, such as histone deacetylases, p300, or CPB (cyclic AMP-responsive element-binding [CREB] protein). It was shown that PIAS-dependent SUMOylation is involved in the repair of DNA double-strand breaks (DSBs) (Zlatanou and Stewart 2010). This function of PIAS proteins in homologous recombination repair (HRR) and nonhomologous end joining (NHEJ) may be mediated through their ability to effectively promote the relocalization of 53BP1 (p53-binding protein 1) and/or BRCA1 (BReast CAncer 1, early-onset) to sites of DNA breaks. Sequestering transcription factors in subnuclear structures has been proposed as another mechanism for PIAS action. The role of SUMOylation in these processes is currently unclear and needs further attention.

PIAS1 knockout mice show increased protection against pathogenic infection, but are otherwise viable (Sharrocks 2006). PIASy-deficient mice display no obvious phenotype with normal distribution of lymphocyte populations, but the absence of PIASy expression had a modest effect on myeloid cell differentiation and Wnt signaling.

In cells derived from *Pias1*-, *Piasx*-, or *Piasy*-mutated mouse lines, the overall SUMOylation patterns of cellular proteins were not reduced as compared to cells from wild-type animals, demonstrating the functional redundancy among the mammalian PIAS proteins at an organismic level.

Drugs

Specific antagonists of PIAS proteins are not available. The engagement of PIAS proteins in different signal pathways suggests that inhibition of PIAS might cause pleiotropic effects. Due to the promiscuous actions of PIAS, the pharmacological reactions resulting from blockade of PIAS functions are currently far from been predictable. Moreover, the response may differ depending on which member of the PIAS family is preferentially targeted.

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Pitocin (Trade Name)

► Oxytocin

PKC

► Protein Kinase C

Placebo Effect

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Synonyms

Placebo response

Definition

The placebo effect is the reduction of a symptom or a change in a physiological parameter, when an inert treatment (the placebo) is administered to a subject who is told that it is an active drug with specific pharmacological properties. The placebo effect, so far considered a nuisance in clinical research when a new treatment has to be tested, has now become a target of scientific investigation to better understand the physiological and neurobiological mechanisms that link a complex mental activity to different functions of the body. Usually, the terms placebo effect and response are used interchangeably by clinical researchers who run clinical trials and by neuroscientists who want to understand the underlying mechanisms. It is important to realize that there is not a single placebo effect but many, which occur through different mechanisms in different conditions, systems, and diseases (Benedetti et al. 2005; Colloca and Benedetti 2005; Colloca et al. 2004).

Basic Mechanisms

Methodological Considerations

The identification of a placebo effect is not easy, and its study is full of drawbacks and pitfalls. In fact, the effect which follows the administration of a placebo can be due to many factors, such as spontaneous remission, i.e., natural history (natural course of a disease or a symptom), regression

to the mean (statistical phenomenon which assumes that individuals tend to receive their initial symptom assessment when the symptom is near its greatest intensity and that their symptom level is likely to be lower when they return for a second assessment), symptom detection ambiguity, and biases. All these phenomena need to be ruled out by means of control groups. The possibility of spontaneous remission can be discarded by means of a no-treatment group, which gives us information about the natural course of a symptom. Regression to the mean can be controlled by using an experimental model in healthy volunteers. Symptom detection ambiguity and biases can be avoided by using objective physiological measurements. It is also important to rule out the possible effects of co-interventions. For example, the mechanical insertion of a needle for the injection of an inert substance may per se induce analgesia, thus leading to erroneous interpretations. Furthermore, the therapeutic outcome of a clinical trial and its interpretation can be affected by the Hawthorne effect, that is, the change in behavior of the patients who are aware of being part of a controlled study (Benedetti et al. 2016).

When all these phenomena are ruled out and the correct methodological approach is used, striking placebo effects can be detected which are mediated by psychophysiological mechanisms worthy of scientific inquiry (de la Fuente-Fernández et al. 2001; Petrovic et al. 2002). Therefore, it is this psychological component that represents the real placebo effect.

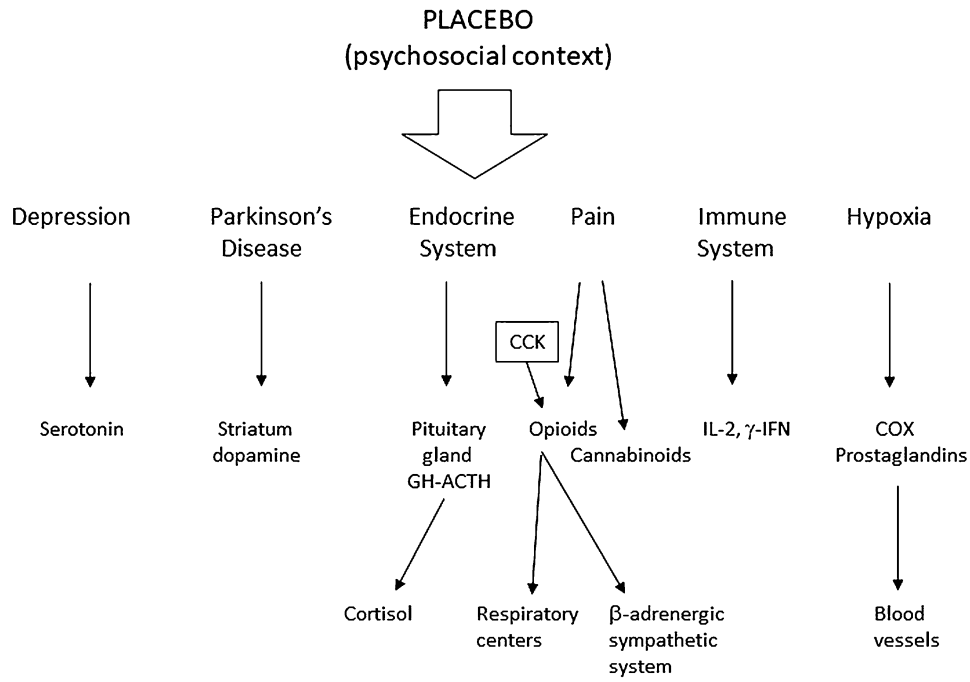
Psychological Mechanisms

The placebo effect is basically a context effect, whereby the psychosocial context (e.g., the therapist's words, the sight of complex machines, and other sensory inputs) around the medical intervention plays a crucial role. Today we know that the context may produce a therapeutic effect through at least two mechanisms: conscious anticipatory processes and unconscious conditioning mechanisms. In the first case, expectation (anticipation of an event; according to expectation theories, expecting an outcome affects the outcome itself) and anticipation of clinical benefit has sometimes been shown to induce a real clinical improvement. In the second case, contextual cues (e.g., color and shape of a pill) may act as a conditioned stimulus that, after repeated associations with an unconditioned stimulus (the active pharmacological agent contained in the pill), is capable alone of inducing a clinical improvement. In the case of pain and Parkinson's disease, it has been shown that expectations play a crucial role, even though a conditioning procedure is performed.

Neurobiology of Placebo Analgesia

The neural mechanisms underlying the placebo effect are only partially understood, and most of our knowledge comes from pain, Parkinson's disease, hypoxia, and immune and endocrine responses, whereas we have only a few pieces of information for other conditions such as depression (Fig. 1). In each of these conditions, different mechanisms seem to take place, so that we cannot talk of a single placebo effect but many.

As to pain and analgesia, in the studies so far performed, there is a general agreement that the endogenous opioid peptide systems play an important role in some circumstances. There are several lines of evidence indicating that placebo analgesia is mediated by a descending pain-modulating circuit which uses endogenous opioids as neuromodulators. This evidence comes from a combination of both imaging and pharmacological studies. In fact, by using positron emission tomography (PET), it was found that the very same regions of the brain in the cerebral cortex and in the brainstem are affected by both a placebo and the opioid agonist remifentanil, thus indicating a related mechanism in placebo-induced and opioid-induced analgesia (Petrovic et al. 2002). In particular, the administration of a placebo induces the activation of the rostral anterior cingulate cortex (rACC), the orbitofrontal cortex (OrbC), and the brainstem. Moreover, there is a significant covariation in activity between the rACC and the lower pons/medulla at the level of the rostral ventromedial medulla (RVM) and a subsignificant covariation between the rACC and the periaqueductal gray (PAG), thus suggesting that the descending rACC/PAG/RVM pain-modulating circuit is involved in placebo analgesia. In another



Placebo Effect, Fig. 1 Cascade of events that may occur during a placebo procedure in different systems and diseases. In pain, both opioid and cannabinoid mediators can be released through the activation of a descending inhibitory network. The respiratory centers may be inhibited by opioid mechanisms as well. The beta-adrenergic sympathetic system is also inhibited during placebo analgesia, although the underlying mechanism is not known (reduction of the pain itself and/or direct action of endogenous opioids). Cholecystokinin (CCK) counteracts the effects of the endogenous opioids, thus antagonizing placebo analgesia, and is also a mediator of the hyperalgesic nocebo effect. Placebos may also affect hormone secretion, like growth hormone (GH), adrenocorticotropic hormone (ACTH), and cortisol, as well as some immune mediators, such as IL-2 and γ-IFN. In Parkinson's disease, placebos induce dopamine release in the striatum, whereas in depression they affect the same brain regions that are affected by serotonin reuptake inhibitors, thus suggesting the involvement of serotonin in the placebo effect in depression. Under hypoxic conditions, placebos may also modulate cyclooxygenase (COX) activity, thereby affecting prostaglandins synthesis

study with functional magnetic resonance imaging (fMRI), it was shown that placebo administration produces a decrease of activity in many regions involved in pain transmission, such as the thalamus and the insula.

The studies with PET and fMRI tell us that placebo analgesia and opioid analgesia share a common neural mechanism and that pain transmission is inhibited by placebos. Recently, a meta-analysis of brain imaging studies has shown that placebo analgesia, while not directly affecting areas involved in pain processing, activates a complex network of areas that influence pain-related regions (Zunhammer et al. 2018).

In support of the involvement of endogenous opioids in this descending circuit, there are several pharmacological studies which show that placebo analgesia is antagonized by the opioid antagonist naloxone. In addition, it has been shown that the endogenous opioid systems have a somatotopic organization, since local placebo analgesic responses in different parts of the body can be blocked selectively by naloxone. A recent PET study used in vivo receptor binding to show that placebos induce the activation of *mu* opioid receptors in different brain areas, like the dorsolateral prefrontal cortex, nucleus accumbens, insula, and rACC.

The placebo-activated endogenous opioids do not act only on pain transmission but on the respiratory centers as well, since a naloxone-reversible placebo respiratory depressant effect has been

described. Likewise, a reduction of beta-adrenergic system activity, which is blocked by naloxone, has been found during placebo analgesia. These findings indicate that the placebo-activated opioid systems have a broad range of action, influencing pain, respiration, and the autonomic nervous system, though it is not known whether they act only through a descending modulating network. The placebo-activated endogenous opioids have also been shown to interact with endogenous substances that are involved in pain transmission. In fact, on the basis of the anti-opioid action of cholecystokinin (CCK), CCK-antagonists have been shown to enhance placebo analgesia, thus suggesting that the placebo-activated opioid systems are counteracted by CCK during a placebo procedure.

It is important to point out that some types of placebo analgesia appear to be insensitive to naloxone, thus suggesting that neuromodulators other than opioids can be involved in some circumstances. For example, if a placebo is given after repeated administrations (preconditioning) of the non-opioid painkiller ketorolac, the placebo analgesic response is not blocked by naloxone but by rimonabant, a CB1 cannabinoid receptor antagonist, which suggests the involvement of endocannabinoids in placebo analgesia.

Furthermore, by using high-altitude hypoxia as a model, it has been found that, similar to aspirin, a placebo can modulate cyclooxygenase activity and the synthesis of prostaglandins and thromboxane in hypobaric hypoxia headache (Benedetti et al. 2014).

Interestingly, although genetic studies are still at the beginning, it has been recently observed that placebo responsiveness is associated with specific genetic polymorphisms in the irritable bowel syndrome (Hall et al. 2012).

Parkinson's Disease

The release of endogenous substances following a placebo procedure is a phenomenon which is not confined to the field of pain, but it is also present in motor disorders, such as Parkinson's disease. As occurs with pain, in this case patients are given an inert substance (placebo) and are told that it is an antiparkinsonian drug that produces an improvement in their motor performance. A study used PET in order to assess the competition between endogenous dopamine and ^{11}C-raclopride for D_2/D_3 receptors, a method that allows identification of endogenous dopamine release (de la Fuente-Fernández et al. 2001). This study shows that placebo-induced expectation of motor improvement activates endogenous dopamine in the striatum of Parkinsonian patients. As this occurs in both the dorsal and ventral striatum, a region involved in reward, it has been argued that the expectation-induced release of dopamine in Parkinson's disease is related to reward mechanisms.

Placebo administration in Parkinson patients affects the activity of the neurons in the subthalamic nucleus, a brain region belonging to the basal ganglia circuitry and whose activity is increased in Parkinson's disease. Verbal suggestions of motor improvement during a placebo procedure are capable of reducing the firing rate and abolishing bursting activity of subthalamic nucleus neurons, and these effects are related to clinical improvement (Benedetti et al. 2005).

Immune and Endocrine Responses

Placebo responses in both the immune and endocrine system can be evoked by pharmacological preconditioning. In fact, after repeated administrations of drugs, if the drug is replaced with a placebo, immune or hormonal responses can be evoked that are similar to those obtained by the previously administered drug. For example, immunosuppressive placebo responses can be induced in humans by repeated administration of cyclosporine A (unconditioned stimulus) associated with a flavored drink (conditioned stimulus), as assessed by interleukin-2 (IL-2) and interferon-γ (IFNγ) mRNA expression, in vitro release of IL-2 and IFNγ, and lymphocyte proliferation. Likewise, if a placebo is given after repeated administrations of sumatriptan, a serotonin agonist of the 5-HT $_{1B/1D}$ receptors that stimulates growth hormone (GH) and inhibits cortisol (glucocorticoids) secretion, a placebo GH increase and a placebo cortisol decrease can be found. These studies support a conditioning mechanism in both immunosuppressive and hormonal placebo responses.

Depression

Depressed patients who receive a placebo treatment show both electrical and metabolic changes in the brain. In the first case, placebos induce electroencephalographic changes in the prefrontal cortex of patients with major depression, particularly in the right hemisphere. In the second case, changes in brain glucose metabolism were measured by using PET in subjects with unipolar depression. Placebo treatments are associated with metabolic increases in the prefrontal, anterior cingulate, premotor, parietal, posterior insula, and posterior cingulate cortex and metabolic decreases in the subgenual cingulate cortex, parahippocampus, and thalamus. Interestingly, these regions are also affected by the selective serotonin reuptake inhibitor, fluoxetine, a result that suggests a possible role for serotonin in placebo-induced antidepressant effects.

Nocebo Effect

The nocebo effect, or response, is a placebo effect in the opposite direction. For example, administration of an inert substance along with verbal suggestions of pain increase may induce a hyperalgesic effect. In this case, anticipatory anxiety may play a fundamental role. Nocebo hyperalgesia has been found to be blocked by proglumide, a non-specific CCK-A/CCK-B receptor antagonist. This suggests that expectation-induced hyperalgesia is mediated, at least in part, by CCK. These effects of proglumide are not antagonized by naloxone; thus endogenous opioids are not involved. Since CCK plays a role in anxiety and negative expectations themselves are anxiogenic, proglumide is likely to act on a CCK-dependent increase of anxiety and pain during the verbally induced negative expectations. Although, mainly due to ethical constraints, the nocebo effect has not been investigated in detail, as has been done for the placebo effect, it shows the powerful effect of the top-down modulation of pain. In other words, cognitive and emotional factors can modulate pain perception in opposite directions.

Nocebo effects represent a source of confusion and misinterpretation in clinical trials, and adverse events described in informed consent forms can actually lead to negative outcomes. For example, in clinical trials for anti-migraine drugs, it has been shown that the adverse events described specifically for each anti-migraine drug out of three different classes (nonsteroid anti-inflammatory, triptans, and anticonvulsants) corresponded to the adverse events observed in each respective placebo arm (Amanzio et al. 2009).

Studies on nocebo effect in depression showed similar effects. Indeed, in a study on the rates of adverse events in serotonin reuptake inhibitor (SSRI) trials, a total of 143 placebo-controlled trials were analyzed showing that the reported rate of adverse events was influenced by their assessment, so that a more systematic assessment led to higher rates in comparison to a less systematic assessment (Rief et al. 2009).

Pharmacological Relevance

Implications for Clinical Trials

According to the classical methodology of clinical trials, any drug must be compared with a placebo in order to assess its effectiveness. If the group that takes the drug shows a larger clinical improvement than the group that takes the placebo, the drug is considered to be effective. However, in light of the recent advances in placebo research, some caution is necessary in the interpretation of some clinical trials. In fact, by considering the complex cascade of biochemical events induced by placebo administration, any drug that is tested in a clinical trial may interfere with these placebo-/expectation-activated mechanisms, thus confounding the interpretation of the outcome of a clinical trial. As we have no a priori knowledge of which substances act on placebo-activated endogenous opioids, dopamine, and serotonin – and indeed almost all drugs might interfere with these neurotransmitters – one way to eliminate this possible pharmacological interference is to make the placebo-activated biochemical pathways "silent." This can be achieved by the hidden administration of drugs.

Hidden Administration of Drugs

It is possible to eliminate the placebo (psychosocial) component and analyze the pharmacodynamic effects of a treatment, free of any

psychological contamination, by administering drugs covertly. In this way, the cascade of biochemical events triggered by a placebo procedure can be eliminated. To eliminate the psychosocial context in which a treatment is given and thus the placebo component of the treatment, the patient is not made aware that a medical therapy is being carried out. To make this possible, drugs are administered through hidden infusions by machines. A hidden drug infusion can be performed through a computer-controlled infusion pump that is pre-programmed to deliver the drug at the desired time. It is crucial that the patient does not know that any drug is being injected, so that he or she does not expect anything. The computer-controlled infusion pump can deliver a drug automatically, without a doctor or nurse in the room and without the patient being aware that a treatment has been started (Colloca et al. 2004).

The analysis of different treatments, either pharmacological or not, in different conditions has shown that an open (expected) therapy that is carried out in full view of the patient is more effective than a hidden one (unexpected). Whereas the hidden injection represents the real pharmacodynamic effect of the drug, free of any psychological contamination, the open injection represents the sum of the pharmacodynamic effect plus the psychological component of the treatment. The latter can be considered to represent the placebo component of the therapy, even though it cannot be called placebo effect, as no placebo has been given. It is important to realize that, by using hidden administration of drugs, it is possible to study the placebo effect without the administration of any placebo.

For example, in a postoperative setting, it has been shown that the analgesic dose used to reduce pain by 50% was higher in hidden compared to open administrations of four different painkillers (buprenorphine, tramadol, ketorolac, metamizol) and the reported pain was higher after a hidden analgesic infusion compared with an open one (Benedetti et al. 2011).

Brain imaging studies have shown different activities for open and hidden administrations of analgesic drugs. Indeed, it has been observed that the open infusion of the analgesic remifentanil (told remifentanil, gets remifentanil) induced stronger analgesic effects than its hidden infusion (told saline, gets remifentanil), and these effects were associated with activity in the dorsolateral prefrontal cortex and pregenual anterior cingulate cortex. Interestingly, the negative expectation of drug interruption (told interruption, gets remifentanil) completely blocked the analgesic effects of remifentanil and was associated with activity in the hippocampus (Bingel et al. 2011).

Prefrontal Control and Placebo Response

A common finding across different neuroimaging studies on placebo analgesia is the predominant activity of prefrontal regions, like the dorsolateral prefrontal cortex, suggesting their crucial role in placebo responses. Data coming from studies on patients' neuroimaging and neuromodulation support this idea.

Since in patients affected by Alzheimer's disease the frontal lobes appear to be severely compromised, this neurodegenerative disorder has been used as a model to test placebo responsiveness. Indeed, in these patients, it has been found that placebo analgesia is positively correlated with cognitive status and functional connectivity between different brain areas, and, conversely, the more impaired the prefrontal activity, the smaller the observed placebo response (Benedetti et al. 2006). More recently, it has been shown that stronger placebo analgesia is associated with increased mean fractional anisotropy values within white matter tracts connecting the PAG with rACC and dorsolateral prefrontal cortex (Stein et al. 2012). Finally, in healthy volunteers, repetitive transcranial magnetic stimulation (rTMS) has been used to inactivate the left and right prefrontal cortex during placebo analgesia, and this prefrontal inactivation resulted in a complete blockade of the placebo response (Krummenacher et al. 2010). Therefore, these studies confirm that the placebo response is directly correlated to prefrontal control: when this activity is abolished, there is no placebo response.

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Placebo Response

▶ Placebo Effect

Plasma ChE

▶ Cholinesterases

Platelet Aggregation Inhibitors

▶ Antiplatelet Drugs

Platelet Inhibitors

▶ Antiplatelet Drugs

Plexins

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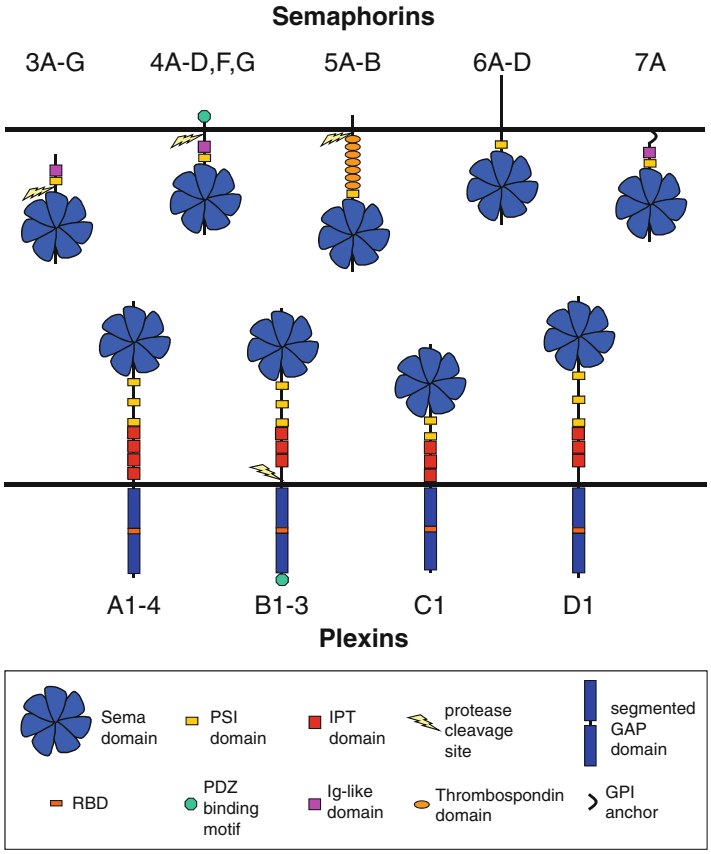
Definition

Plexins comprise an evolutionary conserved and widely expressed family of transmembrane

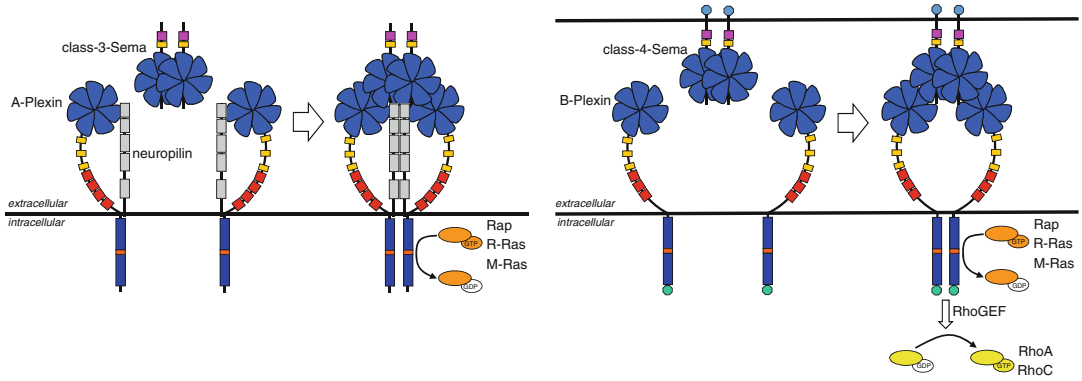


Plexins, Fig. 1 Mammalian plexins and their semaphorin ligands. The mammalian plexins are grouped into four subfamilies (**A**–**D**). The mammalian semaphorin family is divided into five classes (3–7). Both plexins and semaphorins are characterized by the presence of an extracellular sema domain, which contains a seven-blade β-propeller. In addition, all plexins share a highly conserved intracellular moiety that carries a segmented GAP domain and a Rho GTPase binding domain (RBD). Additional domains include PSI (plexin, semaphorin, integrin) domains and IPT (Ig-like, plexins, and transcription factors) domains. The plexins of the B-family have a PDZ binding motif at their C-terminus. Some plexins and semaphorins exhibit protease cleavage sites. While class-3-semaphorins are secreted, classes 4–6 are transmembrane proteins. Class-7-semaphorins are attached to the plasma membrane by a GPI-anchor. Class-5-semaphorins are characterized by seven thrombospondin domains

proteins that serve as receptors for semaphorins. They regulate key cellular functions such as morphology, proliferation, differentiation, and migration and play a pivotal role in cell-cell communication. On the basis of homology, they are grouped into four subfamilies (A–D). Semaphorins are a large family of secreted and membrane-associated proteins. In the mammalian system, five structurally different classes of semaphorins (3–7) have been found (Fig. 1). While the semaphorin-plexin system was initially discovered as a regulator of axonal guidance in the developing nervous system, it has become clear that its physiological and pathophysiological relevance is much larger including the cardiovascular, immune, renal, and bone system as well as cancer (Worzfeld and Offermanns 2014).

Basic Mechanisms

Both plexins and semaphorins are structurally characterized by the presence of an extracellular sema domain, which contains a seven-blade β-propeller (Fig. 1) (Worzfeld and Offermanns 2014). The sema domain of semaphorins mediates



Plexins, Fig. 2 Mechanism of semaphorin-plexin signaling. Binding of a semaphorin homodimer to two plexin monomers induces plexin dimerization and downstream signaling. For class-3-semaphorins, neuropilins are required to stabilize the assembly

Plexins, Table 1 Plexins and their respective ligands

Receptor	Ligand
Plexin-A1	Class-3-semaphorins[a], Sema5A, Sema5B, Sema6C, Sema6D
Plexin-A2	Class-3-semaphorins[a], Sema6A, Sema6B
Plexin-A3	Class-3-semaphorins[a], Sema5A, Sema5B
Plexin-A4	Class-3-semaphorins[a], class-6-semaphorins
Plexin-B1	Sema3C[a], Sema4A, Sema4D
Plexin-B2	Sema4A, Sema4B, Sema4C, Sema4D, Sema4G, angiogenin
Plexin-B3	Sema4A, Sema5A
Plexin-C1	Sema4D, Sema7A, viral semaphorins
Plexin-D1	Class-3-semaphorins[a], Sema3E[b], Sema4A

[a]When plexin is in a complex with neuropilin
[b]Independently of neuropilins. Angiogenin = Angiogenin-1 = Ribonuclease 5 = RNase 5

the formation of semaphorin homodimers (Fig. 2). So far, there is no evidence for semaphorin heterodimers or for functional semaphorin monomers. The extracellular domain of plexins forms an autoinhibited ring-like conformation. In contrast to the sema domain of semaphorins, the sema domain of plexins is monomeric and does not dimerize. The cytoplasmic moieties of plexins (~600 amino acids) are highly conserved and do not show homology to any other known protein. The interaction between semaphorins and plexins is mediated by the sema domains (Table 1). The semaphorin-plexin binding interface is relatively large, and the binding specificity critically relies on insertions in the β-propeller of the sema domains. Each subunit of a semaphorin dimer binds a monomeric plexin, thereby inducing the formation of plexin dimers and plexin downstream signaling (Fig. 2). Class-3-semaphorins require neuropilins as co-receptors, which stabilize the binding of a class-3-semaphorin to its plexin receptor. Other functionally relevant co-receptors for plexins of the B- and D-subfamily include the receptor tyrosine kinases ErbB-2 and Met. Since semaphorins are found both in a membrane-bound form and in a soluble form, plexins can interact with semaphorins on different cells or with semaphorins in solution (Jongbloets and Pasterkamp 2014). In addition to interactions between semaphorins and plexins in *trans* (i.e., the semaphorin and the plexin being on different cells), there is evidence that semaphorins and plexins can also interact in *cis* (i.e., on the same cell). These *cis* interactions can induce plexin signaling in *cis* and/or alter the binding of semaphorins and plexins in *trans*. Furthermore, it has become clear that transmembrane class-4- and class-6-semaphorins can also serve as receptors, rather than ligands, and signal in a reverse manner upon binding of plexins. The semaphorin-plexin system therefore constitutes a bi-directional signaling system.

Activation of plexins results in the induction of a multitude of signaling pathways. Among the central downstream players in plexin signaling are small GTPases (Pascoe et al. 2015). All

plexins contain a GTPase-activating protein (GAP) domain within their cytoplasmic portion, which catalyzes the guanine nucleotide exchange of Rap proteins. This domain also mediates the deactivation of R-Ras and M-Ras; however, plexins do not exhibit direct GAP activity toward R-Ras and M-Ras, and the precise molecular mechanism underlying plexin-mediated R-Ras and M-Ras deactivation remains unclear. Inhibition of Rap and R-Ras by plexins has been suggested to result in the inhibition of integrin signaling. In addition to the GAP domain, the intracellular portion of all plexins carries a Rho GTPase binding domain (RBD) through which Rac1 and Rnd1 can interact with plexins. The functional consequences of these interactions are not entirely understood. Plexins of the B-subfamily carry a PDZ-binding motif at their C-terminus, which mediates a stable interaction with the Rho guanine nucleotide exchange factors (RhoGEFs) LARG and PDZ-RhoGEF. Activation of B-plexins results in the activation of RhoA and RhoC via this pathway. Furthermore, a series of kinases have been implicated as effectors or modulators in signaling of particular plexins. These kinases involve receptor tyrosine protein kinases (Met, ErbB-2), cytosolic tyrosine protein kinases (FAK, Pyk2, Fes, Fyn, Src, Syk), cytosolic serine/threonine protein kinases (PKA, Akt, MAP kinases, Raf, GSK3β), and lipid kinases (PI3K). It has to be noted that plexin signaling is highly versatile and complex and that activation of the same plexin can evoke different and sometimes even opposing cellular responses, depending, e.g., on the association of the plexin with different co-receptors.

Semaphorins and plexins were first described to be involved in the guidance of axonal growth cones during development. Semaphorin 3A (formerly collapsin-1) was in fact one of the first repulsive axonal guidance factors described. Various semaphorins have been shown to induce axonal growth cone repulsion via activation of plexins. However, in some cases, semaphorins can also attract axons. In addition to axon guidance, plexins regulate multiple other aspects of neural circuit assembly, in both the central and peripheral nervous systems, including neuronal morphogenesis, synapse assembly, and synaptic refinement (Koropouli and Kolodkin 2014). Plexins have been suggested to play a role in various neurological diseases, such as Hirschsprung's disease, autism, epilepsy, and neurodegenerative diseases; however, evidence for an involvement of plexins in these diseases is mostly correlative so far (Van Battum et al. 2015). Experiments in animal models of disease demonstrate that interfering with semaphorin-plexin signaling improves regeneration after central nervous system trauma and may be of relevance in the treatment of pathological pain and aberrant fear.

Semaphorins and plexins are of also of vital importance in the cardiovascular system, where they control vascular patterning and remodeling (Corà et al. 2014). Similar to the situation in the nervous system, semaphorin-plexin signaling directs the behavior of specialized endothelial cells at the tip of navigating vascular sprouts. This is the case not only during development but also under pathophysiological conditions at adult stages. For example, class-3-semaphorins, in particular semaphorin 3E, activate Plexin-D1 expressed on endothelial cells to repel growing endothelial sprouts and to regulate endothelial tip cell fate. The anti-angiogenic effect of semaphorin 3E-Plexin-D1 signaling may hold therapeutic potential in microvascular diseases, for example, in patients with diabetic retinopathy, where damage to the microvasculature triggers compensatory hypervascularization. In addition to effects on the vasculature, semaphorins and plexins have also been shown to control multiple aspects of cardiac morphogenesis including myocardial compaction and trabeculation, cardiac-outflow tract development, and autonomic innervation of the heart.

Semaphorins and plexins have been found to be expressed by different cell types of the innate and adaptive immune system, e.g., macrophages, dendritic cells, and T and B lymphocytes, and have emerged as central players in immune cell communication (Nishide and Kumanogoh 2018). They control various functions of immune cells, including immune cell activation, differentiation, motility, migration, and trafficking. As shown

in preclinical animal models of disease, the semaphorin-plexin system is also of major importance in inflammatory and autoimmune diseases. For example, semaphorin 3A promotes the migration of dendritic cells from the periphery to the draining lymph nodes via Plexin-A1, downregulates T cell proliferation via Plexin-A4, and activates macrophages via Plexin-A4. Another example for an important immune semaphorin is semaphorin 4D (also known as CD100), which was the first semaphorin to be characterized for its role in the immune system. Semaphorin 4D expressed on T cells activates Plexin-B1 on microglia to promote neuroinflammation in an experimental autoimmune encephalomyelitis mouse model. Furthermore, semaphorin 4D has been shown to act as a receptor for plexins and to mediate reverse signaling in γδ T cells of the skin and intestinal epithelium; activation of semaphorin 4D supports skin wound healing and ameliorates colitis in mouse models. On B cells and dendritic cells, semaphorin 4D induces its effects via a non-plexin receptor, CD72.

Loss of bone tissue is a hallmark of several bone disorders, in particular of osteoporosis. The semaphorin-plexin system exerts decisive functions in the communication of different types of bone cells and regulates both bone formation and bone resorption (Verlinden et al. 2016). In mice, semaphorin 3A is expressed by osteoblasts and neurons. Through its receptor Plexin-A1, semaphorin 3A promotes bone formation by enhancing the differentiation of osteoblasts and by inhibiting the differentiation of osteoclasts. Alternatively or additionally, semaphorin 3A may increase bone mass indirectly by modulating sensory innervation of bones. Another semaphorin, semaphorin 4D, is expressed on osteoclasts and decreases bone formation by interfering with osteoblast differentiation via activation of Plexin-B1 on osteoblasts.

In the kidney, the semaphorin-plexin system plays an important role both in glomerular and in tubular diseases (Xia and Worzfeld 2016). Several class-3-semaphorins have been found to be expressed in podocytes, and podocyte expression of a particular class-3-semaphorin, semaphorin 3A, is upregulated in diabetic humans and mice. In mice, genetic inactivation of semaphorin 3A or its receptor Plexin-A1 in podocytes, or pharmacological inhibition of semaphorin 3A, ameliorates diabetic nephropathy, presumably through normalization of the slit diaphragm. Class-4-semaphorins and their receptors, B-plexins, have been demonstrated to be expressed in tubular epithelial cells and to be required for kidney repair after acute kidney injury in mice. Moreover, semaphorin 3A has been proposed as a biomarker of tubular injury.

Plexins are of central importance in cancer and can mediate both tumor-promoting and tumor-suppressive effects (Gurrapu and Tamagnone 2019). The expression of several semaphorins and plexins has been found to be dysregulated in human cancers. In various types of cancer cells, plexins control critical cellular properties such as proliferation, apoptosis, migration, invasion, and/or epithelial-to-mesenchymal transition. The semaphorin ligands binding to plexins on cancer cells can be derived from cancer cells (including autocrine signaling loops) or from other cell types in the tumor microenvironment. Conversely, semaphorins expressed by cancer cells can impact on cells of the tumor microenvironment. Among the best-characterized effects of semaphorins in the tumor microenvironment are those on endothelial cells, where semaphorins can induce both anti-angiogenic (class-3-semaphorins) and pro-angiogenic (class-4-, class-5-, and class-6-semaphorins) responses. Moreover, the semaphorin-plexin system conveys information between different types of cells in the tumor microenvironment. For example, semaphorin 4D produced by tumor-associated macrophages acts on endothelial cells to induce tumor angiogenesis. Finally, the ability of the semaphorin-plexin system to signal both in a forward and in a reverse manner potentially enables bi-directional communication among cancer cells or between cancer cells and cells of the tumor microenvironment.

Pharmacological Intervention

The semaphorin-plexin system has been shown to play multiple roles in various organ systems.

Accordingly, most semaphorins and plexins have been validated as potential drug targets in animal models of diseases – including cancer, multiple sclerosis, spinal cord injury, osteoporosis, microvascular diseases, diabetes, and asthma – using genetic (knockout/knockdown) and/or pharmacological approaches (Worzfeld and Offermanns 2014). These pharmacological approaches to block, activate, or modulate semaphorin-plexin signaling comprise small molecules, peptoids, peptides, recombinant proteins (recombinant semaphorins or recombinant plexins acting as ligand traps for semaphorins), and antibodies. Hitherto, the pharmacological agent most far advanced in clinical development is an anti-semaphorin 4D antibody, which has completed phase I clinical trials in patients with advanced solid tumors and in patients with multiple sclerosis. This monoclonal semaphorin 4D antibody, pepinemab (VX15/2503), is currently being tested in further phase I clinical trials in combination with immune checkpoint inhibitors (ipilimumab or nivolumab) in patients with melanoma, head and neck squamous cell carcinoma, and pancreatic or colorectal cancer and in a phase Ib/II clinical trial in combination with immune checkpoint inhibitors (avelumab) in patients with non-small cell lung cancer.

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Polymyxins

► Peptides and Peptidomimetics as Foundations for Drug Discovery

Polypeptide Chain-Binding Proteins

► Chaperones

Posttranslational Protein Modification

► Ubiquitin/Proteasome

Pro-drug

► Prodrugs

Prodrugs

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Synonyms

Pro-drug

Definition

Prodrugs are molecules with little or no pharmacological activity in their own right before they are converted to the active drug. Prodrug strategies can be considered a chemistry-enabled tool to overcome deficiencies in the physicochemical properties of a molecule that limit its formulation options or result in suboptimal bioavailability or safety profile and, consequently, pharmacological response.

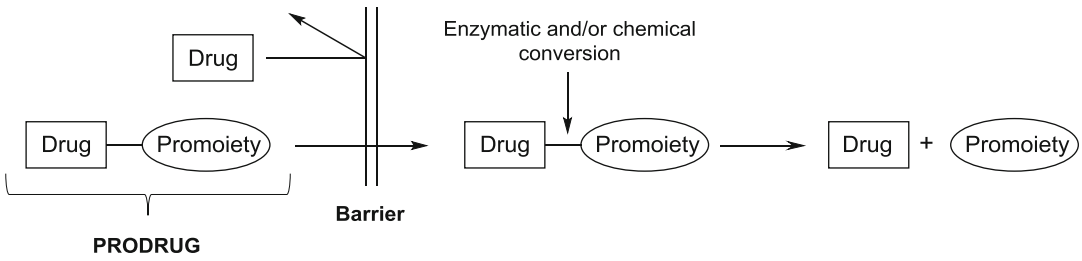
Basic Mechanisms

In most cases, prodrugs are molecules consisting of a chemical group, a promoiety, that is attached to a parent drug via a covalent bond. This attachment must be reversible so that the promoiety is cleaved off by a chemical or enzymatic process or a combination of the two to liberate the pharmacologically active drug in the body (see Fig. 1 for the simplified prodrug concept and Fig. 2 for a representative prodrug example). Some literature uses a term bioprecursor prodrug in order to refer to a more complex prodrug molecule that lack an obvious promoiety but form the active compound through rearrangement such as intramolecular reaction or oxidation (Fig. 2). Codrugs, on the other hand, are inactive molecules that consist of two pharmacologically active drugs that are coupled together, and each is the promoiety for the other. Upon conversion, codrugs liberate both active drugs simultaneously (Fig. 2).

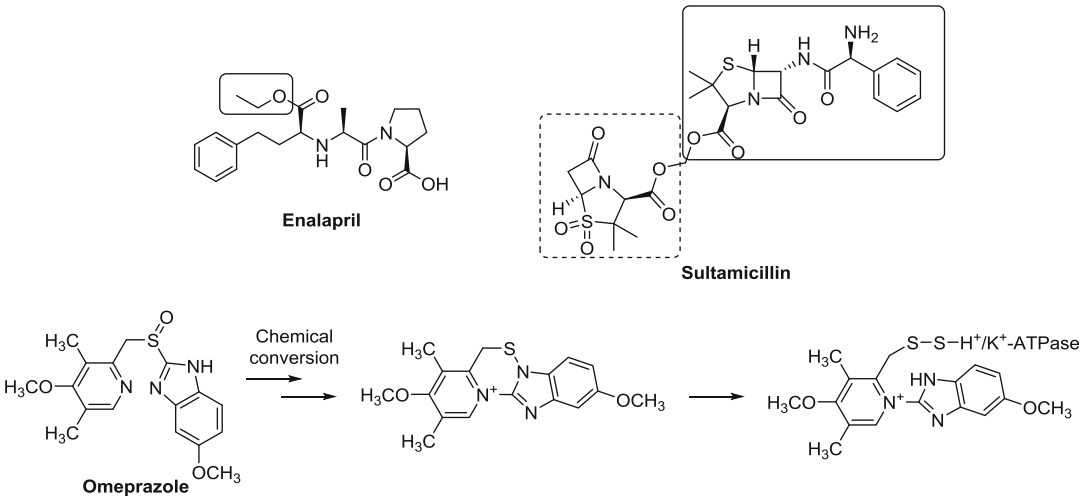
Common functional groups on parent drugs that can be contemplated to chemical modification to prepare prodrugs include such as hydroxyl (-OH), carboxyl (-COOH), as well as basic and acidic NH groups. Prodrugs produced via these modifications include, for example, esters, carbonates, carbamates, amides, phosphates, as well as various *N*-acyl derivatives and *N*-Mannich bases. Also, modification of phosphate (-OPO$(OH)_2$) and phosphonate (-CPO$(OH)_2$) groups has resulted in various experimental and clinically approved phosphate and phosphonate nucleoside prodrugs. Some of the prodrug strategies used in marketed prodrugs are listed in Table 1. It is not surprising that the most common prodrug structures are those requiring a hydrolytic bioconversion in vivo by ubiquitous hydrolases.

Pharmacological Relevance

An increase in the number of druggable targets has substantially expanded the chemical property space for new drug candidates. Consequently, contemporary molecules suffer often from undesirable physicochemical properties that create challenges for their delivery to their biological targets in the body and lead to poor pharmacological response. Also, several drugs already on the market possess suboptimal pharmacology due to same reasons. In these respects, temporary structural modifications by various prodrug strategies can intentionally be used to overcome deficiencies in physicochemical properties such as polarization, electronic factors including ionization constant (i.e., pK_a), topological and steric factors, hydrophobicity and hydrogen bonding that lead to better biopharmaceutical properties such as

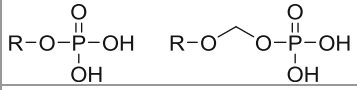
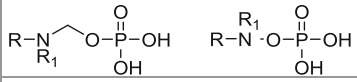
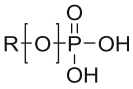
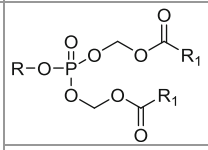
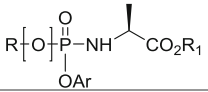


Prodrugs, Fig. 1 A simplified representation of the prodrug concept. The drug-promoiety molecule is the prodrug that is inactive or very weakly active pharmacologically. In broad terms, the barrier can be thought of as any liability for a parent drug that prevents its optimal pharmaceutical or pharmacological performance



Prodrugs, Fig. 2 Examples of prodrugs, bioprecursor prodrugs and codrugs. Enalapril is an ethyl ester prodrug of enalaprilat (promoiety circled). Omeprazole is a bioprecursor prodrug that is converted into its active sulfenamide metabolite, which binds covalently to the proton bump (H^+/K^+-ATPase). Sultamicillin is a codrug, which liberates both the β-lactam antibiotic ampicillin (closed circle) and β-lactamase inhibitor penicillanic acid sulfone (dotted circle)

Prodrugs, Table 1 Examples of prodrug strategies for the most common functional groups on parent pharmacologically active drugs

Functional group	Prodrug strategy		Representative prodrugs
Carboxylic acid R-COOH	R-O-CO-R_1	Esters	Enalapril
	R-O-CO-CH(R_1)-O-CO-R_2	Double esters	Cefuroxime axetil
Hydroxyl R-OH	R-O-CO-R_1	Esters	Abiraterone acetate
	R-O-CO-NR_1R_2	Carbamates	Irinotecan
		Phosphates, phosphonooxymethylethers	Tedizolid phosphate, fospropofol
Amine R-NR_1R_2	R-NR_1-CO-R_2	Amides	Midodrine
	R-NR_1-CO-O-R_2 R-NR_1-CO-O-CH(R_2)-O-CO-R_3	Carbamates	Gabapentin enacarbil
	R-NR_1-CHR_2-O-CO-R_3	*N*-acyloxyalkylamines	Aripiprazole lauroxil
		Phosphates, phosphoramidates	Fosphenytoin, fosaprepitant
Phosphate or phosphonate 		Carbonyloxymethyl	Tenofovir disoproxil
		ProTide phosphoramidates/ phosphoamidates	Sofosbuvir, tenofovir alafenamide

P

Prodrugs, Table 2 Prodrugs can be used to address the following barriers to improve drug delivery and pharmacological response

Formulation and administration
• Inadequate aqueous solubility for liquid dosage forms
• Inadequate shelf-life for solid or liquid dosage forms
• Pain or irritation after local administration
Absorption
• Inadequate dissolution rate due to low aqueous solubility
• Poor membrane permeation and low oral or topical (e.g., dermal, ocular) bioavailability due to poor lipophilicity
• Inadequate stability in the gastrointestinal tract or during the first-pass metabolism
Distribution
• Lack of site-specificity (e.g., poor brain distribution or tumor targeting)
• Need to decrease plasma protein binding or deposition in lipophilic compartments
Metabolism and excretion
• Lack or need of site-specific biotransformation
• Short duration of action
Toxicity
• Lack of site-directed delivery or site-specific transformation
• Need to temporarily mask a reactive, inherently active, functional group

solubility, permeability or partitioning, or better chemical or enzymatic stability, or targeting and, consequently, more favorable pharmacological response (Table 2). Today, the interest in prodrug strategies is evident because approximate 10% of all marketed drugs worldwide can be considered prodrugs.

Improving Solubility

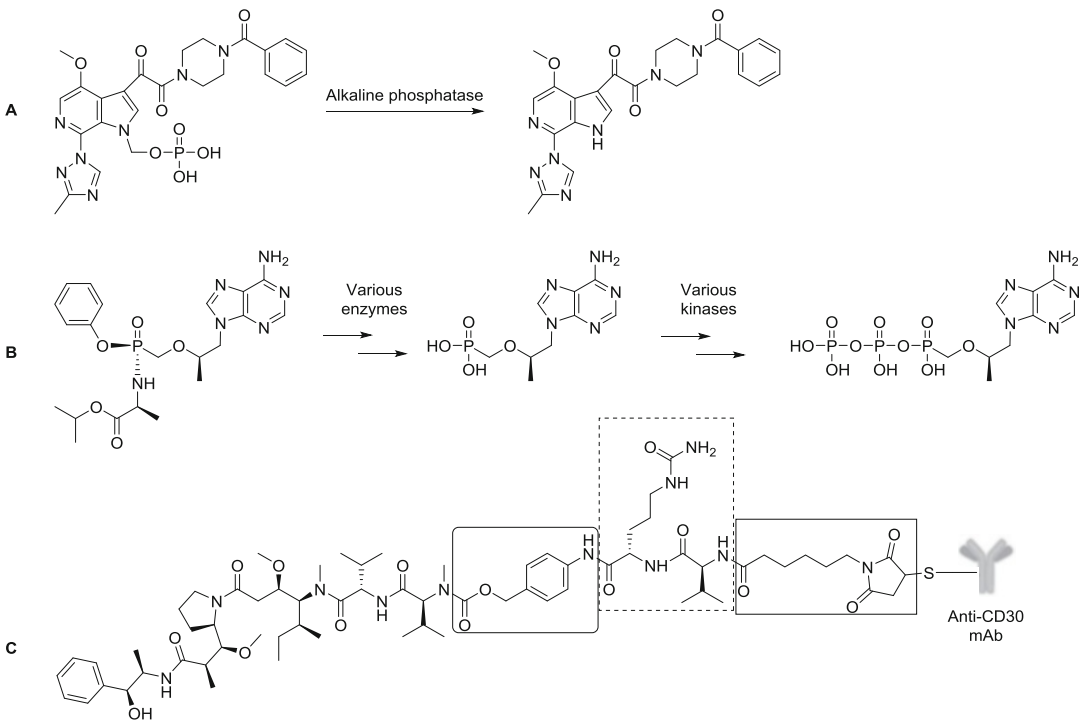
Poorly soluble oral drug may face low and variable oral bioavailability, which leads to unpredictable pharmacological response. Improved solubility can be achieved by attaching an ionized or a polar promoiety, such as phosphate, amino acid or sugar moiety, or by using a promoiety that disrupts the crystal packing of the drug (for example, by blocking intramolecular hydrogen bonding).

Currently, phosphate prodrugs are the most commonly used strategy for improving the aqueous solubility of oral drugs as the increase in solubility imparted by the two negative charges of the phosphate group is often several orders of magnitude (phosphate group has two pK_a values, ca. 2 and 7, which are substantially charged negatively when pH is higher than the pK_a). This is demonstrated by fostemsavir (Fig. 3) that has aqueous solubility of over 500-times higher (>11 mg/ml vs. ~20 μg/ml in the pH range of 2–8 at room temperature) than that of temsavir. The phosphate prodrug strategy successfully overcame dissolution- and solubility-limited absorption as a major barrier to achieving optimal systemic exposure of the parent HIV-1 attachment inhibitor temsavir.

Water-soluble oral prodrugs are usually designed to undergo conversion in proximity to the site of absorption (i.e., typically small intestine) by intestinal enzymes to release the parent drug. Therefore, increasing water-solubility by promoieties is best suited for highly permeable drugs having facile absorption in the intestine after being liberated from the prodrug in order to avoid precipitation.

Improving Permeability

Permeability across any biological membrane is challenging for polar and charged drugs. This usually leads to low and variable absorption and, consequently, to low bioavailability and low exposure for specific target organs. Most frequently enhancement in permeability has been attempted to solve by increasing the lipophilicity of the parent drug by masking its polar and ionized groups. Hydrophilic hydroxyl, carboxyl, phosphate, or amine groups can be converted to the more lipophilic alkyl or aryl esters or *N*-acyl derivatives, which are then converted back to the parent drugs in the body usually by ubiquitous esterase or peptidase activity. As an example, the ethyl ester prodrug oseltamivir is readily absorbed after oral administration resulting in approximately 80% oral bioavailability in humans, whereas its pharmacologically active parent drug oseltamivir carboxylate showed only negligible bioavailability (<5%) in preclinical species. After oral absorption, oseltamivir undergoes bioconversion to its neuraminidase enzyme inhibitor parent drug and ethanol predominantly by human



Prodrugs, Fig. 3 (**a**) Water-soluble prodrug fostemsavir undergoes bioconversion to pharmacologically active temsavir by alkaline phosphatase. (**b**) Tenofovir alafenamide is a lipophilic phosphoramidate prodrug of tenofovir that efficiently delivers this compound to the hepatocytes where, after cleavage by various cytosolic enzymes, it is readily phosphorylated twice to generate the pharmacologically active form. (**c**) Antibody-drug conjugate (ADC) brentuximab vedotin contains a CD30-specific antibody that is attached to an antimitotic agent monomethyl auristatin E (vedotin) via a cathepsin B labile peptide bond that is between the dipeptide valine-citrulline (dotted circle) and *para*-amino benzyloxycarbonyl (closed circle on the left) moieties. The maleimidocaproyl spacer (closed circle on the right) provides space for enzymatic cleavage

carboxylesterase 1. Many successful angiotensin-converting enzyme (ACE) inhibitors, such as enalapril, are also ethyl esters of their respective pharmacologically active carboxylate forms (Fig. 2). More recent ethyl ester prodrugs in clinical use are sacubitril and telotristat etiprate, which are lipophilic prodrugs of the endopeptidase inhibitor sacubitrilat and the serotonin synthesis inhibitor telotristat, respectively.

Much of the prodrug research has recently been focusing on enabling oral bioavailability of various nucleoside analogues that have demonstrated their effectiveness in the treatment of cancer and various infections. To become pharmacologically active, nucleoside analogues must undergo the stepwise addition of phosphate groups to form active nucleoside triphosphates in the body. Because the first phosphorylation step is often limiting, nucleoside analogues are frequently administered as their monophosphorylated forms or are structurally modified to possess a phosphonate moiety, both of which can further be turned into lipophilic prodrug forms. Representative examples are phosphoramidate ProTide prodrugs tenofovir alafenamide (Fig. 3) and sofosbuvir, which are efficiently delivered to the cytosol of hepatocytes after oral administration. After cleavage of promoieties, representative triphosphorylated forms are generated providing inhibitory effect against hepatitis B virus and hepatitis C virus, respectively, for tenofovir alafenamide and sofosbuvir.

Better Targeting

Drug targeting can be achieved in general either by site-directed delivery or site-specific

transformation of a prodrug. While site-directed drug delivery consists of the selective or primary delivery of the intact prodrug to the site of drug action, in site-specific transformation the prodrug can be widely distributed in the body, but it releases the active drug predominantly at the desired site by a selective activation reaction.

Selective delivery can be possible using drug conjugation strategies, wherein the drug is conjugated to a macromolecule carrier that recognizes target-specific markers, such as antigens or receptors. Macromolecules typically cannot penetrate healthy membranes, but because the membranes of cancerous tissues are leaky, macromolecules can enter the tumor microenvironment from the bloodstream. Therefore, macromolecular prodrugs are usually designed to improve drug targeting to a tumor. This has been experimented with various nonbiodegradable polymers such as *N*-(2-hydroxypropyl)methacrylamide (HPMA) and polyethylene glycol (PEG) as well as biodegradable polymers such as polyglutamic acid (PGA), dextrin, and hyaluronic acid that have been used to form macromolecular prodrugs with some anticancer agents and tested in clinical trials. Also, some antibody-drug conjugates (ADCs) consisting of a monoclonal antibody linked to an active drug via a cleavable bond can be regarded as macromolecular prodrugs. For example, brentuximab vedotin consists of a CD30-specific antibody that is attached to an antimitotic agent monomethyl auristatin E (known as vedotin) via the plasma-stable dipeptide valine-citrulline linker (Fig. 3). This linker is designed to be site-selectively cleaved by the proteolytic enzyme cathepsin B once the ADC has been internalized by CD30 expressing tumor cells through endocytosis and then releasing vedotin.

On the other hand, the site-specific transformation of the prodrug can be achieved either by: (i) exploiting endogenous enzymes or physiological conditions of target tissue and/or cells, such as pH or hypoxia, (ii) delivering genes that encode prodrug-activating enzyme into target tissue (e.g., virus-directed enzyme prodrug therapy (VDEPT) and gene-directed enzyme prodrug therapy (GDEPT)), or (iii) delivering prodrug-activating enzyme into target tissue via monoclonal antibodies (e.g., antibody-directed enzyme prodrug therapy (ADEPT)). While most of these strategies are still experimental, a successful example of drug targeting via site-selective transformation can be provided by previously described phosphoramidate prodrugs tenofovir alafenamide and sofosbuvir. These prodrugs are efficiently translocated to the cytosol of their target cells, hepatocytes in the liver where, after removal of promoieties and consequent release of the monophosphate, the monophosphate is readily site-selectively phosphorylated twice to generate the pharmacologically active triphosphate forms.

Another example of where site-specific transformation has resulted in the marketed prodrug is given by the proton pump (H^+/K^+ ATPase) inhibitor omeprazole (Fig. 2). As the weakly basic drug (pK_a is 3.97) omeprazole is not protonated at physiological pH, allowing it to be absorbed and distributed into the secretory canaliculus of the parietal cells. In the acidic conditions of parietal cells (pH of 1–2), omeprazole becomes protonated and accumulates inside the canaliculus of the cell. Protonation also initiates the site-specific chemical transformation of omeprazole into its active metabolite, which binds to the thiol of a cysteine residue of the H^+/K^+ ATPase. This irreversible binding inhibits the ability of parietal cells to secrete gastric acid. The fact that omeprazole and other proton pump inhibitors (e.g., lansoprazole, pantoprazole, and rabeprazole) are only effective on H^+/K^+ ATPases, which contain highly acidic compartments that non-gastric H^+/K^+ ATPases lack, corresponds their excellent safety profiles. Therefore, proton pump inhibitors are transformed into their active species only under highly acidic conditions at the site of action.

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Propionylcholinesterase

► Cholinesterases

Proprotein Convertases

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Synonyms

Proprotein-convertases subtilysin/hexin type 1-9 (PCSK 1-9)

Definition

Proprotein convertases is a family of serine proteases that catalyze the cleavage of specific peptide bonds. This cleavage results in activation of several cellular and extracellular proteins such as hormones, receptors, growth factors, metalloproteinases, and viral and bacterial proteins, among others. Inhibition of the enzymatic activity of PCs constitutes a valuable therapeutic tool for several diseases ranging from cancer, inflammation, viral and bacterial infections to endocrine imbalances, and hypercholesterolemia.

Basic Characteristics

Many proteins are synthesized as inactive precursors, which need to be activated to display full activity. Proteolysis at specific sequences separates the active protein from the prodomain and constitutes one of the main activation mechanisms in mammalian cells. According to their structure, and recognition sequence, the PCs can be classified into kexin-like, pyrolysin-like, or proteinase-like (Seidah and Prat 2012).

The kexin-like subfamily has seven members, PSCK1 to 7, which recognize and cleave at the C-terminus of the sequence K/R-X_n-K/R, where n is 2, 4, or 6. The basic amino acids at the positions indicated (lysine and arginine) are essential for PC recognition. In fact, the former name for the probably best characterized member of PCs, furin (PCSK3), PACE (for paired basic amino acid cleaving enzyme) highlights the specific PC activity. Although the enzymatic activities of these seven kexin-like proteases are somewhat overlapping, each PC shows differential substrate specificity, tissue localization, and patterns of expression. For instance, furin PCSK5 and 6 are ubiquitous, whereas SPCK4 is restricted to the testes and PCSK1 and 2 to the neuroendocrine tissues (Seidah and Prat 2012).

In contrast, PCSK8 and PCSK9 cleave at nonbasic residues and play important roles in cholesterol and lipid metabolism and LDL functionality, respectively. The pyrolysin and proteinase-like recognize the sequences R-X-(L/V/I)-X and V/I/L-F-A-Q, respectively, both essentially nonbasic sequences. PCSK9 is the sole member of the subfamily of proteinase-like PCs and presents a unique behavior regarding the sequence specificity, ability to self-activate by intracellular proteolytic activity, and the lack of substrates, other than itself. After autocatalytic cleavage, the prodomain acts as a chaperone and remains attached to the catalytic site, preventing further enzymatic activity. This PC is also unusual in its crucial role in the regulation of cholesterol levels, its impact on hypercholesterolemia, sepsis, and inflammation. PCSK9 is mainly expressed in the liver, kidney, and small intestine (Seidah and Prat 2012).

Several protein-based and small molecule inhibitors have been designed in order to block the active site of these enzymes. Interference with the stability of furin mRNA, utilizing small hairpin RNA, constitutes the bases of the clinical trial

FANG-VIGIL to prevent furin translation and furthering cancer cell sensitivity to the host's immune system. Other strategies, including nanobodies, have been assayed to abolish PC activities targeting not the active site, but the P domain, necessary for stability (Klein-Szanto and Bassi 2017). Undoubtedly, the development of PCSK9 inhibitors to facilitate the LDLR internalization and cleavage has changed the therapeutic horizons for the treatment of hypercholesterolemia. Two PCSK9 inhibitors are commercially available and used successfully in patients who are refractory to statins or presenting with LDLR mutations in some cases of familial hypercholesterolemia.

PCs and Cardiovascular Diseases

PCs have been implicated in several cardiovascular diseases, such as coronary artery disease (CAD), atherosclerosis, and hyperlipidemia. Furin is associated with the stimulation of several pathways associated with CAD, and its expression increases with the severity of vascular and cardiac valves' lesions. Furthermore, heightened furin expression correlates with negative cardiovascular manifestations observed in patients with type II diabetes (Yakala et al. 2019).

On the other hand, PCSK6 (PACE4) contributes to lower blood pressure. This PC has been identified as the protease that activates the protein Corin, a membrane protease synthesized as a zymogen in the cardiomyocytes. Proteolytic activity of Corin leads to the activation of Atrial Natriuretic Peptide, a key regulator of blood pressure, stimulating diuresis and salt elimination (CHen et al. 2015). PCSK6 inhibition leads to salt-dependent hypertension, precluding it from consideration as a therapeutic target for inhibition. Nevertheless, PCSK6 negative association with hypertension may stimulate new strategies aiming at stabilizing this protein, increasing its expression in cardiomyocytes, or increasing its activity. The design of small molecule agonists specific for this PC may not be out of consideration.

Hypercholesterolemia constitutes a risk factor for the development of atherosclerotic cardiovascular disease (ACVD). Statins are the gold standard for the prevention of ACVD and the treatment of hypercholesterolemia. However, these compounds present serious side effects and may not be well tolerated by a subset of patients. In this context, inhibition of PCSK9 proved to be a successful strategy to decrease the levels of circulating cholesterol associated with low density lipoproteins (LDL-C). Excess of circulating LDL-C binds to its receptor (LDLR) on the surface of cells, prompting their internalization and recycling to the plasma membrane to continue the clearance of LDL-C from circulation. In the presence of PCSK-9, the LDLR remains tightly attached to this PC, not only preventing its recycling, and hence, clearance of LDL-C, but also carrying this receptor to the lysosomes. Once in the lysosomes, LDLR is degraded. PCSK9 inhibitors interfere with this association, stimulating LDLR-mediated internalization of cholesterol and recycling. Two monoclonal antibodies, evolocumab and alirocumab, are already in the market to treat cases of hypercholesterolemia refractory to statins (Seidah 2016).

PCs and Cancer

PCs overexpression has been documented in several cancers including breast, lung, head and neck, ovarian, endometrial, prostate, gastric and colon, among others (Jaaks and Bernasconi 2017). Increased expression of these PCs results in the acquisition of cancerous properties, such as increased proliferation, migration, invasion, and metastasis. PCSK3, PCSK 6, and PCSK5 are the major PCs implicated in cancer.

Inhibition of these PCs in vitro and in vivo decreases the maturation of several substrates necessary for cancer cell proliferation, invasion, and metastasis, including growth factors and their receptors (proliferation, angiogenesis, and lymphangiogenesis), matrix metalloproteinases (invasion and metastasis), and cell adhesion molecules (invasion) (Klein-Szanto and Bassi 2017).

PCs and Viral/Bacterial Infections

Several bacteria produce diseases through the action of exotoxins, extracellular proteins secreted by pathogenic bacteria, capable of producing tissue-specific damage. Most of these exotoxins consist of a catalytically active

A subunit and a membrane-bond B subunit which mediates the attachment and translocation of the A subunit. Once on the surface or in the endoplasmic reticulum of the target cell, the A subunit needs to be separated by limited proteolysis form the B subunit, a process catalyzed by Furin. Free exotoxin A is transported to the nucleus, where it stimulates the transcription of genes, resulting in stalling protein synthesis. Furin activates several exotoxins, including Diphtheria toxin, Pseudomonal exotoxin A, Shiga-like toxins, and anthrax toxins (Braun and Sauter 2019).

Proteolytic activity represents a crucial step for viral maturation, attachment, fusion, infection, and release from the infected cells. Proteins in the envelope or capsid of several viruses, including Papilloma-, Herpes-, Flavi-, Corona-, Paramyxo- (Braun and Sauter 2019), and Retroviruses, contain sequences recognized by PCs. PCs, such as furin, interact with viral proteins during their transit through the secretory pathway, whereas other viral proteins are processed extracellularly. Upon cleavage, these viruses enhanced their pathogenicity and their success in infecting other cells (Sathananthavathi et al. 2019; Izaguirre 2019).

Drugs

PCs play pivotal roles in several physiological and pathological processes. It is not surprising that several strategies came out to inhibit their activity for research and clinical purposes. As the first subfamily of PCs characterized, the kexin-like PCs were extensively studied as targets for inhibition. However, two inhibitors for PCSK9 have completed all the stages of basic and clinical investigation and are now currently used in the treatment of hyperlipidemia.

Inhibitors of Kexin-Like PCs

Small Molecule Inhibitors

These agents usually contain a cleavage site for PCs, RR/KXR, blocking the active site, hence functioning as competitive inhibitors. The high polarity of these molecules and, in some agents, its toxicity, limits their application to research. Examples of these agents include chloromethylketones, ploy-D-arginines, and streptamine derivatives. However, most of these compounds exhibit a K_i in the micromolar range, suggesting their possible future application (Klein-Szanto and Bassi 2017).

One of the first small molecule inhibitors developed was decanoyl-RVKR chloromethyl ketone (Dec-RVKR-CMK) (Angliker 1995). This inhibitor contains the recognition sequence for the kexin-like PCs (RXR/KR) with the C-terminal substitution of a chloromethyl residue that makes this inhibition irreversible by covalent attachment to the PC. In addition, the decanoyl moiety increases the cell permeability for this highly charged tetra peptide. Although this compound is toxic and does not have major selectivity for the different PCs, it represents an invaluable tool for research in the field.

The neuroendocrine PCs, PCSK1 and 2, have been implicated in glucose metabolism, PCSK1 and, in less measure, PCSK 2 activate insulin in the pancreatic β-cells, whereas PCSK2 converts inactive pro-glucagon to glucagon in α cells. A small allosteric inhibitor, RJC00847, has been developed that selectively inhibits PCSK2, but not PCSK1. This inhibitor may represent a lead to develop new strategies to treat some cases of hyperglycemia.

Peptides

Drugs with the active site for the PCs containing a protein scaffold proved to be effective (Ki in the nanomolar order) and less toxic than the small molecules inhibitors. However, their high molecular weight has limited the internalization into the cells. Specific pro-domains from PCs and derivatives of the α1-antitrypsin are representatives of this type of strategies (Jean et al. 1998).

PCSK9 Inhibitors

The developments occurring in the field of inhibition of kexin-like PCs translated into the search for PCSK9 inhibitors. Surprisingly the synthesis and production of anti-PCSK9 antibodies that

Proprotein Convertases, Table 1 Main inhibitors for PCs, their pharmacological mechanism of actions and main uses

Drug	PC inhibited	Mechanism	Clinical/potential uses	Reference
RJC00847	PCSK2	Allosteric inhibition	Hyperglycemia	Yongye et al. (2013)
Dec-RVKR-CMK	Kexin-like PCs	Irreversibly block active site	Research	Angliker (1995)
Antitrypsin Portland	Furin, PC5	Block active site	Viral infections, bacterial, cancer	Jean et al. (1998)
Streptamine and bisguanidines	Furin	Block active site	Anthrax and viral infections	Ramos-Molina et al. (2015)
Evolocumab	PCSK9	Blocking monoclonal antibody	Hypercholesterolemia	Dias et al. (2012)
Alirocumab	PCSK9	Blocking monoclonal antibody	Hypercholesterolemia	Stein et al. (2012)
Piperidine carboxamides	PCSK9	Translation inhibitor	Hypercholesterolemia	Londregan et al. (2018)
Inclisiran	PCSK9	siRNA	Hypercholesterolemia, dyslipidemia	Nishikido and Ray (2018)

completely blocked its functions represented the most far-reaching approach in blocking PC activity. Two antibodies are commercial and currently used in the treatment of hypercholesterolemia with excellent results.

Evolocumab and Alirocumab

Monoclonal antibodies Evolocumab (Rephata) and Alirocumab (Praloent) (Stein et al. 2012) specifically and effectively bind to PCSK9 in the extracellular milieu preventing its binding to the LDLR, increasing LDLR recycling to the membrane, enhancing LDL-C clearance. These drugs are administered as intramuscular injections once or twice a month. Concomitant treatment with statins these antibodies showed a significant reduction in the circulating levels of LDL-C (Stein et al. 2012).

Translator Inhibitors

A new family of drugs, the N-(piperidin-3-yl)-N-(pyridin-2-yl) piperidine/piperazine-1-carboxamides ("piperidine carboxamides") selectively blocks the translation of PCSK9 by preventing the binding of ribosomes to this PCs' mRNA. These new small molecule compounds are formulated as prodrugs, which achieve full activity in the liver, where most PCSK9 is produced. Some of these compounds showed an advance in safety, stability, and potency properties compared with other drugs currently in use. This is a novel approach and may open new venues in the field of small molecule inhibitors (Londregan et al. 2018).

Small Interference RNA

A clinical trial to silence the expression of PCSK9 using injectable siRNA is underway. The drug, Inclisiran, degraded the cellular PCSK9 mRNA, preventing protein synthesis. The side effects seem to be very benign. Furthermore, the drug would require two or three applications per year only, probably reducing the high costs associated with the monoclonal antibodies (Nishikido and Ray 2018) (Table 1).

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Proprotein-Convertases Subtilysin/Hexin Type 1-9 (PCSK 1-9)

► Proprotein Convertases

Pro-resolving Mediators

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Synonyms

Immunoresolvents; Specialized pro-resolving lipid mediators; SPMs

Definition

Pro-resolving mediators are endogenous signaling molecules that act as "stop signals" to terminate an inflammatory reaction when no longer needed to promote the return to a normal tissue state (Serhan 2014). This resolution of inflammation is achieved through both anti-inflammation and an active pro-resolution. The anti-inflammatory actions during the resolution of inflammation include counter-regulating pro-inflammatory signals and limiting immune cell infiltration. At the same time, active pro-resolving actions lead to the apoptosis and efflux of inflammatory cells and the promotion of macrophage uptake mechanisms to clean up the site of inflammation. Hence, anti-inflammation and pro-resolution are not equivalent processes. While both processes aim to limit inflammation, the pro-resolving mediators activate specific receptors to promote processes that facilitate tissue repair and return to homeostasis. Pro-resolving mediators hence act to reprogram the inflammatory response and actively promote tissue repair.

Basic Characteristics

Inflammation is a protective response mounted by the host in response to infection and tissue injury and is normally self-limiting, that is, inflammation

resolves to bring the tissue back to homeostasis. Classically an inflammatory response is hence divided into two phases, the initiation and the resolution phase. In fact, chemical signals produced during the early initiation phase play a pivotal role in turning on the subsequent resolution response (Sugimoto et al. 2016). However, in the case of a failure in the resolution of inflammation, the "stop signals" will lack and the inflammation turns into a chronic response (Bäck et al. 2019).

Pro-resolving mediators exhibit key characteristics allowing them to turn off the inflammatory reaction and subsequently turn on the "cleanup" phase required to return to homeostasis and regain physiological function of the tissue. These characteristics include (1) inhibiting neutrophil trafficking, (2) promoting macrophage differentiation towards an alternatively activated "repair" phenotype (i.e., M1 to M2 switching), (3) promoting neutrophil apoptosis, (4) enhancing phagocytosis of apoptotic cells (efferocytosis) and microbes, and (5) activating structural cells to promote tissue regeneration and repair (Sugimoto et al. 2016). In addition to these fundamental properties, each individual mediator may have other specific actions that are receptor-, cell-, location-, and time-specific.

The mediators involved in resolution of inflammation are a growing class and comprise several chemically distinct molecules that include bioactive lipid mediators as well as resolver proteins and peptides. Gaseous molecules, autacoids like purine, and neuromodulators released by the vagus nerve (Dalli et al. 2017) have also emerged as players in the resolution response.

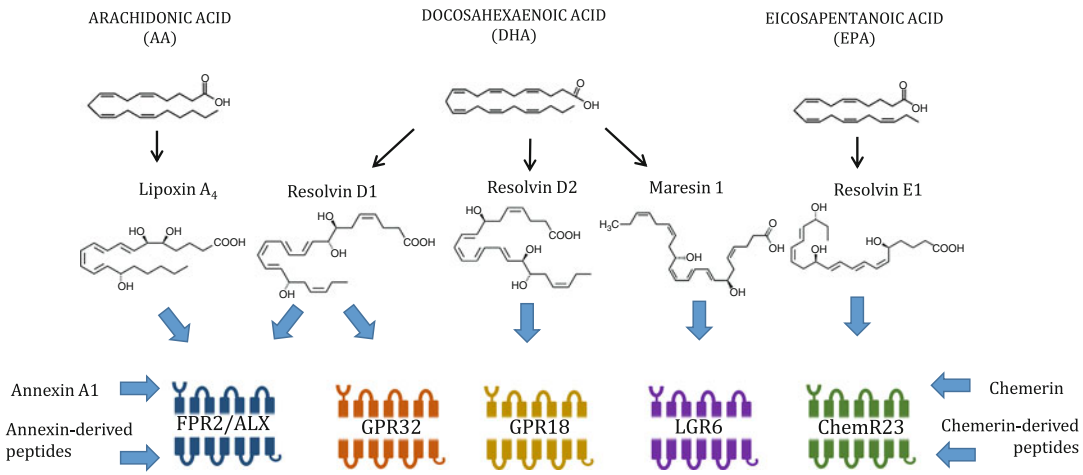
Specialized Pro-resolving Lipid Mediators

The term specialized pro-resolving lipid mediators (SPMs) has been introduced to denote a specific class of pro-resolving mediators formed by enzymatic conversion of polyunsaturated fatty acids (PUFAs). SPMs are comprised of several structurally distinct families, which include the lipoxins from the omega-6 metabolome and resolvins, protectins, and maresins from the omega-3 metabolomes.

Lipid mediators are tightly linked to the initiation, maintenance, and resolution of inflammation. During an inflammatory response, bioactive lipid mediators are produced by variety of cell types, where innate immune cells like macrophages and neutrophils are considered to be their primary source. First, prostaglandins and leukotrienes are produced from the omega-6 PUFA arachidonic acid (AA) by cyclooxygenases (COX) and 5-lipoxygenase (5-LO), respectively. These pro-inflammatory lipid mediators promote the recruitment and activation of immune cells at the inflammatory site to regulate and amplify the inflammatory cascade. Subsequently, AA metabolism is skewed towards production of lipoxins (Fig. 1) via two sequential lipoxygenase steps. This lipid mediator class switch involves upregulation of 15-LO (a key biosynthetic enzyme necessary for the production of lipoxins and other SPMs), transcellular biosynthesis (in particular neutrophil-platelet interactions involving platelet 12-LO), and a change of subcellular 5-LO activity. Whereas 5-LO located on the nuclear membrane primarily converts AA to the pro-inflammatory leukotriene B_4 (LTB_4), a cytoplasmic localization of 5-LO may favor the biosynthesis towards lipoxin A_4 (LXA_4). Furthermore, SPMs can inhibit the cellular translocation of 5-LO to the nucleus, resulting in a pro-resolving amplification loop (Fredman et al. 2014).

In addition to changes in LO metabolism, also an altered COX metabolism will take part in the lipid mediator switch towards SPMs. Of note, aspirin acetylation of COX-2 not only blocks the formation of prostaglandins but also alters the COX-2 enzymatic activity towards intermediates that serve as substrates for stereoisomers of lipoxins, referred to aspirin-triggered lipoxins (ATL). The ATLs share their biological activity with their isomeric counterparts but are more resistant to metabolic inactivation.

Resolvins, protectins, and maresins are synthesized from omega-3 PUFAs (Fig. 1). Resolvins derived from eicosapentaenoic acid (EPA) are indexed with an E (e.g., RvE1; Fig. 1) and are



Pro-resolving Mediators, Fig. 1 Pro-resolving mediators and their receptors. Lipoxin A_4 is derived from arachidonic acid, whereas D- and E-series resolvins are formed from metabolism of the omega-3 polyunsaturated fatty acids docosahexanoic acid (*DHA*) and eicosapentanoic acid (*EPA*), respectively. The figure depicts five receptors for pro-resolving mediator receptors; ALX/FPR2 for lipoxin A_4, D-resolvins, and annexins, GPR32 and GPR18 for D-resolvins, LGR6 for maresin 1, and ChemR23 for E-resolvins and chemerins. (Adapted from Pirault and Bäck 2018)

formed through initial production of 18R-hydroxy-EPA (18-HEPE) by acetylated COX-2 or cytochrome p450, which is then further converted to RvE1 or RvE2 via 5-LO or to RvE3. Docosahexaenoic acid (DHA) is converted to D-series resolvins (RvD1 through RvD6), protectins, and maresins. The metabolism of DHA into SPMs is initiated by 15-LO and then leads to either RvDs via subsequent 5-LO conversion or protectins via its epoxide intermediate. Similar to the lipoxins, D-series resolvins and protectins also include metabolically more stable aspirin-triggered stereoisomers. On the other hand, maresins (e.g., MaR1; Fig. 1) are produced via 12-LO metabolism of DHA.

More recently, peptide-containing SPMs originating from DHA have also recently be uncovered to specifically activate tissue regeneration. These SPMs have been collectively denoted Conjugates in Tissue Regeneration (CTRs) and denoted MCTR, PCTR, and RCTR according to their basic structure being maresin, protectin, or resolvin, respectively (Dalli et al. 2017). Finally, SPM biosynthesis from the omega-3 PUFA docosapentenoic acid (DPA) was discovered. These pro-resolving mediators are produced through similar enzymatic pathway as their DHA counterparts and have consequently been denoted $\text{resolvins}_{\text{n3DPA}}$, $\text{protectins}_{\text{n3DPA}}$, and $\text{maresins}_{\text{n3DPA}}$ (Hansen et al. 2018).

Protein and Peptide Pro-resolving Mediators

In addition to lipid-derived SPMs, several proteins and peptides have emerged as pro-resolving mediators. In particular, annexin A1 (AnxA1) is a 37 kDa protein-induced protein, which is induced by glucocorticoids to mediate anti-inflammatory effects by inhibiting prostaglandin and leukotriene formation through blocking of phospholipase A2 (Perretti and D'acquisto 2009). However, AnxA1's biological activity, as well as that of bioactive peptides that comprise its N-terminal region, has now been extended to promote neutrophil apoptosis and macrophage efferocytosis, contributing to tissue repair while also limiting neutrophil recruitment in vivo (Perretti and D'acquisto 2009), hence fulfilling the abovementioned characteristics of a pro-resolving mediator. Other protein and peptide mediators considered to promote resolution include IL-10 (Bäck et al. 2019), chemerin (Carracedo et al. 2019b), and chemerin-derived peptides, as well

as melanocortins, adrenocorticotropic hormone (ACTH), and galectins.

Pro-resolving Receptor Signaling

The G-protein-coupled receptors (GPRs) identified for pro-resolving mediators represent phylogenetically different families, of which most belong to classes still officially annotated as orphan receptors (Table 1). Apart from the D-series resolvins and LXA_4 being the hitherto sole agonists paired with GPR32 (Pirault and Bäck 2018), the pro-resolving mediators share other GPRs with diverse agonists having a number of more or less specific biological actions. Finally, the molecular structural similarity of the SPMs with pro-inflammatory lipid mediators has particular implication for their receptor signaling, as exemplified by the inhibition of LTB_4 responses by means of RvE1 acting as a partial agonist at the BLT1 receptor (Bäck et al. 2014).

As illustrated in Table 1, the GPRs for pro-resolving mediators represent classes of receptors associated with inflammation (formyl peptide receptors (FPR), chemerin receptors), regeneration (leucine-rich repeat-containing GPRs; LRG), and pain (cannabinoid signaling), all of which are strongly linked to the pro-resolving response. This promiscuity of the GPRs towards their ligands also implicates complex biological agonist interactions. The different ligands may signal differential and even opposite downstream responses through the same receptors by means of, for example, specific receptor binding sites, allosteric modulation, partial agonism/antagonism, conformational changes, and receptor dimerization (Bäck et al. 2014).

Pro-resolving Mediators, Table 1 Receptors for pro-resolving mediators

Receptor family	Receptor	Agonists
Formyl peptide receptors (FPR)	FPR2/ALX	LXA4, ATL, D-series resolvins
		Annexin A1 and annexin-derived peptides
Chemerin receptors	ChemR23	RvE1
		Chemerin and chemerin-derived peptides
Orphan receptors	GPR32	D-series resolvins, LXA4
Orphan receptors, similar to cannabinoid receptors	GPR18	RvD2
		Anandamide
Orphan receptors; leucine-rich repeat-containing GPRs (LGR)	LGR6	MaR1
		R-spondin 1-4

Abbreviations: *GPR* G-protein-coupled receptor, *LXA4* lipoxin A4, *ATL* aspirin-triggered lipoxin, *RvE1* resolvin E1, *RvD2* resolvin D2, *MaR1* maresin 1

Pro-resolving lipoxins, resolvins, and annexins share the FPR2/ALX receptor with pro-inflammatory amyloidogenic and bacterial peptides. Since the downstream receptor signaling differs between different classes of FPR2/ALX agonists (Hanson et al. 2013), the tissue ligand concentrations may determine receptor activation to either resolve or enhance a local inflammatory response (Petri et al. 2015). Another GPR for pro-resolving mediators closely linked to an inflammatory modulation is the chemokine-like receptor ChemR23, also referred to as chemerin receptor 1 and resolvin E1 receptor ERV1 (Laguna-Fernandez et al. 2018). Although chemerin in general is considered to be a pro-inflammatory ligand, chemerin and certain chemerin-derived peptides transduce similar pro-resolving signaling patterns as RvE1 through this receptor (Carracedo et al. 2019b). Lack of ChemR23 increases the pro-inflammatory state of macrophages and abrogates RvE1-induced phagocytosis (Laguna-Fernandez et al. 2018), supporting that ChemR23 primarily acts to resolve inflammation. Nevertheless, in vivo models of genetic ChemR23 deletion have generated contradictory results on inflammatory and metabolic phenotypes, hence reinforcing the complex interaction between signaling of inflammation and its resolution through the same receptor (Carracedo et al. 2019a).

As mentioned above, pro-resolving mediators mediate antinociception, and it is therefore interesting that RvD2 shares the orphan GPR18 with some of the endocannabinoids. In addition to possible antinociceptive effects, GPR18 also transduces RvD2-induced effects in macrophages,

including M2 differentiation and reduced inflammasome activation. The beneficial effects of RvD2 are largely lost in mice lacking GPR18, supporting that GPR18 is directly involved in the pro-resolving response to RvD2 (Pirault and Bäck 2018).

The most recently identified GPR for pro-resolving mediators is LGR6 from the receptor family for R-spondins, which are regulators of the Wnt/β-catenin signaling pathway and mediate regenerative responses. The intracellular signaling response and the phagocytic activity of the pro-resolving mediator MaR1 were enhanced by LGR6 overexpression and diminished by LGR6 knockdown in vitro (Chiang et al. 2019*)*, but the in vivo consequences for of MaR1 signaling through LGR6 remain to be explored.

Taken together, instead of signaling through a common receptor family, the cellular effects of pro-resolving mediators are hence transduced through several GPRs, representing diverse phylogenic and functional classes. The differential and in part opposing downstream signaling of different ligands at these receptors adds another complexity to the overall effects of the pro-resolving mediators, which may be highly dependent on the conditions and the models used to study their effects. This should be taken into consideration when interpreting the somewhat contradictory findings reported from receptor knockout studies and ligand-receptor interaction studies.

Gaseous Pro-resolving Signaling

In addition to pro-resolving mediators of lipid and protein/peptide structures signaling through GPRs, certain gaseous molecules dissolved in extracellular fluids can diffuse over cell membranes to directly affect cellular function to promote the resolution of inflammation (Wallace et al. 2015). In particular, nitric oxide (NO) in addition to its vasoactive effects also serves to regulate immune cells and increase apoptosis of inflammatory cells. Further, hydrogen sulfide (H_2S) alters, for example, intracellular calcium and cAMP and has been shown to reduce inflammatory activation of macrophages and increase efferocytosis (Wallace et al. 2015). Finally. macrophage expression of heme oxygenase-1 (HO-1) is a source of carbon monoxide (CO), which may contribute to limiting leukocyte infiltration, increasing phagocytosis, and suppressing cytokine production (Wallace et al. 2015). In addition, an increase in SPMs has been observed after CO exposure (Chiang et al. 2013), indicating that pro-resolving signaling pathways are interdependent (Bäck et al. 2019).

Pathophysiology and Therapeutic Potential

It is now appreciated that excessive or uncontrolled inflammation is an underlying component of many widely occurring pathologies and diseases. Evidence that such chronic inflammation is associated with a failure in the resolution of inflammation has emerged from studies in, for example, cardiovascular diseases (Bäck et al. 2019; Carracedo et al. 2019a), arthritis (Arnardottir et al. 2016), obesity, periodontal disease, and aging (Arnardottir et al. 2014). Emerging evidence from both human and animal studies suggests that this underlying failed or impaired resolution mechanism may, at least in part, reflect a reduced production of pro-resolving mediators and/or reduced expression or signaling through pro-resolving receptors. Indeed, disturbed balance between pro-resolving and pro-inflammatory mediators is emerging as a potential maker of non-resolving inflammation (Thul et al. 2017).

Pathophysiology and Therapeutic Potential

While current therapeutic approaches treating inflammation by focusing on suppressing or inhibiting pro-inflammatory mediators are effective in relieving the gross signs and symptoms, side effects in terms of possible immunosuppression and increased risk of infections should be considered. Therefore, triggering pro-resolving mediators to prevent the transition into chronic

inflammation without blunting the acute response to injury and infection has the potential to resolve inflammation without causing immunosuppression. Such therapeutic strategies include targeted delivery of pro-resolving mediators and omega-3 PUFA supplementation to enhance SPM formation. Defining appropriate dosage and formulation in clinical trials will be crucial to define the therapeutic potential of pro-resolving mediators.

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Prostaglandin G/H Synthases

► Cyclooxygenases

Prostaglandin-Endoperoxide H Synthases (PGHSs)

► Cyclooxygenases

Prostanoids

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Definition

Prostanoids are a group of biologically active lipid mediators. The group consists of prostaglandins and thromboxane A_2. Prostanoids are not stored in cells. Instead, they are formed ad hoc in the cell via several enzymatic steps after exposure to physical, chemical, or hormonal stimuli and are then released. They play an important role in nearly every tissue and are critically involved in, e.g., inflammatory and hemostatic processes as well as in the regulation of smooth muscle tone. Because of their diverse physiological functions, prostanoids are also of pharmacological relevance, and many important drug classes, in particular nonsteroidal antiphlogistics as well as most non-opioid analgesics, act through inhibition of prostanoid formation.

Basic Characterization

Biosynthesis and Degradation

The decisive step in the biosynthesis of prostanoids is the release of the substrate arachidonic acid by phospholipids of the cell membrane. The formation of arachidonic acid is mainly catalyzed by the cytosolic phospholipase A_2, which translocates to the cell membrane after increase in intracellular Ca^{2+} concentrations. Apart from $cPLA_2$, which is important for the acute release of arachidonic acid, several other PLA_2 isoforms are, in part, induced by chronic cell activation. Arachidonic acid released by PLA_2 is relatively quickly metabolized to prostanoids by cyclooxygenases. In general, the activation of PLA_2-dependent release of arachidonic acid is the rate-limiting step of prostanoid biosynthesis.

Formation of Prostanoids

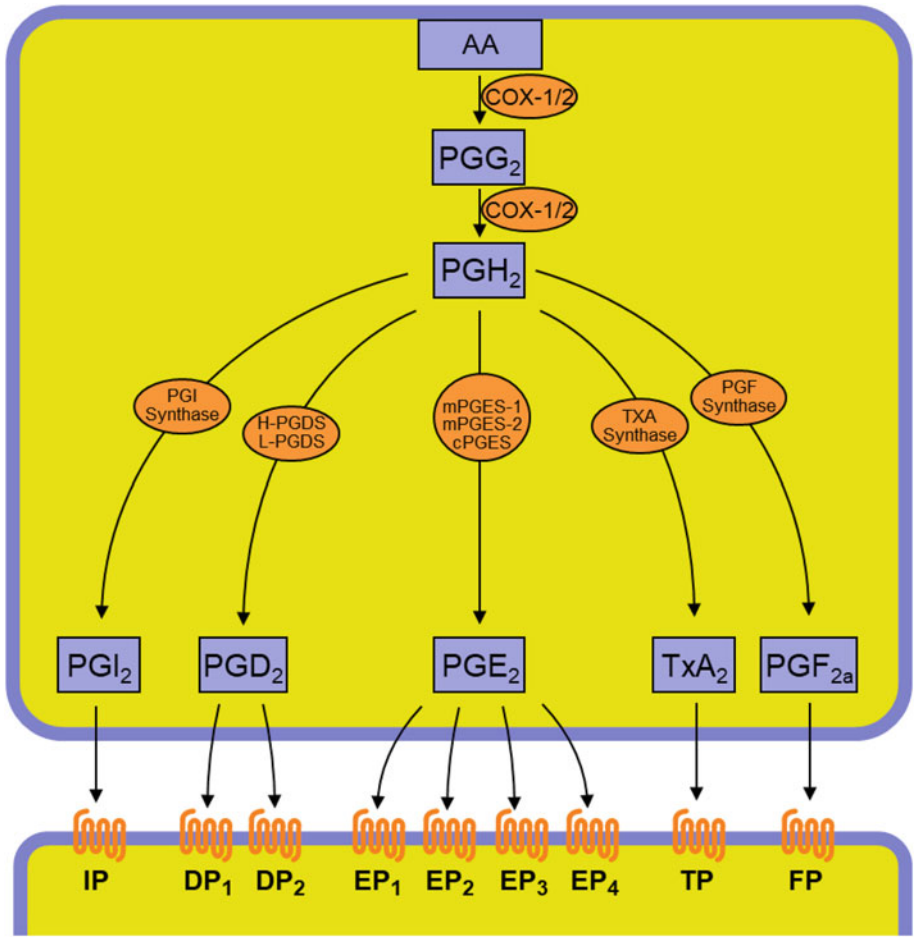
The synthesis of prostanoids from arachidonic acid is initially mediated by cyclooxygenase (COX), which, via the cyclic endoperoxide prostaglandin G_2 (PGG_2), turns arachidonic acid into the progenitor of all prostanoids, prostaglandin H_2 (Fig. 1).

Cyclooxygenase has two isoforms: COX-1 and COX-2. While COX-1 is constitutively expressed in most cells, the expression of COX-2 is induced by various humoral and mechanical factors. COX-2 is also constitutively expressed in different areas of the kidney and the brain as well as in the endothelium of blood vessels.

Cyclooxygenases form PGH_2, which, via different isomerases and synthases, is then further metabolized into the biologically active prostanoids prostaglandin E_2 (PGE_2), prostaglandin I_2 (prostacyclin, PGI_2), prostaglandin D_2 (PGD_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and thromboxane A_2 (TXA_2). Which prostanoids a cell synthetizes depends on the expression of the respective prostanoid synthases.

Many cells are capable of forming PGE_2. Three different PGE_2 synthases are known: two membrane PGE_2 synthases (mPGES-1 and mPGES-2) as well as one cytosolic synthase (cPGES). Similar to COX-2, the PGE_2 synthase mPGES-1 is induced by various stimuli in different tissues, and, in most instances, its presence is associated with COX-2. In contrast to mPGES-1, cPGES and mPGES-2 are constitutively expressed in most tissues and are not primarily coupled to COX-2. Especially cPGES metabolizes mainly PGH_2 generated by COX-1.

PGD_2 formation is induced by two different PGD_2 synthases: As its name already suggests, the hematopoietic prostaglandin D_2 synthase (H-PGDS) is mainly found in the hematopoietic system as well as in the immune system and has been shown to occur in mast cells, TH2 cells, as well as microglia. The so-called lipocalin-type prostaglandin D_2 synthase (L-PGDS) is expressed in the central nervous system and in the heart and testis.



Prostanoids, Fig. 1 Synthesis of several biologically active prostanoids and their receptor targets

According to the current state of knowledge, $PGF_{2\alpha}$, TXA_2, and PGI_2 are each formed by a specific synthase. While TXA_2 and $PGF_{2\alpha}$ synthases are mainly associated with COX-1, the PGI_2 synthase appears to often be co-expressed with COX-2.

Degradation of Prostanoids

Most prostanoids are inactivated rather quickly after their formation, typically within seconds or minutes, and that fact restricts their impact on their immediate site of formation. Some prostanoids, like TXA_2, degrade spontaneously with a half-life of 30 s, while others are broken down very fast by specific enzymes. Prostanoids entering the systemic circulation will get almost completely inactivated during the first passage of the pulmonary circulation.

Effects of Prostanoids

Prostanoids mainly mediate their manifold effects by activating G-protein-coupled receptors (Hirata and Narumiya, 2011; Biringer, 2021; Woodward et al., 2011). While prostanoids like TXA_2 and PGI_2 act through only one receptor, other prostanoids, e.g., PGE_2, have several receptor types for the activation of different G-proteins and secondary signaling transduction pathways (see Table 1).

PGE_2

In most areas of the vascular system, PGE_2 exerts vasodilatory effects by activating G_s-coupled EP_2 and EP_4 receptors. COX-2-dependently formed PGE_2 also plays a special role in

Prostanoids, Table 1 Formation sites, receptors, and effects of some biologically active eicosanoids

Prostanoid	Formation site(s)	Receptor	Expression	Effector	Effects (examples)
Thromboxane A_2 (TXA_2)	Platelets, macrophages	TP	Platelets, smooth muscle	Gq/11/ PLC↑ G12/13, RhoA	Activation of platelets, smooth muscle tone ↑
Prostacyclin (PGI_2)	Vascular endothelium	IP	Platelets, smooth muscle, nociceptive neurons	Gs/AC↑	Inhibition of platelets, smooth muscle tone ↓
Prostaglandin E_2 (PGE_2)	Widely spread	EP_1	Kidney, lung, stomach	Gq/11/ PLC↑	Smooth muscle tone↑, gastric HCO_3 secretion ↑
		EP_2	Uterus, vessels	Gs/AC↑	Vasodilation
		EP_3	Widely spread	Depending on splice variant: Gs/AC↑ Gi/AC↓ $Gq_{/11}$/ PLC↑	Mediating fever reaction, increasing uterus tone, duodenal HCO_3 secretion↑
		EP_4	Widely spread	Gs/AC↑	Keeping open the ductus arteriosus Botalli
Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)	For example, uterus	FP	Smooth muscle, corpus luteum	Gq/11/ PLC↑	Smooth muscle tone ↑
Prostaglandin D_2 (PGD_2)	Mast cells, brain	DP_1	Vessels	Gs/AC↑	Smooth muscle tone ↓
		DP_2	Monocytes, basophils, eosinophils	Gi/AC↓, PLC↑	Chemotaxis

PLC phospholipase C-β; *AC* adenylyl cyclase

keeping open the ductus arteriosus Botalli before birth by activating EP_4 receptors.

PGE_2 is one of the key mediators in acute local inflammatory responses that cause local vasodilation as well as edema formation (Dennis and Norris, 2015; Ricciotti and FitzGerald, 2011; Wang and DuBois, 2018). During inflammatory responses, PGE_2 also sensitizes peripheral nerve endings and has pronociceptive effects on the dorsal horn of the spinal cord. In addition, PGE_2 is the central mediator of fever reaction and, by activating EP_3 receptors in the hypothalamus, leads to changes in the set point of the body temperature.

PGE_2 has inhibitory effects on the differentiation of B-lymphocytes into antibody-producing plasma cells, and it blocks mitogen-induced proliferation of T-lymphocytes.

While leading to contractions of the uterine musculature in the pregnant myometrium, PGE_2 exerts relaxing effects on the nonpregnant uterus.

Together with PGI_2, PGE_2 plays an important role in maintaining the physiological functions of the mucosal membrane in the gastrointestinal tract. PGE_2 has cytoprotective effects, in particular on the gastric mucosa, as it promotes the formation of mucus and bicarbonate as well as encourages the blood flow while at the same time inhibiting the secretion of H^+ ions.

Regarding the kidney, prostanoids such as PGE_2 and PGI_2 have various functions:

- PGE_2 inhibits water resorption through ADH and promotes renal blood flow.

- Together with PGI_2, PGE_2 is also involved in the stimulation of renin release in the context of the tubulo-glomerular feedback mechanism.

TXA_2 and PGI_2

TXA_2 and PGI_2 are antagonistic mediators in the vessel wall.

TXA_2 formation depends on COX-1. It is produced by platelets after activation and leads to vasoconstriction as well as to increased platelet activation. In contrast to TXA_2, the formation of PGI_2 is COX-2-dependent and induced by endothelial cells under the influence of the shear forces of the flowing blood. It counteracts TXA_2 effects by relaxing vascular smooth muscle and inhibiting platelet function. Apart from vascular smooth muscle cells, TXA_2 contracts and PGI_2 relaxes also bronchial and uterine smooth muscle.

Similar to PGE_2, PGI_2 stimulates circulation in the kidney and is involved in the release of renin from cells of the juxtaglomerular apparatus.

Again similar to PGE_2, PGI_2 also plays an important role in acute local inflammatory processes by locally inducing vasodilation and edema formation as well as by sensitizing peripheral nociceptive nerve endings.

PGD_2

PGD_2 is formed and released by, e.g., mast cells. It has vasodilatory effects and inhibits platelet functions. Together with leukotrienes, PGD_2 also induces bronchoconstriction. There are reports of chemotactic effects on eosinophils and TH2-lymphocytes. In the central nervous system, PGD_2 is believed to play a role in sleep induction.

$PGF_{2\alpha}$

$PGF_{2\alpha}$ is formed in the uterus where it induces strong contractions in the normal as well as in the pregnant myometrium. $PGF_{2\alpha}$ also leads to the contraction of smooth muscle cells in the vascular system of pulmonary arteries and veins.

As for the eye, $PGF_{2\alpha}$-mediated contraction of the trabecular network results in improved chamber drainage and, consequently, in reduced intraocular pressure.

Drugs

Due to the very diverse physiological and pathophysiological functions of eicosanoids, inhibition of their formation as well as of the effects they mediate constitute an effective therapeutic approach. In this context, nonsteroidal antiphlogistics are most significant, as they inhibit cyclooxygenases and thereby possess antiphlogistic, antipyretic, and analgesic characteristics, which are all important factors in the treatment of different types of pain and chronic inflammatory diseases. The cyclooxygenase blocker acetylsalicylic acid (aspirin), given at low doses, is an important antiplatelet therapeutic principle and the basis treatment for secondary prophylaxis in cardiovascular disease.

The direct application of prostanoid and prostanoid derivates can also be an effective treatment under certain conditions. However, their systemic administration is often afflicted by a relatively short effect duration and the occurrence of unwanted side effects.

Alprostadil (PGE_1) has strong vasodilatory effects and is injected intraarterially or intravenously when treating advanced stages of chronic arterial occlusion disease. Its vasodilatory effect is also exploited to treat erectile dysfunction. Here, PGE_1 is administered intracavernously or transurethrally. In recent years, however, this principle of action has been mostly substituted by PDE-5 inhibitors, which allow oral administration. PGE_1 can also be utilized to temporally keeping open the ductus arteriosus Botalli in newborn infants.

Epoprostenol, the sodium salt of PGI_2, the PGI_2 derivate iloprost, and the PGI_2-receptor (IP) agonist selexipag all possess strong vasodilatory effects and can be used to treat serious forms of primary pulmonary hypertension. Iloprost is also given in the treatment of advanced stages of thrombangiitis obliterans.

With regard to perinatal care, in particular PGE_2 (dinoprostone) and $PGF_{2\alpha}$ are utilized for their contractile effects in the pregnant uterus. Local administration of PGE_2 as vaginal gel or vaginal pill is used as birth-inducing measure. Uterine atony postnatally can be treated with $PGF_{2\alpha}$.

The PGE_1 derivates misoprostol and gemeprost as well as the PGE_2 derivate

sulprostone are used to induce abortion. Misoprostol affects the acid and mucus secretion in the gastrointestinal tract and can thereby be given together with COX inhibitors to prevent ulcer formation.

Various $PGF_{2\alpha}$ derivates, such as latanoprost, travoprost, tafluprost, or bimatoprost, are of ophthalmological relevance and are used to decrease the intraocular pressure in the treatment of primary open-angle glaucoma.

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Protease-Activated Receptors (PARs)

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Synonyms

Thrombin receptor

Definition

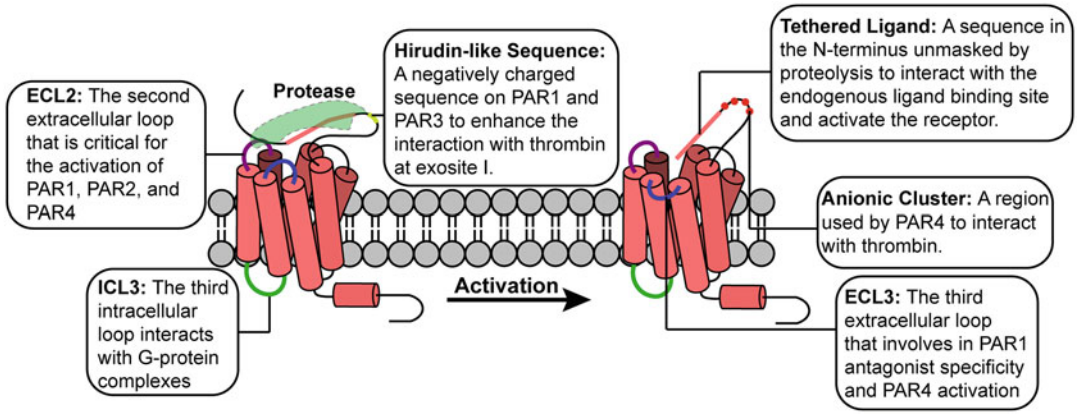
Protease activated receptors (PARs) are a unique set of G-protein coupled receptors (GPCRs) that play a significant role in hemostasis, vascular biology, inflammation, and cancer. There are four members of the PAR family, PAR1-4. PAR1 was discovered 30 years ago and was originally classified as the thrombin receptor on platelets (Vu et al. 1991). Soon after this original discovery, the other family members were identified with important signaling roles in cells such as fibroblasts, myocytes, platelets, neutrophils, and endothelial cells (Adams et al. 2011; Han and Nieman 2020). PARs signal through classical G-protein (Gαq, Gα12/13, and Gαi) and arrestin pathways depending on the cellular context. Understanding how PARs function at the molecular level can be instrumental in developing therapies that target this family of receptors. Here, we will describe unique features of PARs and the pharmacological approaches that have been used to inhibit or modulate PAR activity. By delving into the PAR antagonists that have been developed in preclinical studies, more can be learned to help develop therapies to put into clinical use.

Basic Characteristics

The Tethered Ligand Activation Mechanism

PARs are the primary means by which proteases mediate intracellular signaling. The activation of PARs occurs by cleavage of the N-terminus to produce a tethered ligand that intramolecularly binds to the ligand binding site (Han and Nieman 2020) (Fig. 1). This activation mechanism differs from other GPCRs that are typically activated by a free ligand. Since the sequence of the tethered ligand of each PAR is different, the shape of the endogenous ligand binding sites on the extracellular side of the receptors should also be distinct. The soluble activation peptides mimicking the tethered ligands generally do not cross react between PAR subtypes, which further supports unique binding sites. These data support the concept that it is possible to design peptide mimetics and small molecule antagonists to target each PAR specifically.

The endogenous ligand binding sites have not been fully defined, but there are common themes across PARs. The available structural data from PAR1 and PAR2 in complex with inhibitors do not



Protease-Activated Receptors (PARs), Fig. 1 General mechanism of the tethered ligand-mediated PAR activation. The regions that are critical for the receptor activation and downstream signal transduction are highlighted

reveal how the endogenous tethered ligand interacts with the receptor (Cheng et al. 2017; Zhang et al. 2012). However, experiments with selective point mutations show a key role for extracellular loop 2 (ECL2) in PAR1, PAR2, and PAR4. As additional tools and techniques become available, a more complex model for PAR activation is emerging. For example, hydrogen deuterium exchange studies recently demonstrated an essential role for ECL3 in PAR4 activation (Han et al. 2020a).

The list of proteases that activate PARs continues to grow (Zhao et al. 2014). These proteases rely on a variety of mechanisms such as exosites, cofactors, and membrane localization to ensure efficiency and specificity of substrate proteolysis. For example, PAR1 is an excellent thrombin substrate because it has a hirudin-like sequence that interacts with thrombin's exosite I to increase cleavage efficiency (Han et al. 2020b). In contrast, activated protein C (APC) requires the endothelial protein receptor (EPCR) as a cofactor for efficient cleavage of PAR1. In addition, proteases can have unique recognition sites on PARs that generate a panel of tethered ligands through distinct cleavage sites. This increases the diversity of signaling from individual PAR subtypes within a given cell through biased agonism (Zhao et al. 2014).

Due to the unique, irreversible activation mechanism of PARs, desensitization and internalization are key processes that terminate PAR signaling. Activated PARs do not recycle to the plasma membrane following internalization; they are sent to the lysosome for degradation. These termination events are dependent on G-protein coupled receptor kinase (GRKs), second messenger kinases, and β-arrestin (Grimsey et al. 2014).

Protease Activated Receptor Subtypes

In the 1980s, thrombin was a known potent agonist of platelets but its mechanism of platelet activation was still unknown (Han et al. 2019). In an effort to elucidate the mechanism of thrombin-mediated platelet activation, Vu and colleagues identified PAR1 as the "thrombin receptor" or coagulation factor 2 receptor (F2R) through expression cloning (Vu et al. 1991). Soon thereafter, the other family members were discovered. The amino acid sequence homology between PARs is 27–36% and is primarily in the conserved transmembrane domains similar to most GPCRs. The genes for human PAR1 (F2R), PAR2 (factor 2 receptor like 1 or F2RL1), and PAR3 (F2RL2) are located in a cluster on chromosome 5 (5q13) (Kahn et al. 1998). In contrast, the gene PAR4 (F2RL3) is a more distant relative and is located on chromosome 19p12 (Jin et al. 2016). Although PARs are widely expressed, there are important species differences that impact preclinical drug development (Han et al. 2020b).

PAR1

PAR1 is an efficient thrombin substrate due in large part to a hirudin-like sequence that interacts with thrombin's exosite I to facilitate cleavage of the PAR1 N-terminus at Arg^{41} (Han and Nieman 2020). Once activated by thrombin, PAR1 elicits a rapid signal that is quickly terminated. There is now a growing list of proteases that activate PAR1 including Activated Protein C (APC), Matrix metalloproteases (MMP1 and MMP13), proteinase 3, Cathepsin G, plasmin, elastase, Factor VIIa, and Factor Xa (Zhao et al. 2014). Some of these proteases cleave PAR1 at noncanonical sites, generating unique ligands that give rise to distinct downstream signaling. This is known as biased signaling and can lead to opposite cellular responses. For example, thrombin-mediated activation of PAR1 on endothelial cells results in increased permeability while activation by APC is barrier protective. Furthermore, MMP1 cleavage of PAR1 promotes RhoA activation and p38 phosphorylation (Austin et al. 2013). The diversity in signaling from PAR1 activation has been exploited with allosteric modulators and parmodulins to direct signaling through cytoprotective pathways in preclinical studies. These are discussed below.

PAR2

PAR2 was discovered in 1995 as a trypsin receptor and was the second PAR to be cloned (Yau et al. 2013). PAR2 is widely expressed on the surface of endothelial cells, epithelial cells, fibroblasts, nerves, immune, and inflammatory cells. The expression level of PAR2, however, varies depending on the specific cell type, disease conditions, or even environmental stimuli, such as LPS and dietary fats. Over the years, both the list of the proteases that activate PAR2 and its roles in physiological and pathological conditions continue to grow.

PAR3

The signaling roles of PAR3 remain controversial. In most cells, PAR3 does not signal on its own; it instead serves as a cofactor to modulate the activation and activity from other PARs (Han and Nieman 2020). Given the limited signaling activities of PAR3, it is understudied and only a few pharmacological agents have been developed targeting PAR3.

PAR4

The functional studies of PAR4 have primarily focused on its role in hemostasis and thrombosis since PAR4 was first identified on human platelets and is one of the key receptors mediating thrombin signaling in platelet activation. However, PAR4 is widely expressed in many human tissues such as the kidney, pancreas, lungs, thyroid, and small intestine. The physiological roles of PAR4 in these other tissues are an emerging topic of research.

PAR4 was initially considered a low affinity backup thrombin receptor on platelets because it is a less efficient thrombin substrate (Han et al. 2019). It is now recognized that PAR1 and PAR4 cooperate to mediate thrombin's full signaling profile in platelets. Specifically, PAR1 responds to subnanomolar thrombin and triggers a rapid and transient downstream signal. In contrast, PAR4 requires a ~10-fold higher concentration thrombin for proteolysis and leads to a sustained downstream signal. The sustained signaling from PAR4 results in stable platelet function, increased procoagulant potential, and increased platelet spreading and adhesion (Han et al. 2019). A goal of targeting PAR4 therapeutically is to spare the immediate PAR1 response, but block the sustained PAR4 signaling (Wong et al. 2017). In principle, this would prevent thrombosis and spare hemostasis. An important concept is that although thrombin cleaves both PAR1 and PAR4, the sequences of the unmasked tethered ligands are different. This implies that the location and the shape of the endogenous ligand binding sites of the two receptors are also distinct and can be individually targeted (Hamilton and Trejo 2017).

Drugs

The tethered ligand activation mechanism presents unique challenges to targeting PARs therapeutically. The tethered ligand cannot diffuse from the receptor, which creates an entropy-driven high affinity interaction that is difficult to

block. Three strategies have been used to interfere with PAR signaling (Fig. 2): antibodies that block proteolytic activation, allosteric modulators, and orthosteric antagonists (Tables 1, 2 and 3). Each strategy is discussed below.

Blocking Antibodies

Antibodies that prevent engagement of the protease with the PAR exodomain were the first tools developed to specifically target PAR1 activation and have since been used to inhibit PAR2 and PAR4 (Hung et al. 1992a, b; Hamilton and Trejo 2017; French et al. 2018). The typical strategy is to target an epitope near the protease cleavage site to sterically prevent proteolysis and subsequent receptor activation. However, antibodies that interfere with other recognition sites have also proven successful. For example, antibodies that block the hirudin-like sequence on PAR1 from interacting with thrombin's exosite I inhibit PAR1 activation (Hung et al. 1992a, b). Similarly, blocking the anionic cluster in PAR4 is effective at blocking both mouse and human PAR4 activation (Mumaw et al. 2014). To date, inhibitory antibodies have not been developed for clinical use. However, these reagents have been essential in preclinical models and as laboratory tools to monitor receptor cleavage.

Allosteric Modulators

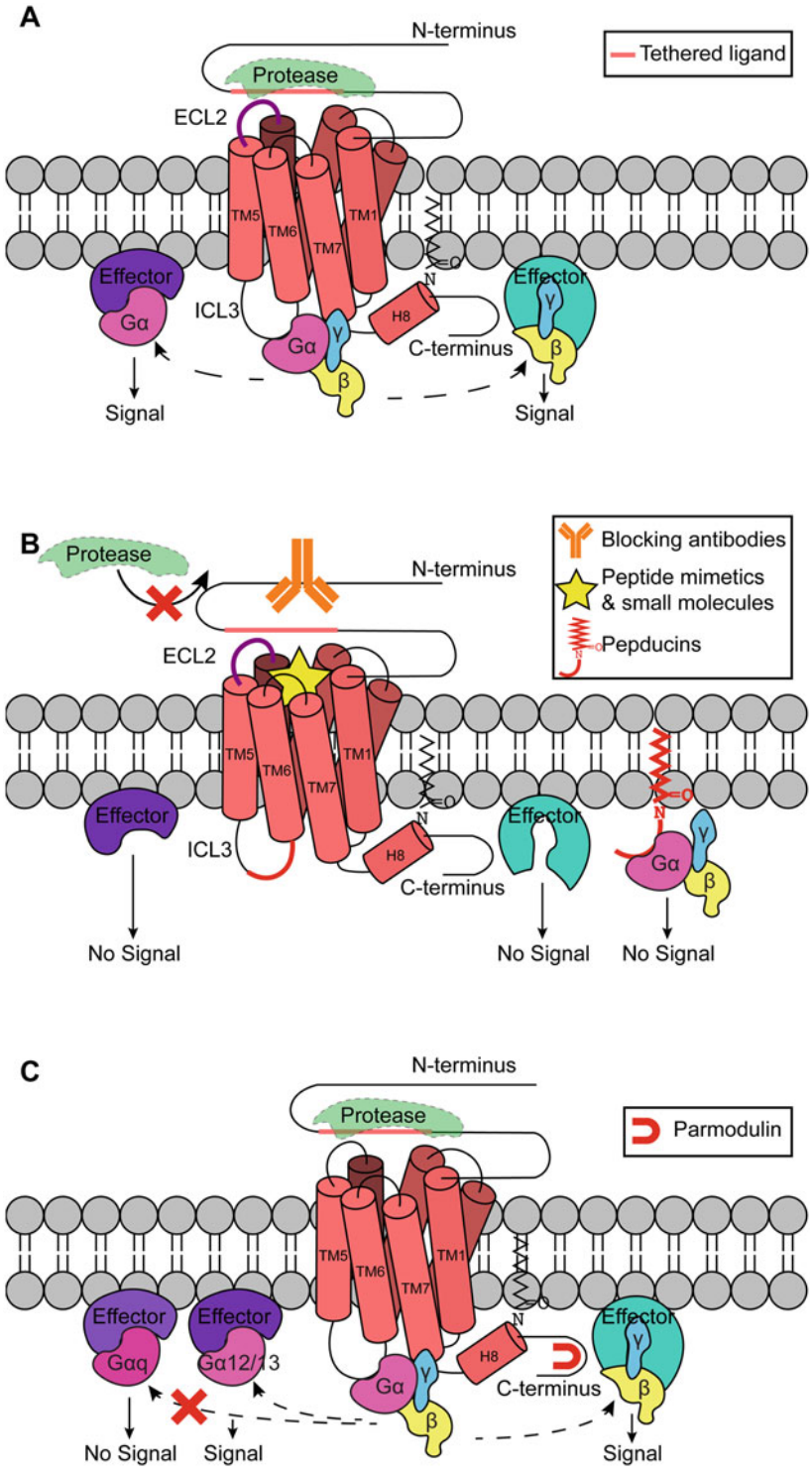
Pepducins are lipidated peptides that mimic intracellular loop 3 (ICL3) of the receptor and disturb the interaction between the receptor and G-proteins (Covic et al. 2002; Hamilton and Trejo 2017). Pepducins have been developed to target PAR1, PAR2, and PAR4. To inhibit signaling, pepducins enter the cell membrane via the lipid moiety, translocate to the intracellular surface, and interfere with G-protein coupling. A PAR1 pepducin, PZ128, has finished two clinical trials. The first trial, NCT01806077, is a Phase 1 study that evaluated the pepducin efficacy in patients with heart disease risk factors (Gurbel et al. 2016). The second trial, NCT02561000, evaluated PZ128 as an antithrombotic therapeutic in the Thrombin Receptor Inhibitory Pepducin-Percutaneous Coronary Intervention study (Rana et al. 2018).

Parmodulins target the 8th helix on the cytoplasmic face of PAR1 (Flaumenhaft and De Ceunynck 2017). Parmodulins do not alter the endogenous ligand binding site located on the extracellular face of the receptor. This property allows some receptor signaling that helps to restrict intracellular calcium flux mediated by the PAR1-Gq axis but has limited effects on RhoA activity by the PAR1-G13 axis (Flaumenhaft and De Ceunynck 2017). Parmodulins are able to block the PAR1-mediated proinflammatory effect in endothelium without altering the APC-mediated cyto-protective pathways.

Small Molecule Antagonists

PAR1

PAR1 antagonists can also be designed by targeting the interaction between the tethered ligand and the ligand binding site. Such antagonists can be divided into: peptidomimetics based on the tethered ligand and nonpeptide small molecules. By modifying the activation peptide at the R1, R2, R3, or R4 position, high affinity PAR1 antagonists such as BMS-200261, RWJ-56110, and RWJ-58259 could be generated (Han and Nieman 2020). These modifications disturb the crucial interaction between the tethered ligand and ligand binding site to produce antagonists that are widely utilized for *in vitro* and *in vivo* experiments. Nonpeptide small molecule inhibitors express improved pharmacokinetic profiles and pharmacodynamic profiles. FR171113 was one of the nonpeptide PAR1 antagonists discovered first, functioning by inhibiting thrombin-or PAR1-AP-induced ERK1/2 activation as well as PAR1-AP- and thrombin-induced guinea pig platelet aggregation *in* vitro (Han and Nieman 2020; Kato et al. 1999). Other nonpeptide small molecule inhibitors include SCH-79797 and its N-methyl analog that are widely used in both *in vitro* and *ex vivo* experiments due to their high specificity and selectivity for PAR1. SCH-79797 also functions as an antagonist in the *in vivo* rodent models (Ahn et al. 2000). However, Lee and Hamilton reported that this molecule drastically changed the morphology and function of platelets independently of PARs (Lee and



Protease-Activated Receptors (PARs), Fig. 2 PAR antagonist mechanism. (**a**) Upon enzymatic cleavage, the tethered ligand activates PARs intramolecularly. G-protein complexes are recruited to the intracellular side

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Hamilton 2013). These effects were seen at SCH-79797 concentrations required for the full inhibitory effect of PAR1, raising major suspicion on the use of the molecule as a PAR1 antagonist.

Vorapaxar (SCH-530348) is a small molecule PAR1 antagonist found to have a significant oral antiplatelet effect (Morrow et al. 2012; Scirica et al. 2012; Tricoci et al. 2012). Voraxapar was able to block human platelet aggregation induced by 10 nM thrombin or 15μM high affinity thrombin receptor activating peptide in preclinical *in vitro* experiments. It plays a major role in the oral antiplatelet effect due to its high oral bioavailability in several species such as the rat and monkey. In May 2014, the FDA approved vorapaxar as a novel antiplatelet therapeutic to treat patients with a history of myocardial infarction or peripheral arterial disease (Baker et al. 2014). To reduce further risk of thrombotic cardiovascular damage, vorapaxar can be administered with aspirin and/or clopidogrel. Although vorapaxar has major oral antiplatelet effects, it has severe limitations such as the increased risk of bleeding in the brain of patients with a history of stroke, transient ischemic attack, intracranial hemorrhage, or a bodyweight <60 kg. These limitations offset any therapeutic benefits, prompting the search for more novel PAR1 antagonists. New PAR1 antagonists are crucial since PAR1 still has potential as a target for therapeutics.

PAR2

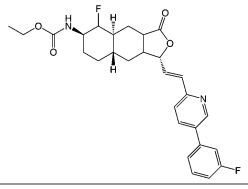
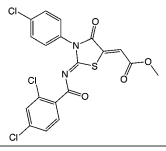
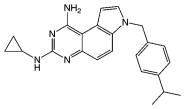
Although PAR2 plays critical roles in inflammation, pain sensation, cancer, and other pathologies, the development of the antagonist targeting PAR2 has not progressed as far as PAR1 (Yau et al. 2013). Two peptide antagonists of PAR2, FSLLRY-NH_2 and LSIGRL-NH_2, blocked trypsin-induced PAR2 signaling but not PAR2-AP (SLIGRL-NH_2)-induced PAR2 activation (Al-Ani et al. 2002). This again highlights that the proteolysis-mediated structural rearrangement of PARs is different from the activation peptide-mediated PAR activation. Several small molecules or peptidomimetic PAR2 antagonists have been reported. The first PAR2 antagonist showed proof of principle, but all have limitations. ENMD-1068 has extremely low potency in vitro (Kelso et al. 2006). K12940 and K-14585 only block the binding of 2-furoyl-LIGRL-NH_2 (a PAR2 peptide agonist) to the receptor but are incapable of inhibiting protease-mediated PAR2 signaling (Goh et al. 2009). Triptolide was not selective to PAR2 and also inhibited neurokinin receptor 1 (NK1) activation (Hoyle et al. 2010).

The first potent, small molecule targeting PAR2, GB88, was released in 2012 and is effective against all known PAR2 activators, including proteases, activation peptides, and nonpeptide agonists (Suen et al. 2012). In additional studies, GB88 and its derivatives including AY117 were shown to be biased ligands (Yau et al. 2013, 2016). GB88 and AY117 were found to inhibit trypsin or 2f-LIGRLO-NH_2-induced calcium mobilization and proinflammatory cytokine release via PAR2 (Yau et al. 2013). This series of PAR2 antagonists also showed anti-inflammatory effect both in vitro and in vivo. Using a similar strategy, Boitano et al. created another PAR2 antagonist, C391, from the PAR2 agonist, 2-furoyl-LIGRLO-NH_2 (Boitano et al. 2015). C391 is a potent and specific PAR2 antagonist that inhibits both PAR2-mediated calcium mobilization and MAPK signaling.

Solving the crystal structure of PAR2 in 2017 led to the most recent breakthrough on PAR2 antagonist development, which yielded the design of the AZ series (Cheng et al. 2017; McIntosh et al. 2020). The crystal structure of PAR2-AZ8838 complex at 2.8 Å uncovered the binding site of this competitive antagonist, while the

Protease-Activated Receptors (PARs), Fig. 2 (continued) of the receptors to further relay the protease signals. (**b**) There are three general strategies for PAR inhibition. Blocking antibodies target the exodomain of PARs to block the protease from interreacting with the receptor. Peptide mimetics and small-molecule compounds are designed to target the ligand binding site, which prevent the interaction between the tethered ligand and PARs. Pepducins act as intracellular modulators that interfere with the G-protein signaling. (**c**) Parmodulin bind to the C-terminus of PARs to redirect PAR signaling

Protease-Activated Receptors (PARs), Table 1 PAR1 antagonists

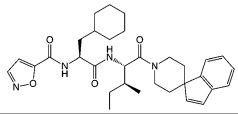
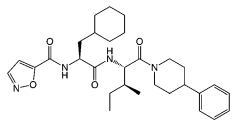
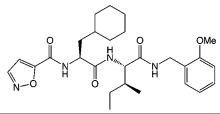
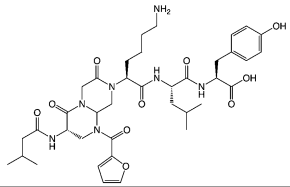
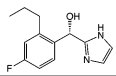
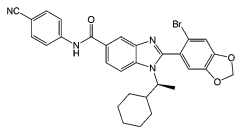
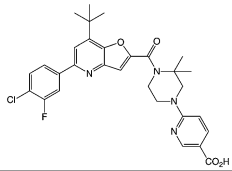
Name	Chemical structure	Mechanism of Action	Comments	Clinical Trial	References
Vorapaxar		Irreversible orthosteric antagonist, compete directly with endogenous tethered ligand	Thrombin $IC_{50} = 10$ nM, PAR1-AP ([^{3}H] haTRAP) $IC_{50} = 15$ nM	FDA approved (2014)	Tricoci et al. (2012), Morrow et al. (2012), Scirica et al. (2012) and Baker et al. (2014)
FR171113		Competitive Antagonist	Thrombin $IC_{50} = 0.29\mu M$, PAR1-AP (SFLLRN) $IC_{50} = 0.15\mu M$		Kato et al. (1999)
SCH-79797		Competitive Antagonist	PAR1-AP ([^{3}H] haTRAP) $IC_{50} = 70$ nM for binding, $IC_{50} = 300$ nM for platelet aggregation		Ahn et al. (2000)

structure of PAR2-AZ3451 at 3.6 Å resolution revealed that AZ3451 served as an allosteric inhibitor for PAR2. So far, only the AZ3451 has been tested in vivo and showed to be a therapeutic agent for osteoarthritis (Cheng et al. 2017; Huang et al. 2019; McIntosh et al. 2020). The PAR2 inhibitor I-287 selectively inhibits Gαq and Gα12/13 signaling while leaving the Gi/o signaling and β-arrestin2 engagement untouched (Avet et al. 2020). Interesting, although only a subset of PAR2 downstream signaling were inhibited, I-287 treatment still showed an anti-inflammatory effect in vivo. Both the observations from the AZ3451 and I-287 highlighted the potential of specifically regulating PAR function using allosteric inhibitors.

PAR4

Until recently, the development of small molecule PAR4 antagonists has received less attention than PAR1 or PAR2. The initial PAR4 antagonists were based on indazole, indole, imidazole, [1,3,4] thiadiazole (IDT), quinoline, and quinoxaline analogues (Hamilton and Trejo 2017; Liu et al. 2021). YD-3 was the first small-molecule PAR4 antagonist that inhibited GYPGKF-mediated activation of washed human platelets at an IC_{50} of 0.13μM (Wu et al. 2000, 2002). Although it showed some selectivity of PAR4 over PAR1, YD-3 has a couple limitations that restricted its preclinical usage. The synthetic strategy of YD-3 is complicated and the synthesis is inefficient since 20% of the final yield is the inactive isomer. Using YD-3 as the lead compound and replacing its indazole core with indole, ML-354 was selected from Vanderbilt University's (VU) sample library as a potent PAR4 antagonist (Temple et al. 2016; Young et al. 2010). Although ML-354 has an IC_{50} of 140 nM to AYPGKF-stimulated washed platelets, this compound has a high toxicity and a poor drug metabolism and pharmacokinetics (DMPK) profile. In 2016, one of the derivatives of ML-354, VU0661259, was reported (Temple et al. 2016). This was the first highly selective PAR4 antagonist that inhibited γ-thrombin-mediated PAR4 activation along with a reasonable pharmacological profile. However, this compound is a mixed competitive/non-competitive PAR4 inhibitor, which limited the in vivo application. The biggest breakthrough on PAR4 antagonist development occurred in 2017, in which Bristol Myers Squibb (BMS) disclosed the patent of BMS-986120 (Wilson et al. 2018; Wong et al. 2017). Containing an IDT core, BMS-986120 is a high selective small-molecule PAR4 antagonist

Protease-Activated Receptors (PARs), Table 2 PAR2 small-molecule antagonists

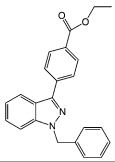
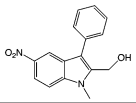
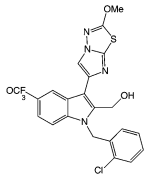
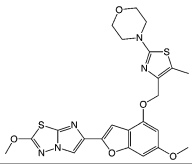
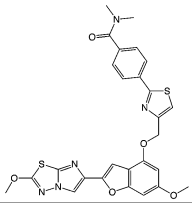
Name	Chemical structure	Mechanism of Action	Comments	Clinical Trial	References
GB88		Biased Ligand	Trypsin $IC_{50} = 2.0\mu M$		Suen et al. (2012) and Yau et al. (2013)
AY117		Biased Ligand	Trypsin $IC_{50} = 2.2\mu M$		Yau et al. (2016)
AY265		Biased Ligand	Trypsin $IC_{50} = 500$ nM		Yau (2013)
C391		Biased Ligand	PAR2-AP (2-at-LIGRL) $IC_{50} = 1.3\mu M$		Boitano et al. (2015)
AZ8838		Competitive Antagonist	Trypsin $IC_{50} = 4.2\mu M$		Cheng et al. (2017)
AZ3451		Alloseric Inhibitor	Trypsin $IC_{50} = 6.6\mu M$		Cheng et al. (2017)
I-287		Alloseric Inhibitor	hTrypsin $IC_{50} = 45$ nM, PAR2-AP (SLIGKV) IC_{50} 390 nM		Avet et al. (2020)

that also shows saturable and reversible binding properties to PAR4. Wong et al. also demonstrated that inhibition of PAR4 is a safer antiplatelet strategy with low bleeding risk and a wider therapeutic window compared to the current standard care. Both BMS-986120 and its derivative, BMS-986141, were in clinical trials and showed promising outcomes as a PAR4 antagonist.

Single Nucleotide Polymorphisms (SNPs)

SNPs have been identified in PAR2 and PAR4 that impact receptor functions. PAR2 has a well-characterized polymorphic variant at residue 240 within ECL2 where phenylalanine is replaced by a serine (PAR2-F240S) (Compton et al. 2000). The minor allele frequency for this polymorphism is 0.084. PAR2-F240S has a significantly decreased sensitivity toward trypsin (~3.7-fold), and PAR2-activation peptides, SLIGKV-NH2 (~2.5-fold) and SLIGRL-NH2(~2.8-fold), and an increased sensitivity to a selective PAR2 agonist, trans-cinnamoyl-LIGRLO-NH2(~4-fold). Furthermore, PAR2-F240S also has constitutive activity (Ma and Burstein 2013). These data point to Phe240 in ECL2 as a key residue in PAR2 activation and this polymorphism may impact the response to PAR2 therapeutics. This has yet to be demonstrated empirically.

Protease-Activated Receptors (PARs), Table 3 PAR4 small-molecule antagonists

Name	Chemical structure	Mechanism of Action	Comments	Clinical Trial	References
YD-3		Competitive Antagonist	First non-peptide PAR4 antagonist. PAR4-AP (GYPGKF) $IC_{50} = 130$ nM		Wu et al. (2000, 2002)
ML-354		Allosteric Modulator	PAR4-AP (AYPGKF) $IC_{50} = 140$ nM		Young et al. (2010)
VU0661259		Mixed Competitive/ Non-competitive Antagonist	PAR4-AP (AYPGKF) $IC_{50} = 180$ nM, γ-Thrombin $IC_{50} = 4.35\mu M$		Temple et al. (2016)
BMS-986120		Reversible competitive Antagonist	PAR4-AP (Ala-(L-4-F-Phe)-Pro-Gly-Trp-Leu-Val-Lys-Asn-Gly) $IC_{50} = 0.56$ nM, γ-Thrombin $IC_{50} = 7.3$ nM	Phase I trial completed	Wong et al. (2017) and Wilson et al. (2018)
BMS-986141		Reversible competitive Antagonist	PAR4-AP (Ala-(L-4-F-Phe)-Pro-Gly-Trp-Leu-Val-Lys-Asn-Gly) $IC_{50} = 0.45$ nM, γ-Thrombin $IC_{50} = 2.1$ nM	Phase II trial completed	Wong et al. (2017)

PAR4 has four well-characterized sequence variants that change the receptor function, A120T, Y157C, F296V, and P310L (Han and Nieman 2020). The PAR4-A120T variant is the only that has correlated with a differential response to an antagonist. This residue is located in the middle of TM2. PAR4-120T is a hyperreactive PAR4 compared to PAR4-120A, which is also associated with a higher platelet reactivity, increased sensitivity to thrombin, resistance to desensitization, and increased *ex vivo* thrombus formation. More importantly, this alternation of PAR4 function between the two sequence variants may impact the efficacy of PAR4 specific inhibitors or even other antiplatelet compounds. The hyperreactive PAR4 (PAR4-120T) not only showed a higher resistance to YD-3, a selective PAR4 antagonist, but also demonstrated less inhibitory effects of the P2Y12 inhibitor and COX2 inhibitor (Han and Nieman 2020). The precise molecular mechanism of how changing a single residue from alanine to threonine in TM2 can alter PAR4 function is not clear. Based on the amide-HDX study, the 120 position is close to the endogenous ligand binding site of PAR4. This may imply that switching threonine to alanine could affect the shape of the ligand binding site, which further impacts the ligand-receptor interaction and the antagonist-receptor interaction (Han et al. 2020a).

Future Directions

The tethered ligand-mediated receptor activation of PARs provides the opportunity to target each receptor individually while also set up a unique challenge for the antagonist to compete with the entropy-driven high affinity between the tethered ligand

and the receptor. Current drug developments of therapeutic targets of PAR1, PAR2, and PAR4 show great potential. Even though the first member of this family, PAR1, was discovered 30 years ago, this is a promising time for PAR research. The PAR1 antagonist vorapaxar was approved by the FDA in 2014. PAR1 pepducin, PZ128, and the PAR4 antagonists BMS-986120 and BMS-986141 showed promise in the preclinical setting.

Looking forward, there is still a lot of work that needs to be done for PAR antagonist development as well as for the molecular mechanism of PARs in general. Large-scale clinical trials are still needed for the PAR4 antagonists to identify the target patient population so the effects of these therapeutics are still up in the air (Han et al. 2019). A better understanding of how the PAR4 polymorphisms influence effectiveness of the therapeutics and how these remedies will be used with existing antiplatelet and anticoagulation therapies are also needed (Han et al. 2019). Also, when targeting PARs therapeutically, it is important to consider their physiological roles in numbers of tissues and the effects of disrupting their signaling pathways (Han et al. 2019). Using PAR1 as an example, the role of PAR1 on hemostasis is always primarily being considered; thus, antagonists targeting PAR1 have been majorly tested as antiplatelet therapies. However, PAR1 is widely expressed in many different tissues and inhibiting PAR1 may affect other tissues. Therefore, when designing PAR inhibitors, blocking potentially protective signaling pathways needs to be balanced with blocking harmful pathways (Han et al. 2019).

Not every drug candidate is suitable enough to be tested in clinical trials. In fact, most of the PAR antagonists are only usable in preclinical studies. Either way, these PAR antagonists still serve as probes to help scientists understand the specific roles of PARs on different tissue as well as enrich the knowledge of the tethered ligand activation mechanism. Even with more than 20 years of study, there is not a full understanding of the contributions of PAR1 and PAR4. Hence, the development of pharmacological tools for PAR research, including more appropriate animal models and more PAR modulators with a different mechanism of actions, is still required.

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Proteases

▶ Nonviral Peptidases

Proteasome Inhibitors

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Synonyms

Bortezomib (Velcade); Carfilzomib (Kyprolis); Ixazomib (Ninlaro)

Definition

Proteasomes are large multicatalytic protein complexes that function in the degradation of proteins into peptides. Drugs that inhibit one or more of the proteasome's catalytic subunits include bortezomib (Velcade), carfilzomib (Kyprolis), and ixazomib (Ninlaro). These drugs are used to treat multiple myeloma and mantle cell lymphoma, and they are being investigated for other diseases.

Mechanism of Action

As their name implies, proteasome inhibitors target the proteasome. More specifically, they target one of the proteolytic subunits within the proteasome, thereby reducing the ability of the proteasome to cleave cellular proteins into peptides (Fricker 2020). While this is ultimately toxic to all cells, some cell types show elevated sensitivity to proteasome inhibition. Sensitive cells include some types of white blood cells, especially those that are abnormal in cancer such as multiple myeloma and mantle cell lymphoma (Gandolfi et al. 2017). The specific downstream pathway that leads from proteasome inhibition to cell death is not known with certainty, and a variety of mechanisms have been proposed (Fricker 2020). These potential mechanisms are discussed below following discussion of the proteasome.

Proteasome Composition and Diversity

Proteasomes exist in a wide variety of forms and are found in virtually every human cell in the body (Collins and Goldberg 2017). The simplest form of the proteasome is termed the "20S proteasome core particle" and consists of two inner rings of β subunits and two outer rings of α subunits. Each ring consists of seven different α or β subunit proteins. The α subunits are named α1 to 7 and are generally the same in every cell in the body with the exception of the α4S subunit that is unique to spermatozoa. In contrast, some of the seven β subunits in each ring are variable with cell type. Three of the seven β subunits are catalytically active as proteases: β1, β2, and β5. In some cell types, especially immune system cells, the three active subunits are replaced with variants: β1i, β2i, and β5i (Goldberg et al. 2002). In thymocytes, another β5 variant is found, named β5t (Murata et al. 2018). Each variant is catalytically active and confers a slight difference to the substrate specificity of the resulting proteasome. The β5, β5i, β5t, and β1i subunits cleave proteins at hydrophobic amino acids, the β1 subunit cleaves at acidic amino acids (Asp or Glu), and the β2 and β2i subunits cleave at basic amino acids (Lys or Arg). The other four β subunits (β3, β4, β6, and β7) are catalytically inactive and are common to proteasomes in all cell types throughout the body. The catalytically inactive β subunits are evolutionarily related to the active subunits but lack either the critical active site threonine or other important residues (Fricker 2020).

In addition to the diversity of the proteasome that arises from differences in the catalytically active β subunits, there are two other sources of diversity in the proteasomes (Morozov and Karpov 2018). One source of diversity is through posttranslational modifications such as phosphorylation, N-acetylation, glycosylation, myristylation, poly-ADP ribosylation, and ubiquitination. These modifications can affect proteasome assembly, stability, activity, and sensitivity to proteasome inhibitors (Morozov and Karpov 2018). Another major source of proteasome diversity is the presence of cap proteins that serve to bind protein substrates and deliver them into the catalytic core of the proteasome (Collins and Goldberg 2017; Morozov and Karpov 2018). There are several different regulatory complexes, and each 20S proteasome core can bind two regulatory complexes, one at each end. These regulatory cap proteins may be identical or different. Furthermore, the 20S core can exist with only one regulatory complex or no complexes (i.e., the free 20S core particle). The largest regulatory complex is the 19S proteasome activator, also known as PA700. The 19S proteasome activator is responsible for the recognition of ubiquitinated proteins, removal of the ubiquitin chains, and the unfolding and translocation of the protein into the 20S core. There are two different 11S proteasome activators, also known as PA28, PA26, and REG, that differ in their

subunits (α/β vs γ). Neither of the 11S proteasome complexes can bind ubiquitinated proteins or use ATP to unfold proteins. Another regulatory subunit is PA200; this also does not bind ubiquitin or ATP. Thus, the diversity of the regulatory complexes has a large impact on the types of cellular proteins cleaved by the proteasome. Only proteasomes with the 19S proteasome activator can bind and unfold ubiquitinated proteins. Proteasomes containing the other regulatory complexes, or those without any regulatory complexes, are thought to primarily cleave peptides and small unfolded or misfolded proteins (Collins and Goldberg 2017; Morozov and Karpov 2018). All proteasome forms are inhibited by the proteasome inhibitors, albeit with difference in potency (Morozov and Karpov 2018).

Downstream Pathways that Contribute to Cell Death

Protein degradation is an important regulatory event that plays an essential role in many processes. Removing unfolded or otherwise damaged proteins is needed to maintain cells in a healthy state. In addition, many cellular pathways include one or more proteins that function as inhibitors to prevent activation of the pathway, and proteasome-mediated removal of the inhibitor protein(s) is required for subsequent activation. As such, there are many possible ways that blocking the proteasome can contribute to cell death. Cellular proteins cleaved by the proteasome that could contribute to cell death upon proteasome inhibition include NF-κB, p53, cyclin, and cyclin-dependent kinases (Heine et al. 2018). Proteasome inhibitors also cause the accumulation of unfolded and misfolded proteins which trigger the unfolded protein response and apoptosis (Zang et al. 2012). The extrinsic caspase-8 pathway and intrinsic caspase-9 pathways have also been proposed to be involved in cell death caused by exposure to a proteasome inhibitor (Gandolfi et al. 2017). While evidence supports each of these pathways, a major problem is that the concentrations of bortezomib required to alter protein levels in each of these studies are generally much higher than the doses used clinically. Studies that incubate cells with high concentrations of bortezomib for 1–2 days do not reflect the concentration and time course of bortezomib in clinical scenarios. For example, subcutaneous administration of bortezomib results in peak plasma levels of ~25–50 nM for 1–2 h, while intravenous injection causes peak plasma levels of ~250–500 nM for only ~5 min, after which the levels rapidly drop as bortezomib distributes to tissues (Schwartz and Davidson 2004).

Because the levels of cellular proteins do not change rapidly with clinically relevant concentrations of the drugs, it is possible that an alternative mechanism accounts for the downstream effects and cell death. One alternative mechanism involves the peptides that are proteasome products, rather than the proteins that are proteasome substrates (Fricker 2020). The levels of proteasome products show rapid changes when cells are exposed to low concentrations of drugs. These proteasome products are peptides formed upon degradation of the protein. Most of these peptides are not stable within cells and are rapidly degraded by cytosolic peptidases. Because of their high turnover, these peptides are rapidly altered by drugs that inhibit the proteasome and block their production. If these peptides are biologically active, they could contribute to the downstream mechanism of action of the proteasome inhibitors (Fricker 2020).

Classes of Drugs

Proteasome inhibitors can be classified by the chemical moiety that binds to the proteasome. The first synthetic compounds designed to inhibit the proteasome contained an aldehyde group that bound to the active site threonine residue in the β1, β2, and/or β5 subunits (Wilk and Orlowski 1983). However, these aldehyde-based inhibitors were not very selective and inhibited a wide range of cellular proteases. Replacing the aldehyde with a boronate residue conferred greater selectivity (Teicher et al. 1999). Alternatively, natural compounds found to inhibit the proteasome contained other chemical moieties, such as epoxyketones or β-lactone γ-lactams, and these groups also conferred selectivity to the proteasome. Synthetic

drugs using each of these active site "warheads" have been developed (Teicher and Anderson 2015). All of these drugs show greatest activity toward the β5 subunit and the related β5i subunit and weaker potency as inhibitors of the β1 or β2 subunits.

Boronates

Bortezomib was the first proteasome inhibitor to be used clinically (Adams and Kauffman 2004). This compound contains a boronate group that binds to the active site threonine on the β subunits. Because the peptide-based backbone of bortezomib is hydrophobic, this compound is most effective at inhibiting the β5 subunit. Bortezomib is an order of magnitude less potent as an inhibitor of the β1 subunit and two orders of magnitude less potent at the β2 subunit.

Another boronate-containing proteasome inhibitor is ixazomib citrate (Gandolfi et al. 2017). This drug was the third proteasome inhibitor to be used clinically. It is an orally administered prodrug that is converted into the active form (ixazomib) by plasma enzymes. In contrast, all other proteasome inhibitor drugs currently on the market require intravenous or subcutaneous administration.

A third boronate-containing proteasome inhibitor is delanzomib (Zangari and Suva 2016). This drug is currently in clinical trials. Delanzomib is related to bortezomib with a peptide-like backbone and a boronate warhead.

Epoxyketones

Epoxomicin is a natural product isolated from *Streptomyces* extracts. This compound was found in a screen for antitumor activity, and further studies found that epoxomicin has anti-inflammatory properties (Hanada et al. 1992). The structure of epoxomicin consists of a peptide backbone with an epoxyketone group. This epoxyketone moiety forms a covalent bond with the active site threonine and leads to irreversible inhibition. While epoxomicin is commonly used in the laboratory to inhibit the proteasome, it was not developed as a drug.

The first clinically useful proteasome inhibitor containing the epoxyketone moiety is carfilzomib (Kuhn et al. 2007). A drug in development, oprozomib, is structurally related to carfilzomib with a peptide-like backbone and an epoxyketone warhead.

Bicyclic β-Lactone γ-Lactams

The first natural product found to inhibit the proteasome was lactacystin. This compound spontaneously hydrolyzes to the active form, clasto-lactacystin β-lactone, which forms a covalent bond with the active site threonine of the proteasome β5 subunit. Although lactacystin is not suitable as a drug, another natural product proteasome inhibitor named marizomib is being developed as a drug. Marizomib also has a bicyclic β-lactone γ-lactam moiety and is an irreversible inhibitor of the proteasome.

Clinical Use (Including Side Effects)

All of the proteasome inhibitor drugs are effective in treating multiple myeloma and mantle cell lymphoma. Bortezomib is typically used in combination with lenalidomide (a thalidomide analogue) and dexamethasone (a glucocorticoid). The three-drug combination with bortezomib is more effective than the two-drug combination lacking bortezomib. Other bortezomib-containing three-drug combinations found to be effective contain dexamethasone and either thalidomide or cyclophosphamide in place of lenalidomide. Likewise, three-drug combinations in which carfilzomib or ixazomib is substituted for bortezomib have also been found to be more effective than the two-drug combinations lacking the proteasome inhibitor. Patients who develop resistance to bortezomib often show responses to carfilzomib or ixazomib (Gandolfi et al. 2017).

Common side effects to bortezomib include peripheral neuropathy, skin rash, fatigue, nausea, vomiting, diarrhea or constipation, and thrombocytopenia. Rarer but potentially serious side effects include heart failure and other cardiovascular events. Carfilzomib and ixazomib produce similar side effects as found with bortezomib, although the relative incidence of each side effect varies among the drugs (Gandolfi et al. 2017).

In addition to the use of proteasome inhibitors for treating multiple myeloma and mantle cell

Proteasome Inhibitors, Table 1 Proteasome inhibitors

Drug	Molecular class	Inhibition	Comment
Bortezomib	Boronate	Reversible	1st proteasome inhibitor drug
Carfilzomib	Epoxyketone	Irreversible	2nd proteasome inhibitor drug
Ixazomib citrate	Boronate	Reversible	Prodrug of ixazomib
Marizomib	β-Lactone γ-lactam	Irreversible	Currently[a] in clinical trials
Oprozomib	Epoxyketone	Irreversible	Currently[a] in clinical trials
Delanzomib	Boronate	Reversible	Currently[a] in clinical trials

[a]in 2020

lymphoma, these drugs are in clinical trials for patients with other types of tumors. For example, marizomib is being tested for glioblastomas based on the ability of this drug to cross the blood-brain barrier (unlike bortezomib). Marizomib was found to be effective in treating glioblastoma in animal studies (Manton et al. 2016). Proteasome inhibitors are also being investigated as immunomodulators (Table 1).

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Protein Degradation

► Ubiquitin/Proteasome

Protein Kinase A Anchoring Proteins

► A-Kinase Anchoring Proteins (AKAPs)

Protein Kinase C

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Synonyms

C kinase; PKC

Definition

Protein kinase C (PKC) is an enzyme family whose members are activated by agonists that cause receptor-mediated generation of lipid second messengers. The activated enzymes transduce information from such agonists by phosphorylating relevant downstream substrates.

Basic Characteristics

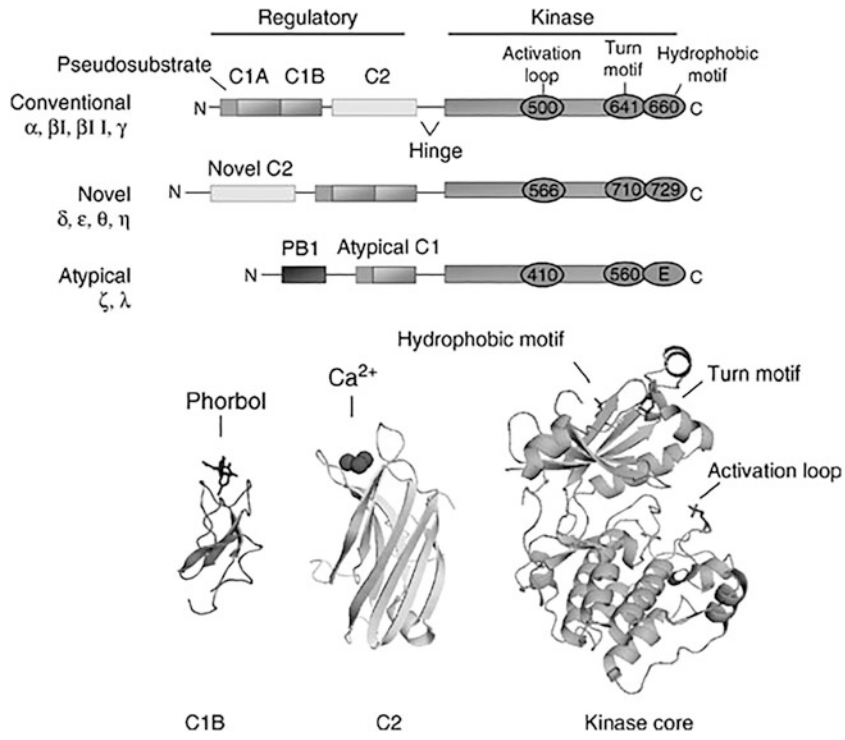
PKC is a family of enzymes whose members play central roles in transducing information from external stimuli to cellular responses. Members of this family of serine/threonine kinases respond to signals that cause lipid hydrolysis. PKC isozymes phosphorylate an abundance of substrates, leading to both short-term cellular responses such as regulation of membrane transport and long-term responses such as memory and learning. They generally function as tumor suppressors, with loss-of-function mutations associated with diverse cancers. Conversely, gain-of-function mutations have been identified in degenerative diseases. Thus, homeostatic control of PKC is essential to avoid pathophysiologies.

The PKC Family

There are nine genes encoding PKC isozymes in mammals, and they fall into three classes: conventional (α, β (with two common splice variants, βI and βII) and γ), novel (δ, ε, η, and θ), and atypical (ζ and ι/λ) PKC isozymes (Newton 2018). As shown in Fig. 1, all isozymes comprise an N-terminal regulatory moiety and a C-terminal kinase core. The regulatory moiety contains two important functional segments: an autoinhibitory pseudosubstrate sequence that allosterically regulates access to the substrate-binding cavity and one or more membrane-targeting modules. It is the nature of the membrane-targeting modules that defines the classes of PKC isozymes. All PKC isozymes have a version of the C1 domain, the diacylglycerol sensor. This domain binds diacylglycerol, the natural agonist, as well as phorbol esters, potent functional analogues, in all isozymes except atypical PKC isozymes. For these isozymes, an impaired ligand-binding pocket does not support the binding of diacylglycerol or phorbol esters, and, as a consequence, the hallmark of atypical PKC isozymes is their complete lack of response to phorbol esters. Rather, these isozymes are regulated by binding protein partners through their PB1 (for Phox and Bem1p) protein interaction domain. Conventional and novel PKC isozymes have a C2 domain. For conventional PKC isozymes, this domain binds phosphatidylinositol-4,5-bisphosphate in the plasma membrane in a Ca^{2+}-dependent manner, serving as a plasma membrane-sensing module. An impaired Ca^{2+}-binding pocket in the novel PKC isozymes makes them unresponsive to Ca^{2+}. In one novel isozyme, PKC δ, phosphorylation on tyrosine controls protein interactions of this domain.

Regulation

PKC isozymes are regulated by three mechanisms: phosphorylation, second messenger binding, and protein–protein interactions. First, a series of ordered phosphorylations renders newly synthesized PKC catalytically competent. The upstream kinase, phosphatidylinositol-dependent kinase-1 (PDK-1), phosphorylates the activation loop of PKC, triggering phosphorylation at two conserved sites in the C-terminus, the turn motif and the hydrophobic motif, by a mechanism that is facilitated by mTORC2. Phosphorylation of the hydrophobic motif occurs via intramolecular autophosphorylation. The positions of these sites on structure of the kinase domain of PKCβII are shown in Fig. 1. These phosphorylations are essential to lock PKC in a stable and autoinhibited



Protein Kinase C, Fig. 1 Domain structure of PKC family members showing regulatory modules (pseudosubstrate sequence and C1, C2, and PB1 domains) and the kinase core. Shown below are the structures of the C1 domain of PKCδ with bound phorbol, the C2 domain of PKCβ with bound Ca^{2+}, and the kinase domain of PKCβII solved by Grant and coworkers (Grodsky et al. 2006) with three processing phosphorylation sites labeled. Figure adapted from (Newton 2003)

conformation. Second, the mature, fully phosphorylated species of PKC is allosterically activated following engagement of the membrane-targeting modules to the membrane. For conventional PKC isozymes, Ca^{2+} pretargets PKC to the membranes by binding the C2 domain and increasing this domain's affinity for anionic lipids. At the membrane, the C1 domain binds diacylglycerol, an event that provides the energy to release the autoinhibitory pseudosubstrate sequence from the substrate-binding cavity. Novel PKC isoforms respond to diacylglycerol alone because their C1 domain has a sufficiently high-affinity binding to diacylglycerol such that pretargeting by the C2 domain is not required. Third, scaffold proteins position PKC near its activators or substrates, allowing specificity in signaling by distinct isoforms.

Signaling by PKC is terminated by concentrations of its ligands dropping to basal levels (i.e., Ca^{2+} and diacylglycerol). Prolonged activation also results in the dephosphorylation and degradation of PKC. Dephosphorylation is controlled, in part, by PHLPP (for PH domain leucine-rich repeat protein phosphatase), which dephosphorylates the hydrophobic motif of conventional and novel PKC isozymes, initiating their downregulation.

Function in Health and Disease

Conventional and novel PKC isozymes generally function to suppress proliferative and survival pathways, with recent evidence converging on roles as tumor suppressors (Newton 2018). Analysis of somatic mutations throughout the PKC family has revealed that the majority are loss of function. In contrast, germ-line mutations that enhance activity are associated with degenerative diseases such as Alzheimer's disease and cerebellar ataxia. For a number of cancers, including pancreatic and colon, patients with high levels of PKC have significantly better prognosis than those with relatively low levels. One exception is PKCι, an

atypical PKC, which functions as an oncogene in cancers such as that of the lung (Yin et al. 2019).

Because the levels of PKC in cells control the amplitude of PKC signaling pathways, defects in PKC regulation that result in altered levels of the kinase are associated with disease. One mechanism that controls the levels of PKC is a quality control pathway by which the phosphatase PHLPP regulates how much newly synthesized PKC progresses to the stable, phosphorylated form: low levels of the phosphatase allow more accumulation of phosphorylated (and thus stable) PKC, whereas high levels promote dephosphorylation and degradation of PKC. This quality control mechanism is particularly important in pancreatic cancer, where patients with low PHLPP and high PKC have considerably better prognosis than ones with high PHLPP and low PKC (Baffi et al. 2019).

Drugs

Activators

Conventional and novel PKC isozymes are potently activated by phorbol esters, heterocyclic compounds found in the milky sap exuded by plants of the Euphorbiaceae family. This sap was used medicinally as a counterirritant and cathartic agent over the millennia; we now know that the active ingredients, phorbol esters, specifically bind to the C1 domain, the diacylglycerol sensor described above. In fact, their ability to recruit PKC to membranes is so effective that phorbol esters cause maximal activation of conventional PKC isozymes, bypassing the requirement for Ca^{2+}. This module is found in a number of other proteins in addition to PKC, so the profound effects of phorbol esters on cells are mediated by other proteins as well. Bryostatins are another class of compounds that bind to the C1 domain and result in acute activation of PKC. Note that these molecules are not readily metabolized and result in the constitutive activation of PKC, triggering PHLPP-mediated dephosphorylation and subsequent downregulation. Thus, while these molecules acutely activate PKC isozymes, they cause the chronic loss of the enzymes. This paradox accounted for confusion as to whether to inhibit or activate PKC in cancer therapies.

Inhibitors

A number of inhibitors directed toward the active site of PKC have been developed (Yin et al. 2019). While these failed in clinical trials for cancer (where, in general, PKC activity should be restored, not inhibited), they may prove useful for neurodegenerative diseases, where inhibiting overly active PKC could be beneficial.

Cross-References

▶ Protein Kinase C

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Protein Phosphatases

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Synonyms

Phosphoprotein phosphatase

Definition

Protein phosphatases are a group of enzymes that catalyze the removal of a phosphate group from substrate proteins, opposing the action of protein kinases.

Basic Mechanisms or Characteristics

Widespread Protein Phosphorylation

The covalent modification of proteins is a common mechanism to control protein function. Dozens of protein covalent modifications are known, with phosphorylation, acetylation, ubiquitination, and methylation being regarded as the most common. Reversible protein phosphorylation was discovered when elucidating the mechanisms that control mammalian glycogen-metabolizing enzymes (Brautigan and Shenolikar 2018) (Moorhead et al. 2007). In eukaryotes, protein phosphorylation occurs predominantly on the hydroxy amino acids, serine (~86%), threonine (~12%), and tyrosine (~2%), generating stable phosphate esters (Brautigan and Shenolikar 2018). Mass spectrometry-based phospho-proteomic studies have revealed that phosphorylation on serine, threonine, and tyrosine residues is also common in prokaryotes. Phosphorylation can occur on several additional amino acid side chains, forming phosphoramidate bonds in phospho-His, phospho-Lys, and phospho-Arg and a phosphorothioate bond with Cys (phospho-Cys). Histidine and aspartate phosphorylation were first discovered in prokaryotes, as part of their two-component basic stimulus-response systems. The recent development of phosphohistidine-specific antibodies has revealed that phosphorylation on histidine is more prevalent in eukaryotes than originally thought. Quantitative mass spectrometry has established that protein phosphorylation occurs on at least 75% of all human proteins with most having multiple phospho-sites (Brautigan and Shenolikar 2018). Given the prevalence of protein kinases and phosphatases across eukaryotes, it is likely that this mechanism of protein regulation is equally widespread in all eukaryotes.

Protein Phosphatases and Kinases

The phosphorylation status of any given amino acid within a protein is governed by the balance of activities of the protein kinase(s) and phosphatase (s) that target that specific residue. With ~98% of human protein phosphorylation on serine and threonine residues, it is no surprise that of the more than over 500 catalogued protein kinases, ~428 are predicted to be serine/threonine-specific, with ~90 being tyrosine-specific protein kinases. Notably, there is a similar number of predicted tyrosine phosphatases (~109) as kinases, but only ~38 serine/threonine phosphatases (Shi 2009). As detailed below, several of the PPP family serine/threonine phosphatase catalytic subunits are used in multiple holoenzyme complexes where the additional subunits provide substrate specificity. Our current knowledge about the number of additional subunits or holoenzyme complexes roughly balances the number of serine/threonine protein phosphatases and kinases (note that most protein kinases and phosphatases have multiple substrates *in vivo*).

The protein kinases belong to one large gene family, while the protein phosphatases are believed to have evolved four times, producing four separate gene families. Like the protein kinases, protein phosphatases were first characterized during the study of glycogen metabolism using in vitro biochemical assays. Extra-mitochondrial serine/threonine protein phosphatase activity was defined as type one (PP1) if abolished by the endogenous inhibitor proteins I-1 or I-2 and called type two if insensitive to I-1 or I-2. Type two activity was further designated PP2A, PP2B, and PP2C if the in vitro activity did not require metal ions, was calcium stimulated, or was dependent on magnesium for activity, respectively (Brautigan and Shenolikar 2018). Subsequent cloning and genomic analysis revealed additional related PPP members, classified as shown in Table 1. PP7 is unique to plants and green algae and contains several insertions in the catalytic region with currently unknown function. Protein phosphatases with EF-hand motifs (PPEFs) are distributed widely in eukaryotes and are also called rdgC (for the *Drosophila* mutant retinal degeneration C). PP2B is also known as

Protein Phosphatases, Table 1 A summary of the protein phosphatases found in humans. PPP, phosphoprotein phosphatase; PPM, protein phosphatase – Mg^{2+}/Mn^{2+} dependent; PTP, protein tyrosine phosphatase; FCP/SCP, transcription factor IIF-associating component of RNA-polymerase II CTD phosphatase and small CTD phosphatase

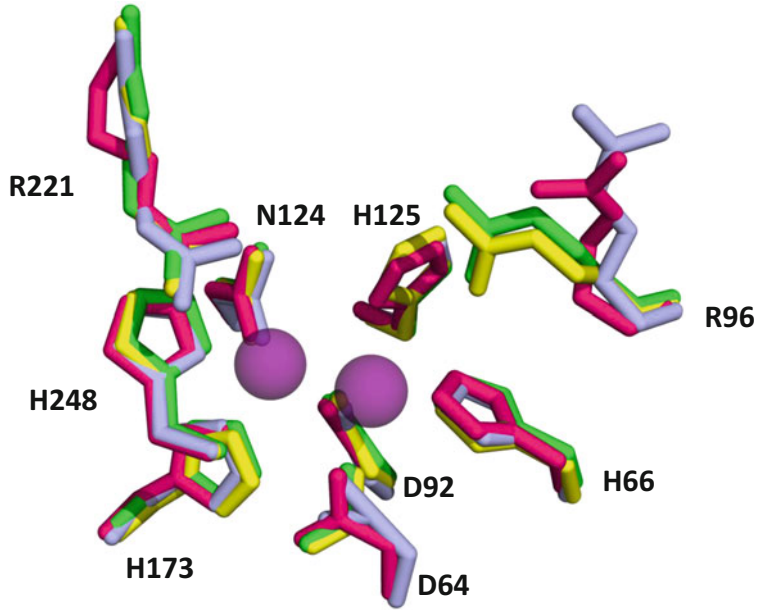
Family	Class	Number of genes	Known regulatory subunits
Serine/threonine phosphatases			
PPP family	PP1	3	>200 (e.g., Repo-Man)
	PP2A	2	Aα, Aβ, B, B′, B′‘, B’“
	PP2B	3	Regulatory B (CNB1), CaM
	PP4	1	R2, R3, R4
	PP5	1	None
	PP6	1	SAP1-3, ANKRD28, 44, 52
	PP7	2	Unknown
PPM family	PP2C	16	None
PTP superfamily (CX_5R)			
Class I PTPs (classic)	Receptor PTP	21	
	Non-receptor PTP	17	
Class I PTPs (DSPs)	MAPKP	11	
	Slingshots	3	
	PRLs	3	
	Atypical DSP	19	
	CDC14	4	
	PTEN	5	
	Myotubularins	16	
Class II PTPs	CDC25s	3	
Class III PTPs	LMWPTP	1	
Asp-based catalysis (DXDXT/V)			
FCP/SCP family	FCP1	1	RAP74 of TFIIF
	SCP	3	
	FCP/SCP-like	4	
HAD family	Chronophin	1	

calcineurin and has been renamed PP3. PP2C has been reclassified to the PPM family.

The discovery of tyrosine phosphorylation and then tyrosine-specific protein kinases led to the discovery of the tyrosine-specific protein phosphatases or phosphotyrosine phosphatases (PTPs). Studies on RNA-polymerase II led to the discovery of an additional serine/threonine phosphatase that controls the dephosphorylation of the C-terminal tail (CTD) of the polymerase. This enzyme was the founding member of a small family of related enzymes termed FCP/SCP (TFIIF-associating CTD phosphatase/small CTD phosphatase). Table 1 is completed with the haloacid dehalogenase or HAD family sharing the same catalytic mechanism as FCP/SCP (Moorhead et al. 2007).

PPP Family of Protein Phosphatases

The phosphoprotein phosphatase family (PPP) are serine/threonine-specific enzymes (Table 1) that all contain a series of completely conserved catalytic residues, several of which coordinate binding of two metal ions (Fig. 1). These metals (thought to be Zn^{2+}, Fe^{2+}, or Mn^{2+}) play a key catalytic role by activating a water molecule for nucleophilic attack on the substrate phosphoryl group, leading to the release of phosphate. A substrate peptide based on the phosphorylation site of the PP5 substrate Cdc37 provided the first clue about the active site plasticity of the PPP enzymes (Oberoi et al. 2016). By altering the phospho-site on Cdc37 to a phospho-mimic and tethering residues 5–20 surrounding this phospho-site to PP5, it was clear that this substrate binds the active site using



Conserved Active Site Residues in PPP

PP1	D64	H66	D92	R96	N124	H125	H173	R221	H248
PP2A	D57	H59	D85	R89	N117	H118	H167	R214	H241
PP2B	D90	H92	D118	R122	N150	H151	H199	R254	H281
PP5	D242	H244	D271	R275	N303	H304	H352	R400	H427

Protein Phosphatases, Fig. 1 Members of the phosphoprotein phosphatase (PPP) family have conserved active sites. Human PP1 (green, PDB code 4MOV), PP2A (light blue, 2 IE4), PP2B (red, 4F0Z), and PP5 (yellow, 1S95) crystal structures were aligned and superimposed using the PyMOL Molecular Graphics System, version 2.2.0 (Schrödinger, LLC). The side chains of nine conserved active site residues were extracted from each structure and drawn in stick representation. Residues on the image are numbered following the canonical human PP1 sequence. The equivalent residues for PP2A, PP2B, and PP5 are tabled below the structure. Purple spheres represent the positions of the two metal ions coordinated in the PP1 active site, which are very similar the positions of metal ions taken in the other structures

all the same conserved active site residues found in all PPP enzymes (with the exception of a single PP5-specific change [N308D]) (Fig. 1). The observation that a number of water molecules occupy the active site with the substrate peptide likely explains how PP5 and other PPPs can accommodate so many substrates with differing side chains, as the water molecules can play a role in molding them to a conserved active site.

As noted above, PPP catalytic enzymes, although serine/threonine-specific, tend to be promiscuous and thus have their activity controlled or directed by additional binding proteins. Research that started with PP1 and PP3 (PP2B) and more recently with PP2A and PP4 have uncovered short linear motifs or SLiMs that direct protein/protein interactions between catalytic and regulatory subunits and help recruit substrates to the active site. Protein phosphatase SLiMs tend to be short (four to eight amino acids), reside in intrinsically disordered regions, and have a few key interacting amino acid side chains. Each will be discussed below, and it is expected that additional SLiMs will be discovered for the remaining PPP enzymes. To date only PP5 and PP7 do not have characterized regulatory subunits, although PP5 does have an N-terminal extension that contributes to its regulation.

PP1

PP1 was originally discovered as the protein phosphatase that resides on mammalian glycogen particles to control the activity of the glycogen metabolic enzymes in response to hormonal signals and cellular needs. PP1 has no affinity for glycogen, and the glycogen-bound enzyme in muscle was found to co-purify with a regulatory protein termed G_M. Identification of a different protein that directs PP1 activity toward smooth muscle myosin (MYPT1) led to the concept of PP1 targeting or regulatory subunits. This in turn led to the identification of a PP1 regulatory subunit binding motif, or SLiM, designated the RVxF motif. This motif is somewhat degenerate ([KR][KR][VI]{FIMYDP}[FW], with amino acids {FIMYDP} not accommodated at the indicated position), is found in nearly all PP1 interactors, and has been characterized in over 200 human proteins. In addition to regulatory proteins, this motif is also found in some PP1 substrates, facilitating direct recruitment of the phosphatase. Additional, much less frequently occurring PP1 binding motifs have now been identified and include the G/SILK, MyPhoNE, and SpiDoC motifs (Brautigan and Shenolikar 2018). PP1 regulatory subunits target PP1 to specific locations in the cell to be in the vicinity of substrates and also control the substrate specificity of the somewhat promiscuous, although serine/threonine-specific, PP1 catalytic subunit.

PP2A/4/6

Phylogenetic analysis of the PPP catalytic subunits from any eukaryotic organism always groups PP2A, PP4, and PP6 together, indicating they are more like each other than any other PPP enzyme and likely evolved from a common ancestral phosphatase. PP2A, PP4, and PP6 are all exquisitely sensitive to okadaic acid, which means that early studies that attributed effects of okadaic acid on cellular processes to PP2A function may have been erroneous, as the effects could have been due to the inhibition of PP4 or PP6 complexes.

PP2A is relatively abundant, regulates an extraordinary range of cellular processes, and is composed of three subunits. The PP2A core enzyme is the catalytic subunit (PP2AC) and a scaffolding A or PR65 subunit, which is composed solely of HEAT repeats. Humans express two versions of each of these subunits. The core then associates with one of several B subunits that belong to four sequence unrelated families: B (also known as B55 or PR55), B′ (B56 or PR61), B″ (PR48/PR72/PR130), and B‴ (PR93/PR110). Each B subunit family protein is expressed from two to five genes with several generating splice variants. It is the B subunits that confer substrate specificity on PP2A holoenzyme complexes. The PP2A catalytic subunit is reversibly methyl-esterified on its carboxy-terminal leucine (as are PP4 and PP6). Methyl-esterification and demethylation are controlled by leucine carboxyl methyltransferase (LCMT-1) and PP2A methylesterase (PME-1), both of which appear to be specific for the enzymes PP2A, PP4, and PP6 (Brautigan and Shenolikar 2018). For PP2A, it is currently thought that this covalent modification controls the association of the PP2A core with B subunits and possibly the incorporation of metal ions into the active site.

PP4 is a predominantly nuclear serine/threonine phosphatase composed of a catalytic subunit (PP4C) and two regulatory subunits (R2 and R3, with A and B versions of R3) (Ueki et al. 2019). R3 is composed of ARM/HEAT repeats and an N-terminal EVH1 domain. It has been established that the EVH1 region recruits PP4 substrates that house the SLiM FXXP. Recent *in silico* and proteomic analysis has uncovered many new potential substrates for PP4 that have the FXXP SLiM (Ueki et al. 2019).

PP6, also a serine/threonine phosphatase, exists as a trimer in cells. Three related human proteins, SAPS1, SAPS2, and SAPS3 (Sit4-associated proteins, also called PPP6R1, PPP6R2, and PPP6R3), associate with the PP6 catalytic subunit. A common N-terminal region of each, known as the SAPS domain, is required to associate with PP6C. Mass spectrometry analysis of PP6 complexes uncovered three ankyrin repeat-containing proteins as regulatory proteins for PP6, and there are three in humans which can form trimers with PP6C and SAPS: ANKRD28, ANKRD44, and ANKRD52 (Brautigan and

Shenolikar 2018). To date no SLiM has been identified for PP6 substrates.

PP3 (PP2B/Calcineurin)

PP3 was originally termed PP2B/calcineurin and identified as a Ca^{2+}-stimulated phosphatase activity *in vitro*. Purification from the skeletal muscle and brain uncovered a catalytic A subunit and Ca^{2+} binding (EF-hand) B subunit. After binding Ca^{2+} PP3 can be further activated by binding Ca^{2+}-calmodulin. In the absence of Ca^{2+}, PP3 is inactive, due to an autoinhibitory region from the A subunit docking at the active site. Upon Ca^{2+} and Ca^{2+}-calmodulin binding, this autoinhibitory peptide is removed from the active site, allowing substrates to dock for dephosphorylation. The best characterized substrate for PP3 is the T-cell transcription factor, NFAT (nuclear factor of activated transcription), which when dephosphorylated shuttles from the cytosol to the nucleus to initiate an immune response. Detailed studies of PP3 and NFAT identified a SLiM on NFAT that binds PP3 (the PIXIXIT motif, where X can be any amino acid). This SLiM has subsequently been identified on most PP3 substrates. A second substrate-recruiting site has also been found in many PP3 substrates and is known as LXVP (Brautigan and Shenolikar 2018).

PP5

PP5 is somewhat unique among the PPP enzymes because it has no known regulatory subunits. This enzyme has an N-terminal extension composed of tetratricopeptide repeats (TPR) that fold back and inhibit the enzyme, with a key glutamate (E76) extending into the active site to act as a phosphomimic. How PP5 and likely all PPP enzymes dock substrates was revealed when the substrate peptide Cdc37 was linked to the tail of PP5 and co-crystalized (described above in the PPP introduction) (Oberoi et al. 2016).

PPM Family of Protein Phosphatases

The PPM enzymes were originally identified and named based on the need to add exogenous Mg^{2+} to enzyme assays to detect their activity. Purification, cloning, and genomic analysis revealed that the PP2C enzymes have a unique sequence, evolving independently of the PPP family enzymes, and they were thus renamed the Mg^{2+}/Mn^{2+}-dependent protein phosphatases (PPM) (Brautigan and Shenolikar 2018). This class also includes the mitochondrial pyruvate dehydrogenase phosphatases. Like the PPP enzymes, the metal ions activate a water molecule to drive catalysis. To date, no regulatory or accessory subunits are known for PPM enzymes, but most have additional domains outside of the catalytic core. There are 16 members (Table 1) in the human family, and most plant genomes have many more (e.g., there are more than 80 PPMs in *Arabidopsis thaliana*).

PTP Family of Protein Phosphatases

With the discovery of tyrosine phosphorylation in 1979 and the identification of tyrosine-specific protein kinases, the search began for tyrosine-specific protein phosphatases. The first, PTP1B, was discovered in 1988 and is now known to counter insulin and leptin signaling. Members of the PTP family (Table 1) all share a conserved PTP domain and catalytic signature designated CX_5R, with the C and R being critical for catalysis (Moorhead et al. 2007). Subsequent cloning and genomic analysis identified many more PTP family members, including the first mitogen-activated protein kinase phosphatase (MAPKP) that was the founding member of the dual-specificity phosphatase subfamily known as the DUSPs (Table 1). They are considered "dual-specificity" because all protein phosphatases characterized before this were specific for serine/threonine or tyrosine, while these enzymes could dephosphorylate the TxY motif of MAPK activation loops. Members of this subfamily are placed here based on amino acid sequence. Characterization has confirmed they are phosphatases, although many act on non-protein substrates (RNA, glycogen, starch, phosphoinositides; Moorhead et al. 2007) or dephosphorylate serine or threonine residues on proteins.

Aspartate-Based Family of Protein Phosphatases

Eukaryotic RNA-polymerase II is hyper-phosphorylated in a C-terminal repeat of the sequence YSPTSPS (on serine 2 and 5) during

elongation of transcripts and is dephosphorylated to initiate another round of transcription. A biochemical search for an activity that would dephosphorylate the C-terminal tail of RNA-polymerase II identified FCP, a serine/threonine phosphatase (Moorhead et al. 2007). The human genome has seven related, but smaller, versions of FCP designated SCP (small CTD phosphatase). FCP/SCP have no additional subunits, but do have accessory domains outside the catalytic core. To date, their only known substrate is RNA-polymerase II. Unlike the other protein phosphatases, FCP/SCP catalysis is driven by aspartates in the motif DXDXT/V.

Chronophin is also an aspartate-based protein phosphatase and belongs to the HAD family of proteins (Table 1). To date, chronophin's only known substrate is cofilin, a regulator of actin dynamics. It is postulated that other HAD family members could be protein phosphatases, although these enzymes are currently only known to target cellular metabolites, not proteins.

Pharmacological Intervention

The biological importance of the PPP family phosphatases is highlighted by the widespread production of an enormous array of potent and chemically diverse small-molecule inhibitors of PPP enzymes by a wide range of organisms. Some degree of specificity exists between inhibitors (microcystin, nodularin, okadaic acid, tautomycin, fostriecin, and others) and the PPP enzymes they target. Potent inhibition has been described for PP1, PP2A, PP4, PP5, and PP6, and only very weak inhibition of PP3 (PP2B) and PP7 (Brautigan and Shenolikar 2018). These molecules have no effect on PPM, PTP, and aspartate-based family enzymes. As such, they have proven to be useful research tools for implicating specific types of protein phosphatases in specific cellular events (although caution needs to be taken when interpreting these results (Brautigan and Shenolikar 2018)) and for locking the phospho-status of proteins in extracts for analysis. In addition, conjugation of microcystin to affinity matrices has facilitated the purification and analysis of microcystin-sensitive protein phosphatase complexes.

From a pharmaceutical industry perspective, the serine/threonine protein phosphatases were initially not thought to be ideal drug targets due to their broad substrate specificities. This view is changing, with the realization that the activity of holoenzyme complexes can be controlled with high specificity by targeting the regulatory rather than the catalytic subunits. This can include activating or inhibiting activity against a specific substrate, as required. PP3 (PP2B), in contrast, can be directly inhibited by two natural products that target the active site and have proven to be invaluable for organ transplants. These two bacterially produced molecules, FK506 and cyclosporin, bind the proteins FKBP12 and cyclophilin, respectively, and the drug-protein complexes in turn bind PP3 (PP2B). This abolishes the ability of PP3 (PP2B) to dephosphorylate the key T-cell transcription factor NFAT, which prevents its translocation to the nucleus and initiation of an immunogenic gene response, thereby preventing organ rejection.

Aberrant tyrosine phosphorylation has been linked to multiple human diseases, including cancer, diabetes, neurodegenerative and infectious diseases, and autoimmune disorders, and the tyrosine kinases have been key therapeutic targets for decades. Their counterpart PTPs have also been linked to a wide range of diseases and thus represent novel drug targets. The PTPs all share a common catalytic mechanism requiring the signature C and R amino acids of the CX_5R motif. The PTPs have, to some degree, evolved unique active sites to accommodate novel substrates that can be exploited for drug targeting. One recent novel approach to develop selective inhibitors of PTPs was to develop bivalent ligands that dock the active site and a secondary binding pocket (He et al. 2013). Although no PTP inhibitors are in the clinic as yet, selective inhibitors have been produced for PTP1B, TC-PTP, SHP2, Lyp, mPTPA, HePTP, PTPβ, PTPγ, MKP-3, CD45, Cdc25, YopH, PTPRO, VHR, MKP-1, and mPTPB (He et al. 2013; Stanford and Bottini 2017).

As we gain a better appreciation of the physiological significance and complexity of phosphatase regulation, these previously "undruggable" enzymes have become more approachable and now represent a significant pool of molecular targets that can be exploited therapeutically.

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Protein S-Nitrosylation

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Synonyms

S-Nitrosation

Definition

Protein S-nitro(sy)lation is a reversible posttranslational modification, resulting from the covalent attachment of a nitric oxide (NO)-related group to the thiol (or, perhaps more properly, thiolate anion, S^-) of a specific cysteine residue. Similar to other posttranslational modifications, S-nitrosylation can regulate the enzymatic activity, conformation, binding properties, or intracellular localization of a protein. In the setting of normal/low levels of NO, the resulting protein S-nitrosylation mediates physiological signaling pathways, often critical for cellular homeostasis and survival. However, high levels of NO, as seen in many disease or inflammatory states, can result in aberrant protein S-nitrosylation of cysteine residues not normally modified in healthy cells, resulting in dysfunctional or deleterious cell function. Note that the term "S-nitros*ylation*" was adopted to reflect the biological actions of NO on redox-sensitive proteins, akin to phospho*rylation* and methy*lation*, through modification of the sulfur (S) atom of a cysteine residue. Nitrosylation can also refer to direct addition of NO to a metal center, such as ferrous heme in a metalloprotein. In addition, another form of reactive nitrogen species (RNS), peroxynitrite ($ONOO^-$), can induce nitration of tyrosine residues, also affecting protein function and structure (Ischiropoulos et al. 1992). Thus, metal nitrosylation and tyrosine nitration are clearly distinct from S-nitrosylation of cysteine residues. The first protein whose activity was shown to be regulated by S-nitrosothiol formation on critical cysteine residues (and thus to be S-nitrosylated) was the *N*-methyl-D-aspartate (NMDA)-type of glutamate receptor (NMDAR) in the brain (Lipton et al. 1993).

Basic Characteristics

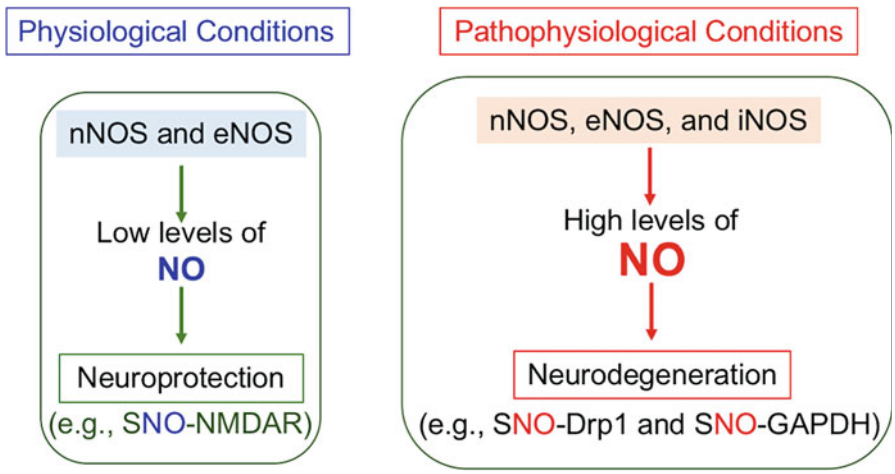
Generation of NO in Mammalian Cells

Many mammalian cells constitutively produce moderate but sufficient levels of NO to regulate physiological processes such as dilation of blood vessels, cardiac contractility, and memory formation in the brain. NO production in mammalian cells entails activation of a family of NO synthases (NOSs) that converts L-arginine to

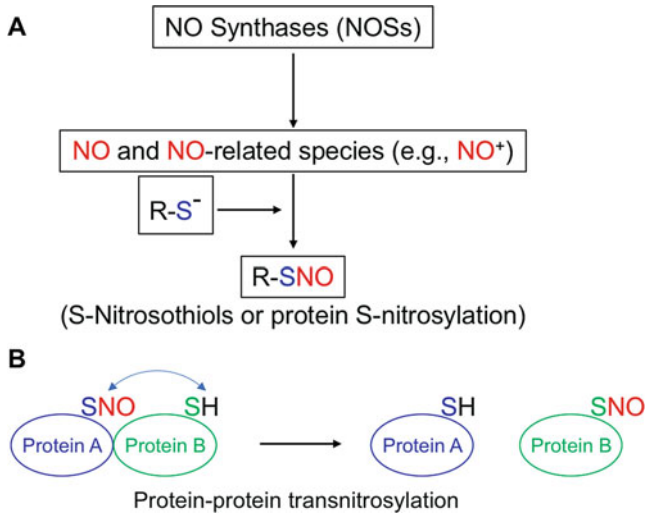
L-citrulline and NO (in the presence of low arginine levels, reactive oxygen species (ROS) are produced instead of RNS). Three NOS members exist in mammals: neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS, NOS3, or cNOS). nNOS and eNOS activities are stimulated by intracellular calcium, whereas iNOS generates NO in a calcium-independent manner. For example, in the case of nNOS in the brain, mild activation of NMDARs at neuronal synapses triggers calcium influx that moderately activates nNOS, which exists in a protein complex tethered to the NMDAR via PDZ domains in PSD-95. The physiological production of NO supports synaptic plasticity and neuronal survival through various protein S-nitrosylation reactions, although S-nitrosylation-independent effects of NO (e.g., via activation of guanylate cyclase and formation of guanosine-3′,5′-monophosphate [cGMP]) also mediate various biological effects (Fig. 1). In contrast, excessive activation of NMDARs, particularly extrasynaptic receptors, causes pathological production of RNS including NO, contributing to aberrant protein S-nitrosylation reactions that can result in protein misfolding, mitochondrial dysfunction, transcriptional dysregulation, synaptic damage, and other neurotoxic events, thus contributing to various neurodegenerative diseases (Nakamura et al. 2013). Consistent with these findings, genetic ablation of nNOS ameliorates neuropathological features seen in mouse models of not only NMDA toxicity but also ischemia and Parkinson's disease (PD).

Protein S-Nitrosylation

As delineated above, protein S-nitrosylation results from the targeted covalent attachment of NO-related species to a specific thiol group (RSH or, more properly, thiolate anion, RS^-), forming an S-nitrosothiol (R-SNO or SNO-protein) (Fig. 2a). The chemical intermediate thought to be involved in S-nitrosothiol formation is nitrosonium cation (NO^+), which is not, however, present in a free



Protein S-Nitrosylation, Fig. 1 NO-dependent signaling pathways under physiological and pathophysiological conditions. At physiological levels, NO generally supports normal neuronal signaling and function, in many cases enhancing neuronal survival in the brain. In contrast, excessive production of NO from iNOS, eNOS, or nNOS can contribute to protein misfolding, mitochondrial/bioenergetic dysfunction, ER stress, synaptic injury, and other forms of neuronal damage in neurodegenerative disorders. Physiologically produced NO provides neuroprotection in part through protein S-nitrosylation of the NMDAR (forming SNO-NMDAR). Additionally, non-S-nitrosylation pathways, such as activation of soluble guanylate cyclase (sGC) to produce cGMP, may mediate some neuroprotective actions of NO. Under pathophysiological conditions, high levels of NO contribute to neurodegeneration via aberrant protein S-nitrosylation (e.g., formation of SNO-Drp1 and SNO-GAPDH). Notably, a full S-nitrosylation motif (SNO motif) is typically needed near the target cysteine to form an S-nitrosothiol at physiological levels of NO (see main text for details); however, excessive production of NO-related species can cause aberrant S-nitrosylation even if a partial SNO motif is present and potentially in the absence of nitrosylating enzymes



Protein S-Nitrosylation, Fig. 2 Mechanisms for production of S-nitrosylated proteins. (**a**) In mammalian cells, NO synthases (NOSs) produce NO, allowing subsequent formation of NO-related species. Protein S-nitrosylation emerges from a chemical reaction between thiolate anion (RS^-) and NO-related species that have an S-nitrosylating activity (such as NO^+). (**b**) Protein-protein transnitrosylation entails transfer of an NO group from one protein to another protein. Importantly, direct protein-protein interaction is required for transnitrosylation reactions to occur, because such a protein complex provides a platform for enzymatic denitrosylation from one protein and S-nitrosylation of another recipient protein

state at physiological pH. For donation of NO-related species in cells, a transition metal is probably required to facilitate electron transfer. As an alternative mechanism for R-SNO formation, direct chemical reaction between a thiyl radical (RS·) and ·NO has been suggested (Tannenbaum and White 2006).

Protein S-nitrosylation can also be accomplished via transfer of an NO-related species from one thiol group to another thiol (Nakamura et al. 2013). This process is known as trans(S-) nitrosylation and likely represents a key mechanism for generation of SNO-proteins in vivo. In general, S-nitrosylating enzymes (or nitrosylases) facilitate donation of NO-related species to a protein, while denitrosylating enzymes catalyze removal of NO. Several enzymes that participate in other reactions have been reported to mediate denitrosylation as well (e.g., S-nitrosoglutathione reductase (GSNOR) and thioredoxin) (Sengupta and Holmgren 2013; Benhar et al. 2009; Smith and Marletta 2012). In the case of trans-nitrosylation, the protein donating NO acts as the nitrosylase, and the protein accepting NO as the denitrosylase (Fig. 2b).

Other chemical reactions can occur following protein S-nitrosylation. For example, if S-nitrosylation occurs on one of vicinal thiols, it will facilitate disulfide bond formation (Cho et al. 2009; Nakamura et al. 2013). Chemically speaking, although dependent on the local redox milieu, the NO group in the R-SNO is often a good "leaving group." As NO leaves, it may facilitate reaction of ROS with the remaining free sulfhydryl group to produce additional oxidation products, such as sulfenic (R-SOH), sulfinic ($R-SO_2H$), and sulfonic ($R-SO_3H$) acid derivatives (Nakamura et al. 2013). Importantly, these other oxidation products may also promote aberrant protein activity, misfolding, or aggregation. In fact, since the higher oxidation states (i.e., $R-SO_3H$) represent irreversible reactions, the abnormal effect on the target protein may be permanent.

Specificity of Protein S-Nitrosylation

S-Nitrosylating and denitrosylating enzymes facilitate protein S-nitrosylation and denitrosylation, respectively, and the local redox environment also exerts a strong influence, as alluded to above. Several additional factors have been proposed for

specific targeting of S-nitrosothiol (-SNO) formation to a specific cysteine residue (Hess et al. 2005; Lipton et al. 1993; Nakamura et al. 2013; Smith and Marletta 2012). A major determinant of SNO specificity involves the presence of nucleophilic amino acid residues (e.g., lysine, histidine, aspartate, and glutamate) flanking or in proximity to the target cysteine, constituting a "SNO motif," which facilitates reaction of the target thiol with an NO-related species (Hess et al. 2005; Stamler et al. 1997). In this scenario, sidechains of the nucleophilic amino acids (typically acidic or basic) lower the pKa of the target cysteine thiol to enhance SNO formation (Stamler et al. 1997; Hess et al. 2005).

While a full SNO motif is typically required for a target cysteine to form an S-nitrosothiol at physiologically low levels of NO-related species, excessively high levels of NO, as occur in neurodegenerative disorders, can result in S-nitrosylation of cysteine residues that have only a partial SNO motif, thus promoting "aberrant" protein S-nitrosylation under these conditions. Intramembrane localization of a target protein can also affect specific SNO formation. In this regard, the hydrophobic environment present in lipid membranes can promote nitrosylation by NO or other hydrophobic NO-related species. Additionally, localization of a target cysteine near NOS can facilitate S-nitrosylation because of the local increase in NO concentration.

As outlined above, transnitrosylation represents an important mechanism for cellular SNO formation and transfer from one protein to another. In this case, precisely regulated transfer of the NO proceeds only when a specific protein-protein interaction occurs (Fig. 2b). For example, interaction of a SNO-protein with its binding partner protein can lead to specific S-nitrosylation of the acceptor protein and denitrosylation of the donor protein.

Examples of Neuroprotective Signaling Pathways Triggered by S-Nitrosylation in the Brain: S-Nitrosylation of NMDARs

Physiological levels of NO typically afford neuroprotection. For example, addition of an NO groups can act as neuroprotective agent via S-nitrosylation of NMDARs, DJ-1, or caspases. Here, we review the effect of S-nitrosylation of NMDARs. Hyperactivation of NMDARs (especially extrasynaptic, as opposed to synaptic, NMDARs) has been shown to trigger ROS/RNS generation and to activate downstream enzymatic cascades that contribute to neuronal and synaptic damage in a wide range of neurodegenerative diseases, including Alzheimer's disease (AD), PD, and stroke. Our group and subsequently others have shown that physiological levels of NO-related species can prevent excessive activation of this receptor via S-nitrosylation of multiple NMDAR subunits, thus mediating the neuroprotective action of NO (Choi et al. 2000; Lipton et al. 1993; Lipton 2006, 2007). In contrast, a decrease in S-nitrosylation of NMDARs has been reported to exacerbate neuropathology in a mouse model of prion disorders.

NO-related species can S-nitrosylate NMDARs at multiple cysteine residues on different NMDAR subunits (Lipton 2006, 2007; Lipton et al. 1993). These include Cys744 and Cys798 on the GluN1 (formerly termed NR1) subunit, and Cys87, Cys320, and Cys399 on the GluN2A (formerly NR2A) subunit. Note that high ambient oxygen levels facilitate the formation of an intramolecular disulfide bond between Cys744 and Cys798 on GluN1, thus preventing S-nitrosylation of these sites; however, under relatively hypoxic conditions, as seen in the normal brain, the free thiol groups on these GluN1 cysteines are maintained. This renders these GluN1 cysteine residues vulnerable to S-nitrosylation, as observed in the X-ray crystal structure of GluN1. Notably, S-nitrosylation of Cys744 and Cys798 on GluN1 by themselves exerts little effect on the electrophysiological activity of the NMDAR; however, formation of S-nitrosothiol on the GluN1 subunit increases the sensitivity of cysteine residues on GluN2A to S-nitrosylation (i.e., at Cys87, Cys320, and Cys399). Hence, Cys744 and Cys798 on GluN1 act as a redox sensor via an apparent allosteric influence on GluN2A nitrosylation status (Choi et al. 2000; Lipton et al. 1993; Lipton 2006, 2007). Poly-S-nitrosylation of the GluN2A subunit inhibits excessive NMDAR activity, thus limiting ion influx, and representing a negative feedback mechanism for the NMDAR-nNOS-NO pathway (Choi et al. 2000).

Examples of Neurodegenerative Signaling Pathways Triggered by Aberrant S-Nitrosylation in the Brain: S-Nitrosylation of Drp1 and GAPDH

As alluded to above, excessive production of NO-related species can result in aberrant S-nitrosylation of a large number of proteins. In the brain, this contributes to the development of neurodegenerative pathology, including mitochondrial dysfunction, protein misfolding, and synaptic damage. Aberrantly S-nitrosylated proteins include SNO-Drp1 (dynamin-related protein 1), SNO-GAPDH (glyceraldehyde-3-phophate dehydrogenase), SNO-PDI (protein disulfide isomerase), SNO-MMP9 (matrix metallopeptidase 9), SNO-MEF2C (myocyte enhancer factor 2C), SNO-PINK1 (PTEN-induced kinase 1), SNO-Parkin, and SNO-XIAP (X-linked inhibitor of apoptosis) to name but a few. In this chapter, we highlight the pathological consequences of SNO-Drp1 and SNO-GAPDH, as these SNO-proteins may contribute to the pathogenesis of multiple neurodegenerative diseases (Hess et al. 2005; Nakamura et al. 2013; Hara et al. 2006).

In neurons, efficient energy production from healthy mitochondria is critical to the maintenance of membrane potential and synaptic transmission. Mitochondria are known to constantly undergo the processes of fission and fusion, collectively termed mitochondrial dynamics. Mitochondrial dynamics regulate the size, shape, number, and intracellular distribution of mitochondria. Moreover, mitochondrial fusion and fission can influence ROS generation, apoptosis, bioenergetics, and mitophagy. Hence, well-balanced mitochondrial dynamics represents a critical cellular process for normal synaptic function and neuronal survival. In a many neurodegenerative diseases, including AD, PD, and Huntington's disease (HD), abnormally fragmented, small-sized mitochondria have been observed, consistent with the notion that abnormal mitochondrial dynamics participate in the pathogenesis of the disease.

As a GTPase, Drp1 functions as a GTP-dependent mitochondrial fission protein. Our group and subsequently others found that oligomeric Aβ peptide or mutant huntingtin (mHtt) induces nitrosative stress, thus causing aberrant S-nitrosylation of Drp1 (forming SNO-Drp1) at Cys644 in both human brain and experimental models of AD and HD (Cho et al. 2009). S-Nitrosylation of Drp1 hyperactivates its GTPase activity, leading to pathologically fragmented mitochondria with damage cristae that are bioenergetically compromised. In contrast, expression of non-nitrosylatable mutant Drp1 prevents excessive mitochondrial fragmentation and synaptic damage in neurons exposed to oligomeric Aβ or mHtt, supporting the notion that SNO-Drp1 plays a causal role in the pathogenesis of AD and HD. Moreover, Drp1 has now been shown to be aberrantly S-nitrosylated in human brains with various neurodegenerative disorders (Cho et al. 2009). These results support our hypothesis that SNO-Drp1-dependent mitochondrial fragmentation mediates a signaling pathway common to several neurodegenerative diseases that leads to compromised mitochondrial bioenergetics, thus contributing to synaptic damage and neuronal loss.

As a second example of aberrant protein S-nitrosylation in neurodegenerative disorders, we next consider SNO-GAPDH. GAPDH is well characterized as a key enzyme in glycolysis, but the protein also plays an important role in transcriptional regulation and apoptosis. S-Nitrosylation of GAPDH mediates a neurotoxic effect of NO not only via inhibition of GAPDH catalytic activity but also by increased binding to the ubiquitin E3 ligase Siah1 (Hara et al. 2006). When S-nitrosylated, GAPDH interacts with Siah1, and the SNO-GAPDH/Siah1 complex translocates to the nucleus because of the nuclear localization signal of Siah1. Once in the nucleus, Siah1 enhances degradation of nuclear proteins, augmenting an apoptotic cascade. In mouse models, this SNO-GAPDH pathway appears to contribute to the neuropathogenesis of PD, HD, stroke, and possibly other neurodegenerative diseases (Hara et al. 2006).

Drugs Affecting Protein S-Nitrosylation

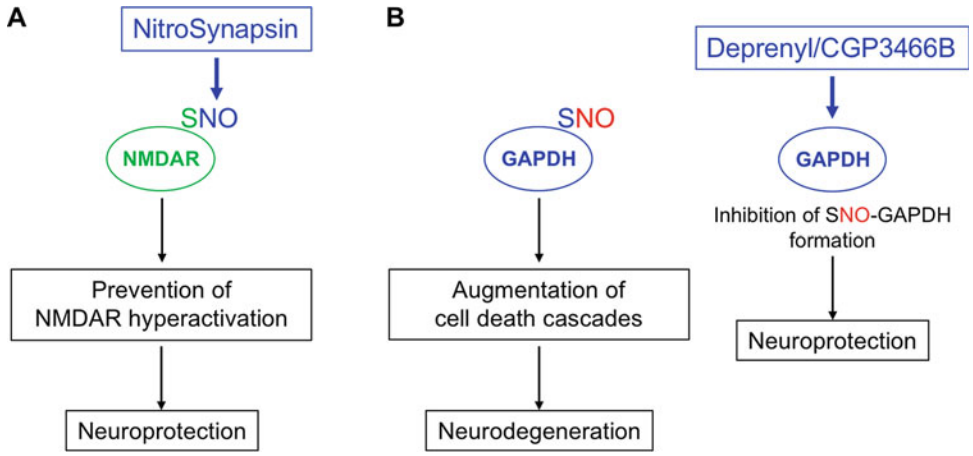
Recent studies with mass spectrometry-based S-nitrosoproteomic techniques have identified several hundreds of S-nitrosylated proteins not only under pathophysiological conditions but also in normal cells (Doulias et al. 2013). Intriguingly,

although many proteins can be aberrantly modified by S-nitrosylation in neurodegenerative brains, in several cases, prevention of S-nitrosylation of just a single protein, such as PDI or Drp1, can mitigate neuropathological features such as protein misfolding or mitochondrial impairment, respectively (Cho et al. 2009). Additionally, pharmacological interventions that affect either physiological or aberrant S-nitrosylation pathways can be harnessed to protect neurons. Two such approaches are highlighted here that selectively regulate SNO-NMDAR and SNO-GAPDH formation, thus potentially protecting neurons in neurodegenerative disorders.

NitroSynapsin

Our group recently developed a neuroprotective small compound, named "NitroSynapsin" that can protect neurons via dual allosteric action on the NMDAR. First, the aminoadamantane moiety of the drug functions as an *u*ncompetitive *f*ast-*o*ff rate antagonist of the NMDAR-associated ion channel (termed "UFO" type of inhibition), similar to our findings with the FDA-approved aminoadamantane drug, memantine. Second, the new drug is a chemical adduct between the aminoadamantane moiety and a nitro group, which functions as a "warhead" on the aminoadamantane that can S-nitrosylate the NMDAR, thus providing additional efficacy for inhibiting excessive receptor activation (Fig. 3a). Critically, the aminoadamantane group serves to target the nitro group to the NMDAR because, like memantine, the aminoadamantane moiety is an "open-channel blocker," meaning that it prefers to bind to excessively open/pathologically-over-activated channels. This mechanism of action also tends to spare physiological/shorter opening of channel during synaptic transmission. Unlike other known NMDAR antagonists that indiscriminately inhibit all NMDARs with high affinity and thus block synaptic activity, the unique features of memantine and NitroSynapsin prevent the drugs from blocking normal synaptic function, thereby manifesting fewer clinical side effects (Lipton 2006, 2007).

Additionally, our group demonstrated that the FDA-approved drug, memantine, as well as NitroSynapsin, preferentially blocks NMDARs at extrasynaptic sites because these ion channels tend



Protein S-Nitrosylation, Fig. 3 NitroSynapsin and deprenyl/CGP3466B show neuroprotective activities via manipulation of protein S-nitrosylation. (**a**) NitroSynapsin contains a nitro group (-NO_2) linked to the memantine moiety. This allows NitroSynapsin to serve as an S-nitrosylating agent specifically targeted to NMDARs. Accordingly, NitroSynapsin can provide increased inhibition of hyperactivated NMDARs via S-nitrosylation of the receptor in addition to memantine-dependent blockage of the ion channel activity. (**b**) CGP3466B (a deprenyl derivative) affords neuroprotection at least in part via inhibition of SNO-GAPDH formation. S-Nitrosylation of GAPDH initiates the formation of a protein complex with Siah1, enhancing its nuclear localization (due to the nuclear localization signal of Siah1) and stimulating cell death pathways. Because CGP3466B (TCH346 or omigapil) directly binds GAPDH and selectively blocks S-nitrosylation of the protein, this drug can serve as a neuroprotective agent

to be excessively/tonically open (i.e., hyperactivated) in neurodegenerative disorders, in contrast to the physiological/phasic activity of synaptic receptors (Lipton 2006, 2007). Since excessive activation of NMDARs can contribute to the pathogenesis of a number of neurodegenerative diseases, this type of drug action affords a degree of neuroprotection. Unfortunately, memantine is positively charged, and as neurons become ill, they depolarize and are relatively more positively charged, thus repelling memantine out of NMDAR-operated ion channels. NitroSynapsin has the ability to compensate for this shortcoming by offering a second site of inhibitory action at the S-nitrosylation sites on the NMDAR. Thus, the dual action of NitroSynapsin at NMDARs affords superior performance to memantine in several models of neurodegenerative and neurodevelopmental disorders (Lipton 2006, 2007). In the future, clinical trials will be needed to see if the potential disease-modifying activity of NitroSynapsin can be realized in humans.

Deprenyl and CGP3466B (TCH346 or Omigapil)

GAPDH has been reported to be a direct target of the putative neuroprotective compound, deprenyl (Selegiline®). Clinically, deprenyl is claimed to delay the progression of symptoms in early PD and AD. The mechanism of action of deprenyl was initially thought to involve selective inhibition of monoamine oxidase-B (MAO-B). However, the deprenyl derivative, CGP3466B (TCH346 or Omigapil), which exhibits virtually no MAO-B inhibition, displayed neuroprotective activity equivalent to or even more potent than deprenyl itself in cell-based and animal models of PD. The findings in these and other studies are consistent with the notion that deprenyl (and related compounds) acts as a protective drug through blockade of GAPDH-dependent cell death pathways; alternatively, deprenyl may exert antioxidant effects or possibly even increase catecholamine content rather than inhibiting MAO-B enzymatic activity. Further along these lines, Solomon Snyder's group more recently found direct binding of deprenyl or CGP3466B to GAPDH that prevents formation of SNO-GAPDH, thereby inhibiting the interaction of GAPDH and Siah1; this effect blocks nuclear translocation of GAPDH (Hara et al. 2006) (Fig. 3b). Based on these encouraging preclinical results, human clinical trials with CGP3466B were then conducted for PD and amyotrophic lateral sclerosis (ALS). Unfortunately, although CGP3466B proved to be nontoxic and nonaddictive in phase I trials, the drug failed to show neuroprotective effects in either disease. In summary, although the neuroprotective action of deprenyl and CGP3466B may in part arise from its ability to block the SNO-GAPDH-Siah pathway, drug efficacy in human clinical trials remains unproven.

Disclosure of Conflicts of Interest

S.A.L. is the inventor on worldwide patents for the use of memantine and NitroSynapsin for neurodegenerative disorders. Per guidelines of Harvard University, his former institution where the work was initially performed, S.A.L. participates in a royalty-sharing agreement with his former institution Boston Children's Hospital/Harvard Medical School, which licensed the drug memantine (Namenda®) to Forest Laboratories, Inc./Allergan. S.A.L. is also a scientific co-founder of Adamas Pharmaceuticals, Inc., which developed or co-developed the FDA-approved drugs NamendaXR®, Namzaric®, and Gocovri®.

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Protein Tyrosine Kinases

► Tyrosine Kinases

Proteinases

► Nonviral Peptidases

Proteolytic Enzymes

► Nonviral Peptidases

Protocidal Drugs

► Antiprotozoal Drugs

Proton-Sensing GPCRs

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Synonyms

GPR4; Ovarian cancer G protein-coupled receptor 1 (OGR1, GPR68); T cell death-associated gene 8 (TDAG8, GPR65)

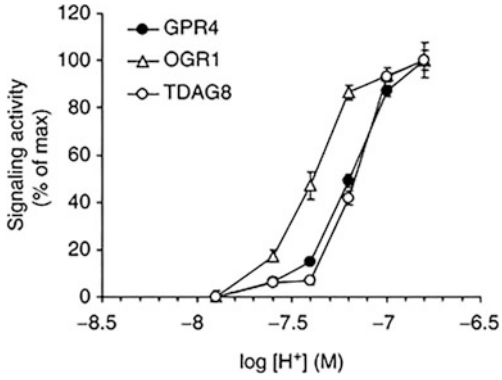
Definition

GPR4, OGR1, and TDAG8 are GPCRs regulated by extracellular pH in the physiological range. These receptors are silent at pH 7.8 and fully activated at pH 6.8.

Basic Characteristics

The receptors GPR4, OGR1, and TDAG8 form a subfamily of GPCRs distantly related to purinergic and angiotensin receptors. These receptors are activated by neutral to slightly acidic extracellular pH (Ludwig et al. 2003; Wang et al. 2004), stimulating second messenger formation as effectively as other receptors activated by, e.g., monoamines, lipids, or peptides, suggesting that protons are the genuine activating ligands (Seuwen et al. 2006). A fourth molecule, the receptor G2A (GPR132), is also related to this group. However, recent data suggest that G2A is a receptor for oxidized fatty acids and N-acyl amino acids (Foster et al. 2019). G2A does not exhibit structural elements required for pH sensing (see below), and activation by acidic pH could not be confirmed (Seuwen et al. 2006; Foster et al. 2019).

The response of GPR4, OGR1, and TDAG8 to extracellular pH in recombinant cell systems is shown in Fig. 1. OGR1 appears slightly more sensitive to extracellular protons than GPR4 and TDAG8. In the experimental setup used,



Proton-Sensing GPCRs, Fig. 1 pH-dependent activation of recombinant receptors expressed in host cells. GPR4 was stably expressed in Hela cells and OGR1 and TDAG8 in CCL39 hamster fibroblasts. For OGR1, formation of inositol phosphates was measured, and for GPR4 and TDAG8, cAMP formation was determined. Data were normalized to maximal stimulation (100%). Untransfected host cells show no pH-dependent activation of second messenger formation. Experimental details are as described in (Ludwig et al. 2003)

half-maximal activation of receptors was observed at pH 7.45 ± 0.04 for OGR1 ($n = 14$), pH 7.18 ± 0.02 for GPR4 ($n = 3$), and pH 7.15 ± 0.02 for TDAG8 ($n = 6$). As described in (Ludwig et al. 2003), the pH values refer to buffers adjusted at room temperature. To obtain pH at 37 °C, 0.15 pH units should be subtracted.

To date, we could not identify other GPCRs showing a similar pH-dependent response. Without exception, all receptors studied so far in our hands show a rather stable signal output over the extracellular pH range of 6.5–7.8 following activation by their cognate ligand (Seuwen et al. 2006). However, the existence of other pH-sensing GPCRs is not excluded.

Structure-Function

OGR1 and GPR4 exhibit a cluster of histidines exposed to the extracellular space, and these amino acids are conserved across mammalian species. Site-directed mutagenesis experiments confirmed the importance of five histidine residues for normal pH sensing of the receptor OGR1 (Ludwig et al. 2003). A similar study confirmed the importance of the histidine cluster for pH sensing in GPR4 (Liu et al. 2010).

No detailed studies are yet available for TDAG8. In the latter receptor, three of the histidine residues shown to be relevant in OGR1 are replaced by basic amino acids. It is possible that these Arg and Lys residues, in the context of other surrounding amino acids in TDAG8, can function in pH sensing (Seuwen et al. 2006).

Crystal structures for the pH-sensing GPCRs are not yet available.

Potential Physiological Roles of pH-Sensing Receptors

The three pH-sensing receptors are found in vertebrates – orthologues for OGR1 and GPR4 are detected in zebrafish, chicken, and in all sequenced mammalian species, but not in *Drosophila*, *C. elegans*, or *Plasmodium*. TDAG8 is not found in zebrafish, but in the mammalian species analyzed to date. The receptors thus appear to be relevant for physiological processes developed relatively late in evolution.

Surprisingly little is known today about the molecular mechanisms governing pH homeostasis of higher organisms. Overall control is achieved through regulation of respiration and renal handling of bicarbonate and protons. The bone plays an important role as a reserve for hydroxyl ions, bicarbonate, and phosphate. Cardiovascular function and immune responses are influenced by acidosis.

Indeed, since the discovery of the pH-sensing properties of GPR4, OGR1, and TDAG8, the receptors have been linked to regulation of pH homeostasis in specific ways. A short summary of pertinent information is given in the following (see also Table 1).

The first data on mice deficient in GPR4, OGR1, and TDAG8 demonstrated that animals were viable and fertile, showing no overt phenotypes. Yang et al. (Yang et al. 2007) reported that adult $GPR4^{-/-}$ mice appear normal; however, increased perinatal lethality was observed which was associated with vascular abnormalities. Indeed, GPR4 is expressed in endothelial cells, and experiments on aortic rings ex vivo showed that microvessel outgrowth was less sensitive to acidic pH in preparations obtained from $GPR4^{-/-}$ mice compared to control. A later study by Wyder

Proton-Sensing GPCRs, Table 1 GPR4 and TDAG8 are Gs-coupled GPCRs; they stimulate cAMP formation inside cells. OGR1 is Gq-coupled, leading to activation of phosphoinositide turnover and release of calcium from intracellular stores (Ludwig et al. 2003; Wang et al. 2004). All receptors were also shown to activate signal transduction through G_{13}/rho activation. Prominent sites of expression, examples for suggested physiological roles (not comprehensive), and currently available pharmacological tools are indicated

Name	Chromosomal location	Signal transduction	Expression and physiological roles	Pharmacological tools
GPR4	19q13.2–q13.3	cAMP ↑ (G_s)	Endothelial cells Angiogensis (Yang et al. 2007; Wyder et al. 2011) Kidney function (Sun et al. 2010) Respiration (Kumar et al. 2015)	Antagonists cpd **3b** (Fukuda et al. 2016) cpd **13** (Velcicky et al. 2017; Sanderlin et al. 2019)
OGR1 (GPR68)	14q31	IP_3/Ca^{2+} ($G_{q/11}$)	Osteoblasts, osteoclasts, smooth muscle cells Bone metabolism (Krieger et al. 2016; Parry et al. 2016) Vascular function (Tomura et al. 2005; Xu et al. 2018) Mechanosensing (Xu et al. 2018; Wei et al. 2018)	Agonist Ogerin (Huang et al. 2015)
TDAG8 (GPR65)	14q31–q32.1	cAMP ↑ (G_s)	Immune system Inflammation (Okajima 2013; Jostins et al. 2012; Lassen et al. 2016; Tcymbarevich et al. 2019; Ma et al. 2017)	Agonist BTB09089 (Onozawa et al. 2012; Ma et al. 2017)

et al. (Wyder et al. 2011) showed a significantly reduced angiogenic response to VEGF-A (vascular endothelial growth factor A), but not to bFGF (basic fibroblast growth factor), in $GPR4^{-/-}$ animals.

Interestingly, GPR4 was also linked to renal acid handling (Sun et al. 2010) and to control of respiration in response to elevated CO_2 (Kumar et al. 2015), two important mechanisms controlling pH homeostasis.

OGR1 is expressed at low but relevant levels in many tissues including notably kidney, bone, and the nervous system (Ludwig et al. 2003 and references cited therein). We and others demonstrated pH-dependent signaling of the receptor in bone-forming osteoblasts as well as in bone-resorbing osteoclasts (Ludwig et al. 2003; Krieger et al. 2016). OGR1-deficient mice show a normally developed skeleton; however, there is a slight increase in bone volume detectable in growing animals, indicating a shift in the bone formation / resorption ratio (Krieger et al. 2016). Additional work is required to assess the response of the skeleton of $OGR1^{-/-}$ mice to chronic acidosis and hormonal changes. Further supporting an involvement of OGR1 in bone metabolism is the observation in humans of loss of function mutations in the OGR1 gene that lead to amelogenesis imperfecta, a disease of teeth characterized by abnormal enamel formation (Parry et al. 2016).

Expression and function of OGR1 were also described in human aortic smooth muscle cells, and the receptor was reported to control acid-induced PGI2 production (Tomura et al. 2005). PGI2 is a vasodilating mediator and may contribute to acidosis-induced vasorelaxation. Recent publications also described machanosensing properties for OGR1, and the receptor may directly transduce effects of shear stress in the peripheral vascular system (Xu et al. 2018; Wei et al. 2018). Importantly, mechanosensing through OGR1 works only at acidic pH, and the concept of co-incidence detection of shear stress and acidic pH was therefore suggested (Xu et al. 2018; Wei et al. 2018). Tissue acidosis and mechanical signals may explain how OGR1 stimulates TGFbeta secretion and fibrosis in mouse models of intestinal bowel disease (Hutter et al. 2018).

TDAG8 appears restricted to the immune system. As tissue inflammation is usually followed

by local hypoxia and acidosis, the pH-sensing property of TDAG8 appeared of particular interest in this context. While initial exploration of mice deficient in TDAG8 failed to unravel a prominent immune-related phenotype, later studies applying specific challenges suggested that TDAG8 inhibits inflammation, which is in line with the coupling of the receptor to cAMP production (Okajima 2013). TDAG8 attracted more attention when GWAS studies in humans linked the gene with high scores to inflammatory bowel disease and other immune disorders (Jostins et al. 2012). The receptor was thereafter shown to be required for normal lysosomal function and clearance of bacteria by macrophages (Lassen et al. 2016). In a DSS colitis model, TDAG8 deficiency enhanced macrophage and neutrophil infiltration into the colon and expression of pro-inflammatory mediators (Tcymbarevich et al. 2019).

In addition to their physiological roles in pH homeostasis, the proton-sensing receptors likely play an important role in cancer. Solid tumors are typically characterized by hypoxia and acidosis, and OGR1, GPR4, and TDAG8 sense low extracellular pH and appear to exert effects on tumor cell survival, proliferation, and motility, but also regulating angiogenesis and immune cell infiltration (Justus et al. 2013; Damaghi et al. 2013; Wiley et al. 2019).

Drugs

Despite the fact that the pH-sensing GPCRs represent attractive targets for drug development, limited information is available to date on modulators of receptor function. A first publication describing BTB09089, an agonist of TDAG8, appeared in 2012 (Onozawa et al. 2012). BTB09089 is able to activate cAMP formation dependent on TDAG8 in the slightly alkaline pH range, where the receptor is normally silent or only weakly active. Potency and efficacy of the molecule is modest, reaching $<30\%$ of the effect of low pH stimulation at a concentration of >10 microM. However, BTB09089 is specific against GPR4 and OGR1 as well as other GPCRs and was shown to inhibit cytokine production in primary murine T cells and macrophages. The molecule is today used to probe the function of TDAG8 in in vitro and in vivo studies (Ma et al. 2017).

Using screening of receptor activity in recombinant yeast, Huang et al. (Huang et al. 2015) identified agonist activity of some benzodiazepine molecules on OGR1. Receptor homology modeling and ligand docking led to the identification of ogerin, a positive allosteric modulator of OGR1, active in the micromolar range. Ogerin is specific against other GPCRs, and importantly, it could be shown to work in a murine in vivo model of fear conditioning (Huang et al. 2015).

For GPR4, potent and specific inhibitors were identified. First, Fukuda et al. described a potent ($IC_{50} = 67$ nanoM) and fully efficacious blocker of GPR4-dependent signaling and demonstrated that the molecule had impressive effects reducing mortality in a murine myocardial infarct model (Fukuda et al. 2016). Velcicki et al. later reported potent and highly selective GPR4 inhibitors with effects on nociception, inflammation, and angiogenesis (Velcicky et al. 2017; Sanderlin et al. 2019).

Conclusions

The data available to date identify GPR4, OGR1, and TDAG8, but not G2A, as genuine pH-sensing receptors. The receptors have been shown to influence processes linked to regulation of pH homeostasis and response to acidosis, such as bone metabolism, kidney function, respiration, and inflammation.

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Pseudocholinesterase

► Cholinesterases

P

Psychedelic Drugs

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Synonyms

Classic hallucinogen; Hallucinogen; Psychotomimetic; Serotonergic hallucinogen

Definition

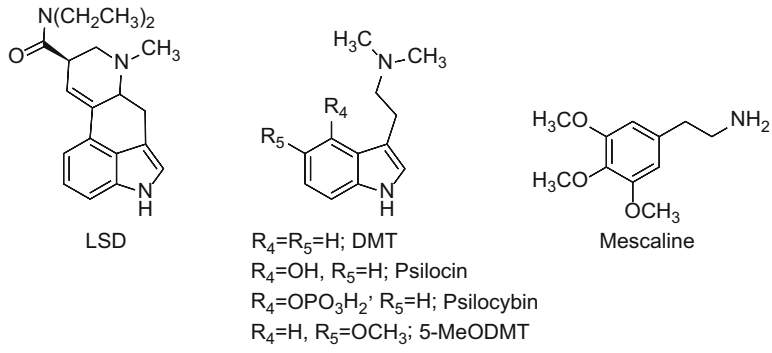
Psychedelic drugs are substances that can produce changes in thoughts, feelings, mood, and alterations in sensory perception that are not experienced otherwise except during dreaming or at times of religious exultation. They do not have reinforcing properties and do not lead to addiction or dependence. At high doses they lead to a breakdown in the perception of time, space, and selfhood. Although "hallucinogen" has been used to refer to any exogenous substance that produces sensory distortions (e.g., cannabis, PCP, MDMA), the term "psychedelic" is reserved for a subclass of hallucinogenic agents that primarily mediate their effects through activation of the serotonin 5-HT_{2A} receptor. There are three different main families of psychedelics based upon structure (Fig. 1).

Background

These substances have been used by humans back through antiquity. References to *Soma* in the Rigveda, the holy literature of India, indicate it was an important ritual drink in ancient India. Its identity is lost today, but descriptions of its effects suggest it was probably some kind of psychedelic drink. Similarly, the Eleusinian mysteries of ancient Greece employed a drink known as κψκεον (*kykeon*) that was a central component in an annual ritual held in the village of Eleusis (Wasson et al. 1978). The ceremony was held each year in the fall for about 2,000 years! Although the composition of *kykeon* remains unknown today, what little was written about the ceremony indicates it was very powerful and suggests that *kykeon* also was some sort of hallucinogenic concoction.

In Mesoamerica, certain mushrooms were used in ritual and healing contexts and were known by the Aztecs as *teonanacatl*, or flesh of the gods. Although Spanish missionaries attempted to destroy all records of the use of these mushrooms, a great number of "mushroom stones" were ultimately recovered, although their significance remained unknown for many years. In 1956 New York banker and amateur mycologist R. Gordon Wasson traveled to Mexico and participated in a mushroom ceremony presided over by Mazatec Curandera Maria Sabina, who taught them about the uses and effects of the mushroom. The description of this trip and ceremony was published in *Life Magazine* in 1957 and ultimately led to the popularization of psychedelic mushrooms in Western society. Psilocybin was subsequently isolated and identified as the active component of these mushrooms by Albert Hofmann in 1959 (Hofmann et al. 1959). Psilocybin is orally active, with a typical dose of 10–30 mg and a duration of effect of 4–6 h.

Mescaline is a naturally occurring psychedelic produced by the peyote cactus *Lophophora*



Psychedelic Drugs, Fig. 1 Structures of commonly used psychedelic drugs. Ergolines are represented by LSD. Tryptamines are represented by psilocybin and DMT. Phenethylamines are represented by mescaline. Whereas ergolines have broad affinity for several monoamine receptor systems, receptor effects of tryptamines are largely restricted to serotonin receptors, and phenethylamines are mainly agonists of 5-HT_2 receptors

williamsii that is native to Mexico and Southwestern Texas. It occurs to the extent of about 0.4% in the fresh cactus. There is archeological evidence that peyote has been used for more than 5,000 years. Today, peyote is the sacrament for the Native American Church (NAC), where the tops (buttons) of the cactus are generally chewed or boiled in water to produce a psychoactive tea. Typically, NAC ceremonies are directed by a Roadman, last all night sitting around a fire with singing and chanting, with the effects of the peyote lasting between 8 and 12 h. The principal psychoactive alkaloid in the peyote cactus, mescaline, was identified by Arthur Heffter in 1898 (Heffter 1898), and its total synthesis reported by Späth in 1919 (Späth 1919). Mescaline is orally active, with a typical dose of 200–400 mg and a duration of effect of 8–12 h.

Another naturally occurring psychedelic is *N,N*-dimethyltryptamine (DMT), which is found in a number of plants including the Amazonian plant *Psychotria viridis*. Prolonged boiling of leaves from *Psychotria* together with the pounded stems of the vine *Banisteriopsis caapi* results in a tisane called ayahuasca. Ayahuasca (hoasca tea) has been used by natives in Amazonia as a religious sacrament whose use may stretch back to the beginnings of the Inca civilization of Peru. Ayahuasca serves as the focus of two syncretic churches, the Santo Daime, founded in the 1930s, and the União do Vegetal, founded in 1961. Although both religions had their origins in the Amazon, there are numerous adherents throughout the world, and the use of ayahuasca by these religions is recognized in Brazil and is protected in the United States under the Religious Freedom Restoration Act (RFRA). Although DMT is a powerful psychedelic, it is not orally active as a result of its first-pass degradation by monoamine oxidase A (MAO-A) in the liver. *Banisteriopsis* vines contain beta-carboline alkaloids such as harmaline, harmine, and tetrahydroharmine, which are reversible MAO inhibitors. Thus, ingestion of the mixture leads to inhibition of MAO-A, preventing degradation of DMT in the liver, and allowing it to produce biological effects, with a duration of action of 4–6 h.

The most potent and perhaps most well-known psychedelic is lysergic acid *N,N*-diethylamide (LSD, LSD-25). It is a semisynthetic molecule first synthesized by Sandoz natural product chemist Albert Hofmann in 1938 (Nichols 2018). Pharmacological studies failed to reveal anything of interest, but 5 years later, in 1943, Dr. Hofmann decided to prepare another sample of LSD, believing that the pharmacology department must have missed something important in their first screening of the molecule. Hofmann was motivated by what he referred to as a "peculiar presentiment." On Friday April 16, 1943, he somehow accidentally ingested a small amount of LSD, which produced remarkable psychic effects. The following Monday, in a self-experiment, he deliberately ingested a solution containing 0.25 mg of LSD tartrate, realizing that no compound then known would produce significant effects at that small dose. Surprisingly, the effects of this dose were very profound. Communicating the results of his self-experiment, his research director found the report somewhat incredulous. Nevertheless, several colleagues at Sandoz ingested doses of 0.08 mg of LSD, which confirmed the remarkable potency and unusual psychological properties of LSD. A typical research dose of LSD was 0.1 mg of the tartrate salt, with a duration of effect of 8–10 h.

Mechanism of Action

There is overwhelming evidence that an agonist or partial agonist action at 5-HT_{2A} receptors expressed in certain cortical regions of the brain is the key mechanism of action for psychedelics (Nichols 2016). Although this conclusion was initially based on in vitro and animal models, the ability of selective 5-HT_{2A} receptor antagonists to block the psychoactive effects of psilocybin and LSD in humans has validated this receptor as the primary target for mediating psychoactive behaviors in humans (e.g., see Vollenweider et al. 1998). Rapid tolerance, called tachyphylaxis, develops to repeated use of most psychedelics such that ingestion of LSD on a daily basis results essentially in a complete lack

of effect by the third or fourth day. Cross tolerance occurs between several of these drugs, emphasizing that they have a similar or identical mechanism of action.

Early mechanistic studies showed that iontophoretic application of LSD to slices of rat brain cortex led to production of excitatory postsynaptic currents (EPSCs) in cortical pyramidal cells (Aghajanian and Marek 1997). Following these EPSCs, there was a slowly developing series of asynchronous depolarizations that appeared to be a result of glutamate diffusion from other cells (Aghajanian and Marek 1998). Beique et al. (2007) then described a population of cells in deeper layers of the cortex that responded to direct application of a psychedelic by generation of action potentials, which released glutamate from their projection fields.

Subsequently, Martin and Nichols (2016) developed a novel method for cell sorting of whole neurons and identified a small population of neurons in deeper layers of the cortex that had higher expression of 5-HT_{2A} receptor mRNA and which became transcriptionally active following systemic administration of a psychedelic in comparison to the bulk of pyramidal neurons that did not. Transcriptional activity and the rapid expression of Immediate Early Genes, as was found in this neuronal population, is generally accepted as a marker for neuronal depolarization. Further, they found that psychedelics also lead to rapid transcriptional activation within small subsets of inhibitory GABA interneurons and astrocytes. The patterns of gene response in a given cell type differed between distinct brain regions. An important brain area that included these cells, which Martin and Nichols named the trigger population, was the claustrum, the most highly connected brain area. Immunohistochemical studies have shown that whereas 5-HT_{2A} receptors are densely expressed on apical dendrites of pyramidal cells in layer 5 of the cortex, the highest expression of 5-HT_{2A} receptors is reported to be in the claustrum (Pazos et al. 1985). Therefore, the acute effects of psychedelics in the brain are rapidly to activate subpopulations of excitatory, inhibitory, and glial cells in differential patterns depending on brain region. These complex cellular responses likely underlie the observed brain network connectivity changes as described below.

Recently, a 2.9 Å resolution x-ray crystal structure was published showing LSD bound within the human serotonin 5-HT_{2B} receptor, which is a close homolog of the 5-HT_{2A} receptor (Wacker et al. 2017). Their results further underscored that LSD is a functionally selective ligand, with the ability to activate not only canonical G protein intracellular signaling pathways but also to promote the recruitment of β-arrestin2. They discovered through receptor kinetics measurements that LSD has a very slow association with the receptor and an even slower dissociation rate. These unusual receptor kinetics were traced to a distinct structural element within both the 5-HT_{2A} and 5-HT_{2B} receptors, extracellular loop 2 (EL2), which connects the top of receptor helix 4 with the top of helix 5. A crucial residue in EL2 L209 (in 5-HT_{2B}) or L229 (in 5-HT_{2A}) "wedges" itself into the orthosteric binding pocket to essentially trap LSD within the receptor. When this leucine was mutated to the much smaller alanine, LSD readily associated and dissociated with the receptor. Molecular dynamics simulations were used to illustrate the effect of this mutation on the relative flexibility of EL2 in the unbound and LSD-bound state of the receptor.

Importantly, they showed that the residence time of LSD in the receptor greatly influenced the ability of the receptor to recruit β-arrestin2. That is, the longer LSD remained bound in the receptor, the greater were the potency and efficacy of the receptor to recruit β-arrestin2. Thus, under in vivo conditions, it would be expected that signaling through the 5-HT_{2A} receptor by LSD would be highly arrestin biased, relative to G protein signaling. The ability of the wild-type receptor to sequester LSD for a long period of time also could be relevant to the long duration of action for this drug.

Based on the crystal structure of LSD within the 5-HT_{2B} receptor, as well as structures of several related ergolines, McCorvy et al. (2018) have given detailed rationales to explain agonist and antagonist activity, as well as potential explanations for ligand bias (G protein vs arrestin

signaling) for the ergoline class of psychedelic. In particular, residues Thr3.37 in helix 3, ala/ser5.46 in helix 5, and Leu7.35 in helix 7 appear to be particularly important. At the present time, there are no crystal structures of other psychedelics bound in the 5-HT_{2A} receptor, and the overall signaling characteristics of ligand-receptor ensembles have not yet been well characterized.

Anti-inflammatory Properties

Yu and colleagues in 2008 made the surprising discovery that psychedelics had potent anti-inflammatory effects (Yu et al. 2008). Their first report examined the ability of several psychedelics in a cell-culture model of vascular cells to prevent inflammation initiated by TNF-α. They found that although several psychedelics were anti-inflammatory, a particular drug related in structure to mescaline, (*R*)-2,5-dimethoxy-4-iodoamphetamine, (*R*)-DOI, had super-potent effects about 100-fold greater than a typical steroidal anti-inflammatory in vascular cells. These effects were blocked by a specific antagonist for the 5-HT_{2A} receptor, indicating that they were being mediated by the same receptor that is the target for its behavioral actions in the brain.

A subsequent study translated this discovery to the whole animal, where (*R*)-DOI was found to block potently the systemic effects of TNF-α (Nau et al. 2013). Since then, (*R*)-DOI has been shown to have therapeutic efficacy to treat inflammation in several models of human diseases including cardiovascular and metabolic disease, and asthma, at sub-behavioral doses of drug (Nau et al. 2015; Flanagan et al. 2019a, b). Although precise mechanisms remain largely unknown, it is believed that activation of the 5-HT_{2A} receptor by psychedelics recruits specific anti-inflammatory pathways to the receptor that serotonin does not. These serve not only to block TNF-α-related pathways, but to also prevent innate immune cells like macrophages and adaptive immune cells like T-helper cells from activating and secreting pro-inflammatory molecules, in addition to preventing recruitment of key immune cells like eosinophils and Th2 cells to sites of inflammation (Flanagan and Nichols 2018). The full therapeutic potential of psychedelics to treat inflammatory-related diseases remains to be determined in human clinical testing.

Clinical Uses

Shortly after its discovery, LSD was hailed as a new breakthrough for psychiatry. From 1950 to the early 1960s, there were approximately 1,000 publications, describing treatment of more than 40,000 patients for a variety of different conditions (Grinspoon and Bakalar 1979). The most promising were the use of LSD to treat alcohol use disorder and treatment of terminal cancer patients. The latter indication was perhaps the most well documented as efficacious. The Controlled Substances Act of 1970 (CSA) placed all psychedelics into the most restrictive drug category, Schedule 1, which was a significant deterrent to further research. The last clinical program with LSD ended in 1976, and no further clinical studies were carried out for almost 20 years.

Clinical studies finally resumed, with Dr. Rick Strassman's 1994 and 1996 reports on the effects of intravenous DMT administration in humans. These studies were widely reported and led to both a book and a movie based on the results. Strassman had finally demonstrated that it was possible to obtain FDA approval to administer psychedelic agents to humans.

Then, in 2006 Dr. Roland Griffiths and colleagues reported on a study of 36 hallucinogen-naïve volunteers who were administered an oral dose of 30 mg/70 kg of psilocybin. Thirty-three percent of the volunteers rated the psilocybin experience as being the single most spiritually significant experience of their lives, with an additional 38% rating it to be among the top five most spiritually significant experiences. Neither the Strassman nor the Griffiths study focused on therapeutic efficacy, however.

The first study of therapeutic efficacy was reported by Moreno et al. in 1997 (Moreno and Delgado 1997), where 9 subjects suffering from obsessive-compulsive disorder were administered a total of 29 psilocybin doses. Marked decreases

in OCD symptoms of variable degrees were observed in all subjects during one or more of the testing sessions. These results were tantalizing, but unfortunately not conclusive.

Then, in 2011, Dr. Charles Grob reported results from a clinical study of psilocybin-assisted psychotherapy in 12 adults with advanced-stage cancer and anxiety (Grob et al. 2011). The State-Trait Anxiety Inventory trait anxiety subscale demonstrated a significant reduction in anxiety at 1 and 3 months after treatment. The Beck Depression Inventory revealed an improvement of mood that reached significance at 6 months.

Gasser et al. (2014) studied LSD-assisted psychotherapy in 12 patients with anxiety associated with life-threatening diseases. Patients received 200 μg of LSD free base in a double-blind randomized placebo-controlled study. At the 2-month follow-up, positive trends were found with the State-Trait Anxiety Inventory (STAI) in reductions in trait anxiety ($p = 0.033$) with an effect size of 1.1, and state anxiety was significantly reduced ($p = 0.021$) with an effect size of 1.2, with no acute or chronic adverse effects persisting beyond 1 day after treatment or treatment-related serious adverse events. STAI reductions were sustained for 12 months.

Subsequently, in 2016, two studies were published describing psilocybin-assisted psychotherapy in cancer patients with a terminal diagnosis. One study, by Dr. Steven Ross and his collaborators at New York University, reported on 29 patients with cancer-related anxiety and depression who were randomly assigned and received treatment with single-dose psilocybin (0.3 mg/kg) or niacin placebo along with psychotherapy (Ross et al. 2016). At the 6.5-month follow-up, psilocybin was associated with enduring anxiolytic and antidepressant effects (approximately 60–80% of participants continued with clinically significant reductions in depression or anxiety), sustained benefits in existential distress and quality of life, as well as improved attitudes toward death. Similarly, in the same year, Griffiths and colleagues at Johns Hopkins University reported on the effects of psilocybin in 51 cancer patients with life-threatening diagnoses and symptoms of depression and/or anxiety (Griffiths et al. 2016). High-dose psilocybin (22 or 30 mg/70 kg) produced large decreases in clinician- and self-rated measures of depressed mood and anxiety, along with increases in quality of life, life meaning, and optimism and decreases in death anxiety. At 6-month follow-up, these changes were sustained, with about 80% of participants continuing to show clinically significant decreases in depressed mood and anxiety.

In addition to relieving depression and anxiety in cancer patients, psilocybin-assisted psychotherapy also proved efficacious in treating alcohol use disorder and nicotine addiction. A 2014 study of psilocybin-assisted therapy by Johnson and colleagues found that 12 of 15 participants (80%) showed 7-day point prevalence abstinence at 6-month follow-up, which far exceeds abstinence rates of around 35% with the best therapies available (Johnson et al. 2014). Similarly, Bogenschutz et al. (2015) studied psilocybin coupled with motivational enhancement therapy in ten subjects with AUD and reported that abstinence increased significantly following psilocybin administration ($p < 0.05$). Gains were largely maintained at follow-up to 36 weeks.

The combination of these studies, and particularly the remarkable efficacy of psilocybin-assisted therapy in cancer patients, has led to a rejuvenation of interest in the potential medical value of psychedelics. Media stories and interviews with clinicians have recently increased dramatically in frequency, and numerous books have also recently appeared, all further stimulating the public's interest in psychedelics. Newer studies now underway are examining psilocybin-assisted therapy for obsessive-compulsive disorder, anorexia, and eating disorders, and much larger studies of alcohol use disorder and nicotine as well as cocaine addiction.

As a result of these very promising clinical results, the FDA has granted breakthrough therapy status for psilocybin-assisted therapy in treating major depressive disorder (MDD) and treatment-resistant depression and has also granted breakthrough therapy status to the use of MDMA-assisted therapy for the treatment of post-traumatic stress disorder (PTSD). If Phase 3 clinical studies prove definitive, one might expect

FDA approval for psilocybin and MDMA to occur in a time frame of perhaps 2–4 years (i.e., 2022–2024).

Effects of Psychedelics on Functional Brain Dynamics

Within about the past 10–15 years, powerful brain imaging technologies, including BOLD-fMRI, EEG, and magnetoencephalography (MEG), coupled with improved computational power, have led to important insights into the effects of psychedelics on brain function. Resting-state brain activities are organized into multiple large-scale functional networks, called resting state networks (RSNs). Numerous studies have revealed that acute administration of psychedelics leads to a reorganization of connectivity within and between resting-state networks, generally showing decreased connectivity within resting-state networks and increased connectivity across such networks. Reduced broadband oscillatory power has been observed following the administration of LSD and psilocybin, especially in the alpha (8–12 Hz) band.

Connectivity analysis has identified sets of regions that are essential for enabling efficient communication between key cortical regions. These "brain hubs" are embedded within central anatomical networks and participate in functional roles across a range of cognitive and affective tasks with widespread dynamic coupling within and across functional networks. These brain hubs are crucial for integrating information that serves as the basis for various aspects of complex cognitive function. Psychedelics acutely reduce the stability and integrity of these well-established brain networks and simultaneously reduce the degree of segregation between them. One of the fundamental large-scale networks where psychedelics (LSD, psilocybin, DMT) have been shown to decrease functional integrity is known as the default mode network (DMN). They increase its functional connectivity with other brain systems and reduce frontoparietal connectivity. Psychedelics also disrupt the internal integration of multiple brain hubs that normally do not have strong connections to each other. After the normal RSN organization is disrupted, however, strong, topologically long-range functional connections emerge that are not present in the normal state.

In summary, activation of 5-HT_{2A} receptors occurs in various important brain hubs, including cortical regions, leading primarily to a reduction in the threshold for cortical cell depolarization. However, the molecular nature of how neurons and cells respond to psychedelics beyond simple reductions in membrane potentials is complex and region dependent. A small subset of excitatory trigger neurons in deeper layers of the cortex depolarizes and initiates recurrent activity that spreads throughout specific regions, which then recruit subpopulations of inhibitory and non-neuronal cells to a supercritical state. That leads to generation of amplifying neuronal avalanches and destabilization of local network hubs, with subsequent changes in global connectivity and ultimately profound alterations in perception and cognition. It remains a significant challenge to understand how these changes in both cellular and molecular processes and global connectivity lead to therapeutic improvement of certain psychiatric disorders, but many laboratories are now focused on answering these questions.

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Psycho Analeptics

▶ Antidepressant Drugs

Psycho Energizers

▶ Antidepressant Drugs

Psychomotor Stimulant Drugs

► Psychostimulants

Psychostimulants

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Synonyms

Psychomotor stimulant drugs; Stimulant drugs; Stimulants

Definition

Psychostimulants are drugs that substantially influence cognitive and affective functioning and behaviors. Effects are increased motivational desire, agitation, heightened vigilance, euphoria, hyperactivity, and decreased sleepiness as well as appetite. Traditionally, mainly cocaine and amphetamines as well as other drugs (like MDMA or methamphetamine) are considered psychostimulants. However, nicotine, caffeine, and even certain antidepressants and modafinil could be included in this class. Methylphenidate and amphetamine (like dextroamphetamine) are widely used for the treatment of attention deficit/hyperactivity disorder (ADHD). In the recent years, long-acting formulations of methylphenidate (like OROS methylphenidate among others) or prodrugs (like lisdexamfetamine) came into focus and are mainly used in treatment of adult ADHD. Various formulations (like chewable, liquid, or transdermal) are available (Cortese et al. 2017).

Mechanism of Action

General Remarks

The final pathway of psychostimulants on the behavioral level is an increased mobilization of the normal fight/flight/fright reaction that is mediated by the biogene amines epinephrine, norepinephrine, serotonin, and dopamine. The most widespread extraclinically used psychostimulant is ecstasy (3,4-methylenedioxymethamphetamine, MDMA), which also exhibits perceptual distortions due to 5-HT $_{2A}$-receptor agonism like lysergic acid diethylamide (LSD).

The main target of action of methylphenidate, the most widespread clinically used psychostimulant, is the dopamine transporter (DAT); its inhibition increases intrasynaptic dopamine concentrations. The subcortical dopamine system (mesolimbic and nigrostriatal parts) mediates the unconditioned and conditioned responses toward reinforcement.

Within the striatum, dopamine terminals have direct synaptic contacts with the spines of striatal neurons. These synaptic contacts appear to provide the anatomical substrate for both compartmental (synaptic) and volume (extrasynaptic) transmission. The DAT is located intra- or perisynaptically, suggesting that dopamine has a limited ability to escape the striatal synapse. Moreover, dopamine D2 and, to a lesser extent, D1 receptors are located postsynaptically from dopamine terminals, suggesting compartmental transmission. However, there is a significant cohort of D1 and D2 receptors that are not directly opposed to dopamine terminals, suggesting some component of volume transmission within the striatum. Therefore, tonic and phasic changes in dopamine transmission are critical in producing behavioral effects associated with the striatum. In contrast, in the cerebral cortex, volume transmission appears to be more critical in mediating the effects of dopamine. DAT density is reduced in the frontal cortex (FC) relative to the striatum, and it is localized extrasynaptically. Moreover, D1-immunoreactivity within FC is virtually never opposed to tyrosine hydroxylase immunopositive terminals suggesting that the effect of dopamine on D1 receptors is by volume transmission. This hypothesis has direct relevance to the neurochemistry of ADHD because cognitive functions known to be affected in this disorder, namely, working memory and inhibitory control, are sensitive to manipulations of D1

receptor-mediated dopamine transmission (Taylor and Jentsch 2001; Grace 2001). Thus, the tonic component might be more critical for the behavioral functions of the FC.

Several classes of drugs modulate the firing rates or patterns of midbrain dopamine neurons by direct, monosynaptic, or indirect, polysynaptic, inputs to the cell bodies within the ventral mesencephalon (i.e., nicotine and opiates). In contrast, amphetamine, cocaine, and methylphenidate act at the level of the dopamine terminal interfering with normal processes of transmitter packaging, release, reuptake, and metabolism.

Methylphenidate, Amphetamine, and Cocaine

Methylphenidate like cocaine largely acts by blocking reuptake of monoamines into the presynaptic terminal. Methylphenidate administration in therapeutic dose produces an increase in the steady-state (tonic) levels of monoamines within the synaptic cleft. Thus, DAT inhibitors, such as methylphenidate, increase extracellular levels of monoamines. In contrast, they decrease the concentrations of the monoamine metabolites that depend upon monoamine oxidase (MAO), that is, HVA, but not catecholamine-*o*-methyltransferase (COMT), because reuptake by the transporter is required for the formation of these metabolites. By stimulating presynaptic autoreceptors, methylphenidate-induced increase in dopamine transmission can also reduce monoamine synthesis, inhibit monoamine neuron firing, and reduce subsequent phasic dopamine release.

The pharmacology of amphetamine is considerably more complex. It does not only block monoamine reuptake but also directly inhibits the vesicular monoamine transporter, causing an increase in cytosolic but not vesicular dopamine concentration. This may lead to reverse transport of the amines via the membrane-bound transporters. Further mechanisms of amphetamine action are direct MAO inhibition and indirect release of both dopamine and serotonin in the striatum.

Mild increases in tonic dopamine release – as a consequence of the administration of both methylphenidate and amphetamine – could have important impact on subsequent phasic release by feedback mechanisms (lowering the concentration).

As pointed out before, there are some major differences between the striatal and cortical dopamine terminals (Table 1).

Therefore, dopamine transporter inhibitors exhibit less effect in the FC. There, dopamine seems to be reuptaken by the norepinephrine transporter, which dopamine actually has a higher affinity for than norepinephrine itself.

Amphetamine administration produces a marked increase in cortical dopamine, norepinephrine, and serotonin release that is impulse independent. Methylphenidate can produce significant increases in dopamine and norepinephrine release (Table 2).

Dopaminergic mechanisms within the ventral striatum (i.e., nucleus accumbens) subserve the ability of amphetamine and methylphenidate in low to moderate doses to increase locomotor activity. In contrast, very low dosages in animals seem to cause hypoactivity presumably by stimulation of autoreceptors, a finding that would be compatible with the clinical impression that methylphenidate might be useful in some patients with mania.

At low doses, both psychostimulants could theoretically stimulate tonic, extracellular levels of monoamines, and the small increase in steady-state levels would produce feedback inhibition of further release by stimulating presynaptic autoreceptors. While this mechanism is clearly an important one for the normal regulation of monoamine neurotransmission, there is no direct evidence to support the notion that the doses used clinically to treat ADHD are low enough to have primarily presynaptic effects. However, alterations in phasic dopamine release could produce net reductions in dopamine release under putatively

Psychostimulants, Table 1 Properties of cortical versus striatal dopamine system

	Cortical	Striatal
Synthesis-regulating autoreceptors	0	++
Impulse-regulating autoreceptors	0	++
DAT expression	0/+	++
DAT localization	Extrasynaptical	Synaptical

Psychostimulants, Table 2 Influence of the psychostimulants amphetamine, cocaine, and methylphenidate on the different biogene amines

	Amphetamine	Cocaine	Methylphenidate
Dopamine	++	++	++
Norepinephrine	++	++	+
Serotonin	+	++	(+)

altered tonic dopaminergic conditions that might occur in ADHD and that might explain the beneficial effects of methylphenidate in ADHD.

Repeated intermittent exposure to stimulants can produce sensitization, where subsequent drug exposures produce increased behavioral and neurochemical responses. The ability of the drug and ultimately of related stimuli to elicit behavior may be increased with repeated administration or intake of the drug. Dopaminergic sensitization within the amygdala has also been found after repeated exposure to amphetamine, and this can enhance appetitive and aversive learning even after drug cessation.

Cocaine- and amphetamine-regulated transcript (CART) peptides are putative neurotransmitters that are found in those dopaminergic brain regions. Stimulants seem to alter CART mRNA and peptide levels (Jaworski and Jones 2006).

Besides the dopaminergic system, the noradrenergic nucleus, locus coeruleus (LC), may be another structure involved in the mode of action of psychostimulants. Electrophysiological recordings from this area demonstrate a relationship between behavior and response of the LC to targets versus distractors in an attention task. Baseline firing shows a constant increase paralleling the conditions from drowsiness to hyperarousal. In contrast, phasic responses are maximal in an optimal alert attentive state and minimal in drowsiness as well as hyperarousal that may be associated with poor cognitive performance due to high distractibility. Methylphenidate and also amphetamine (although relatively lower in affinity) are also shown to exert direct effects on the noradrenaline (norepinephrine) transporter (NET).

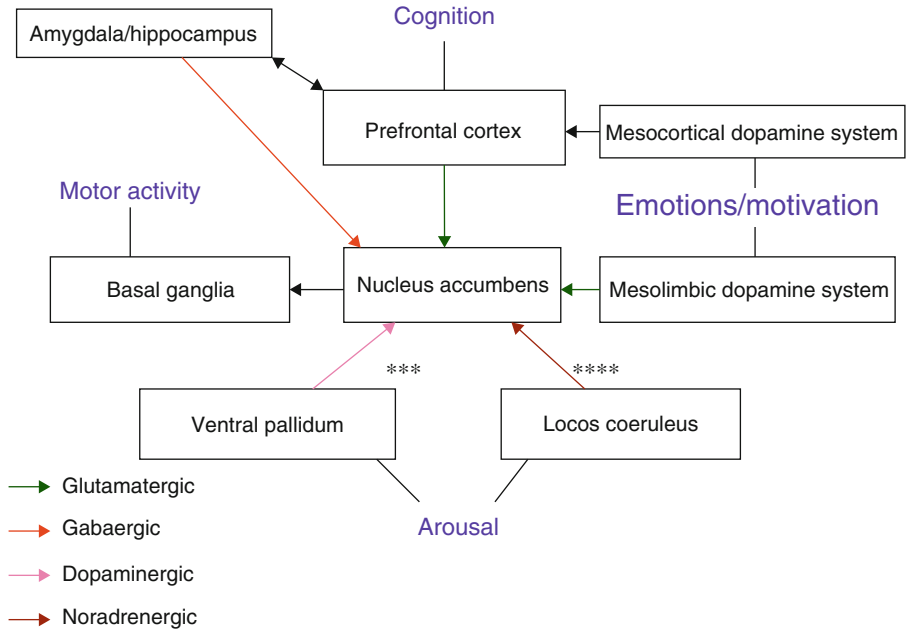
Dose-dependent glutamate effects of methylphenidate linked to behavioral improvement of attention (low dose) and aggression and elevated locomotor activity (high dose) via adrenergic modulation of N-methyl-D-aspartate receptors (NMDAR) and alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPAR) in the prefrontal cortex are also suggested following initial animal data.

Also, immunogenetic effects of psychostimulant administration are researched. First evidence hints at a DAT-genotype-associated effect of methylphenidate on DAT-antibody levels in children with ADHD. Regulatory effects in basal ganglia genes are also reported after psychostimulant use.

Cerebellum alterations have also been linked to ADHD pathology. The cerebellum is a further possible target for psychostimulants since it is not only important for motor coordination but also for processing cognitive situations. Dopamine levels within the dense dopamine projections to the cerebellum could be increased by psychostimulants accounting for registered cerebellar volume changes associated with stimulant treatment in youth with ADHD.

Several mechanisms, postulated in excessive use of especially (meth)amphetamines, are discussed to bring out neurotoxic effects of psychostimulants. Mitochondrial dysfunction, oxidative stress, and excitotoxicity have been laid out, and in the recent years, effects on neuroinflammation, blood-brain barrier (BBB) dysfunction, and impaired neurogenesis are being suggested (Goncalves et al. 2014).

In summary, main structures involved in the action of psychostimulants can be divided into cortical (mainly prefrontal cortex) and subcortical (basal ganglia and related structures, LC and cerebellum) ones. Figure 1 gives a schematic overview of the connections between these structures, omitting the cerebellum due to lack of precise information.



Psychostimulants, Fig. 1 The nucleus accumbens as a major intersection of cognitive, motor, and arousal processes

Modafinil

Modafinil, as its R-enantiomer armodafinil, is a wake-promoting psychostimulant drug. It reduces excessive daytime sleepiness due to narcolepsy, obstructive sleep apnea, and shift workers disorder. It is used off label to treat ADHD or in chronic fatigue. There have been a limited number of clinical trials in ADHD, and results point at modafinil being a reasonable treatment alternative to amphetamine-type stimulants in terms of symptom reduction and tolerability in children and adolescents. Stimulant side effects, including potential addiction, are modest compared to amphetamines. Of note, serious rashes and multi-organ hypersensitivity reactions form very rare, but severe, possible side effects in children and to a lesser degree in adults in post-marketing experience.

Modafinil shows procognitive effects comparable to low-dose amphetamine-type psychostimulants. The exact mechanisms of action of modafinil are not well understood. It inhibits the DAT and lesser so NET with minor affinity to the receptor and therefore enhances tonic rather than phasic dopamine activity. It has been suggested that modafinil increases wakefulness by activating α_1 noradrenergic receptors and via downstream effects that lead to the release of orexin, histamine, 5HT, and glutamate. Also, it may act by modulating the GABAergic tone that might lead to an increased dopamine release in the nucleus accumbens. There is also evidence modafinil elicits anticraving effects via metabotropic glutamate receptor pathways after cocaine administration in rats via increasing extracellular glutamate (Minzenberg and Carter 2008) (Gibson et al. 2006).

Nicotine

Nicotine is the main psychoactive ingredient of tobacco and is responsible for the stimulant effects and abuse/addiction that may result from tobacco use. Cigarette smoking rapidly (in about 3 s!) delivers pulses of nicotine into the bloodstream. Its initial effects are caused by its activation of nicotinic acetylcholine (nACh) receptors. nACh receptors are ligand-gated ion channels and pre- and postsynaptically located. Reinforcement depends on an intact mesolimbic dopamine system (VTA). nACh receptors on VTA dopamine neurons are normally activated by cholinergic innervation from the laterodorsal tegmental nucleus or the pedunculopontine nucleus.

In the last decade, the use of e-cigarettes or vaping became a well-received mode of nicotine consumption. Its clinical use (i.e., for tobacco cessation) is discussed controversially as there is growing evidence of severe side effects (Werner et al. 2020).

Clinical Use

The main indication for certain psychostimulants is ADHD in children, adolescents, and adults (Faraone et al. 2015; Okie 2006). Research shows that the clinical effect and benefit are dramatic even in adults. About 60% of adult patients receiving stimulant medication showed moderate-to-marked improvement, as compared with 10% of those receiving placebo. The core symptoms of hyperactivity, inattention, mood lability, temper, disorganization, stress sensitivity, and impulsivity have been shown to respond to treatment with stimulant medications. Further, there is evidence that severe problems following ADHD (like psychiatric comorbidity and criminality rates) are reduced with stimulant treatment. Patterns of use have changed over the recent years. In the USA methylphenidate (generally considered standard first-line treatment in ADHD) is being outdone by amphetamine in all ages, and psychostimulants are more frequently prescribed to adults than to children. Also more women are prescribed stimulant medication than men.

Other clinical indications include hypersomnia and weight loss. Psychostimulants are also widely discussed to elicit specific neuro-enhancing effects (Spencer et al. 2015).

The characteristic behavioral effects of acute and chronic psychomotor stimulant drugs are locomotor activation, stereotypy, and conditioned reward and stimulus-reward learning. The most important brain regions involved in these effects are summarized in Table 3.

Psychostimulants, Table 3 Acute effects of psychostimulants and the brain regions that are mainly involved in these effects

Acute effects	Mainly involved brain region
Locomotion	Ventral striatum (i.e., nucleus accumbens)
Motor stereotypy	Dorsal striatum (i.e., caudate putamen)
Reinforcement	Ventral striatum
Conditioned reward	Ventral striatum/amygdala
Stimulus-reward learning	Amygdala

Each of these processes depends upon increases in dopaminergic transmission within the striatum and, possibly, amygdala. Moreover, neurochemical actions within the FC may contribute to the ability to modulate some of these basic motivational processes. This data is based primarily on studies in rodents given systemic injections of moderate-to-high doses of stimulants, and it is not known whether lower doses applied orally would produce similar behavioral and neurochemical effects. Nevertheless, when given acutely or chronically to animals, stimulants appear to alter the neurochemistry of the striatum in such a way as to augment the control of behavior by conditioned or unconditioned stimuli associated with reinforcement processes. These effects may be consistent with the suggestion that amphetamine or methylphenidate may exert some of their beneficial clinical effects by augmenting conditioned reinforcement and stimulus-reward associations that could enhance aspects of task performance.

A simple decrease in striatal dopamine transmission produced by even low clinical doses of the drugs according to current data seems an untenable hypothesis. However, if the tonic control of phasic release by dopamine is abnormally low in ADHD, then a high phasic dopamine response may be associated with ADHD and treatment efficacy. Moreover, alterations in corticolimbic tonic/phasic balance may also provide an explanation of why chronically administered psychostimulants in ADHD produce a behavioral profile and response associated with subcortical dopamine neuronal hyperactivity and cortical hypoactivity, though issues of dose and route of administration should be considered in this context.

Another theory for the action of stimulant drugs in ADHD involves effects on nonstriatal monoamine systems. Frontal cortical dopamine,

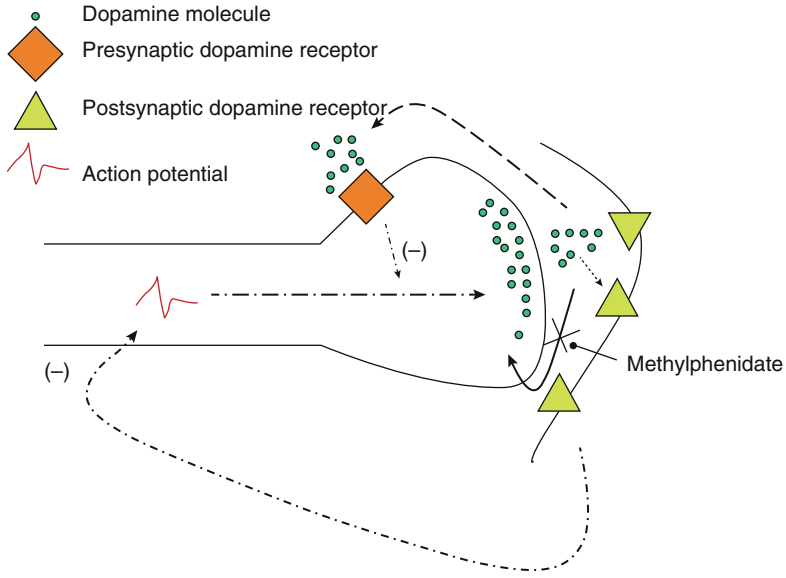
norepinephrine, and serotonin are clearly important in cognitive functioning and impulse control. These neurotransmitters directly modulate reward-related behaviors associated with the striatal dopamine system. Moreover, the amygdala may be pharmacologically influenced leading to enhanced associative learning and recall. Thus, the net behavioral effects of stimulant drugs may promote changes in reward-motivated behaviors and impulsivity, as well as neuromodulation of inhibitory control (FC), working memory, and incentive learning (amygdala).

Dysfunction of cortical-subcortical dopamine systems is associated with an impaired inhibitory control after chronic drug administration.

Since there is some evidence that the dopaminergic system might also play an important role in the pathophysiology of depression, methylphenidate has also been successfully used as an augmentation strategy in the treatment of depressive disorders and reduces risk of comorbid depressive episodes in ADHD. Effects here seem to be rather symptom specific (i.e., to counter lack of drive and in cognitive emotional processing) than genuinely antidepressant as recently underlined by meta-analytic data (Mcintyre et al. 2017). In ADHD patients research goes into whether depressiveness might be a rare stimulant-induced side effect.

Figure 2 (Jaworski and Jones 2006) illustrates the acute and chronic actions of methylphenidate. By blocking DAT, methylphenidate causes an accumulation of dopamine in the synaptic cleft. Although this may initially increase the stimulation of postsynaptic DA receptors, in the long term, the consequence is rather a downregulation of dopamine release. First, there is feedback inhibition of dopamine neuron firing to decreased spike-dependent dopamine release. Second, much larger quantities of dopamine are enabled to escape from the cleft and accumulate in the extrasynaptic space. Presynaptic receptors are stimulated and thus firing rate is reduced. The amount of phasic dopamine that can be released is subsequently diminished.

Moreover, no significant differences could be found between the efficacy of methylphenidate and amphetamine. Methylphenidate is faster

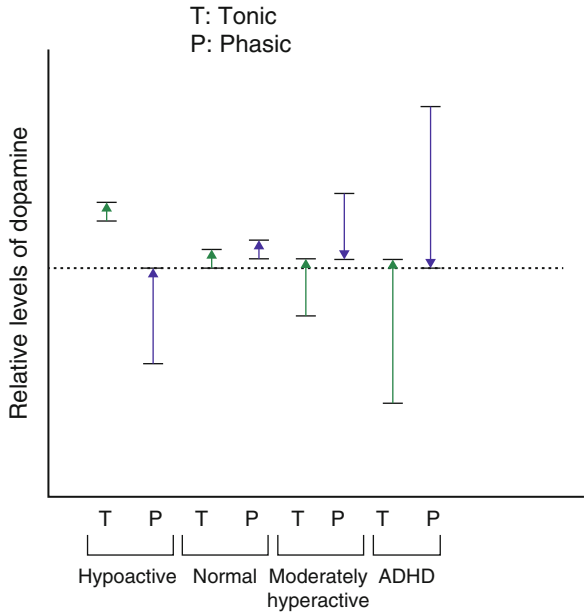


Psychostimulants, Fig. 2 Dopamine molecules have two different possible targets. Both ways are initially increased by DAT inhibition caused by methylphenidate: pre- and postsynaptic dopamine receptors. Stimulation of postsynaptic receptors results in inhibition of presynaptic action potential generation. On the other hand, presynaptic receptor stimulation leads to a transmission inhibition of action potentials. Therefore, both mechanisms are responsible for a decrease in vesicular depletion of dopamine into the synaptic cleft (adapted from Jaworski and Jones 2006)

metabolized and seems to be associated with fewer side effects regarding appetite loss and insomnia. From a clinical point of view, dosage and route of administration are the most important features influencing the spectrum of effects and side effects. ADHD drugs and cardiovascular risk (especially via increased heart rate, blood pressure, and to a lesser degree QTc elongation) has recently become a major issue with regard to sudden death. Although risk of severe cardiovascular events is considered very low (Martinez-Raga et al. 2013), selective and restricted use to clear-cut diagnoses are advocated, and side effects are regularly to be monitored.

An important clinical clue connected with the difference between tonic and phasic dopamine release is the so-called rate dependence of psychostimulant action. That means it depends on the actual dopaminergic state (tonic and phasic) how an individual will react to psychostimulants. Figure 3 illustrates this rule by some examples (Jaworski and Jones 2006). The arrows represent the response of each component to methylphenidate for each of the classes of subjects tested, with the horizontal dashed line representing the baseline tonic and phasic levels present in control individuals. Summarizing, methylphenidate tends to normalize dopamine transmission regardless what the baseline rate is.

Despite their clinical use, psychostimulants are strongly reinforcing, and their long-term use is linked to potential abuse and addiction, especially when they are rapidly administered. Nevertheless, long-term use is rather associated with emotional and motivational than with physical dependence. This is also true for cocaine and (meth)amphetamine. Methylphenidate might also be abused, although it is far less potent, possibly due to its specific mode of action (see above). Two drugs with low, if at all, abuse potential that are used in ADHD are atomoxetine (Gibson et al. 2006), a noradrenaline reuptake inhibitor, and bupropion, a dopamine and noradrenaline reuptake inhibitor. By their mode of action, they would rather fit into the group of antidepressants than psychostimulants.



Psychostimulants, Fig. 3 Effect of methylphenidate depending on baseline tonic (T) and phasic (P) dopamine levels. In a "normal" state, only minimal changes are noted (which points to a rather low abuse potential). From a "hypoactive" state, methylphenidate increases both T and P levels. However, this is much more true for the strongly lowered P tone. In contrast, in moderately hyperactive states and ADHD, T levels are increased, and P levels are decreased, respectively, correlating with the baseline levels (adapted from Jaworski and Jones 2006)

Nicotine differs from cocaine in that it is powerfully reinforcing in the absence of subjective euphoria. The high incidence of cancerogenicity associated with long-term tobacco use is associated with compounds other than nicotine that are also contained in tobacco. Main short-term effects of nicotine are increased alertness, muscle relaxation, nausea, and increased psychomotor activation. Typical withdrawal symptoms include dysphoria, increased appetite, hyperventilation, and concentration difficulties. If the α4β2 nicotinic partial agonist varenicline that is used for smoking cessation is also to be regarded as a stimulant drug still remains to be elucidated.

Cross-References

► Synaptic Transmission

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Psychotomimetic

► Psychedelic Drugs

PT-141

► Bremelanotide

Pulmonary Arterial Hypertension

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Synonyms

Pulmonary vascular disease; Pulmonary vascular remodeling

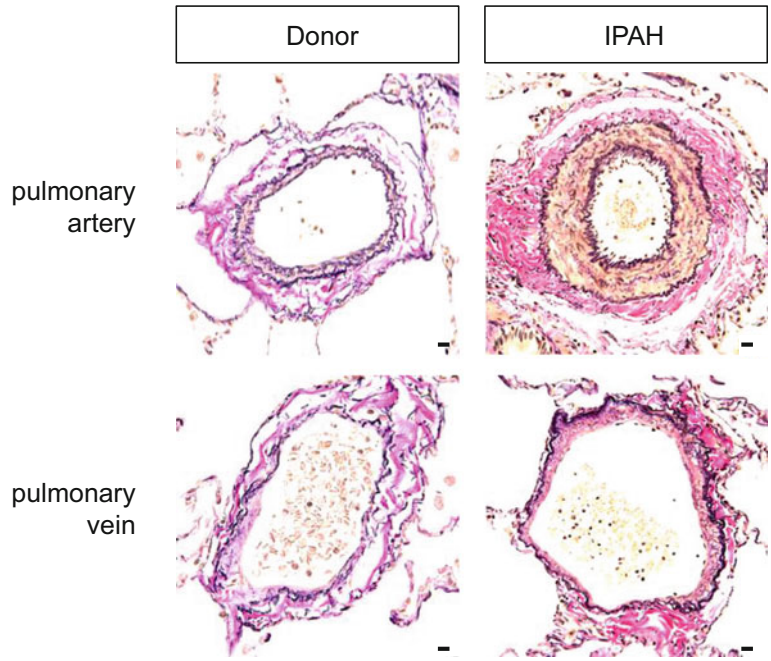
Definition

Pulmonary arterial hypertension (PAH) is a life-threatening lung disease clinically defined by a mean pulmonary artery pressure (mPAP) above 20 mmHg at rest in the presence of a pulmonary capillary wedge pressure equal or below 15 mmHg assessed by right heart catheterization (Galiè et al. 2019). Loss of patency of the lung vasculature through dysbalanced vasoactive mediators and obstructive remodeling of the pulmonary vascular bed account for a rise in pulmonary vascular resistance and mPAP, leading to progressive functional decline over time and, ultimately, right ventricular heart failure and death. The central histopathologic feature underlying PAH disease is a progressive narrowing of the pulmonary vasculature through inward remodeling of small pulmonary arterioles for which there are limited therapies and no cure (Humbert et al. 2019). This occlusive vasculopathy is characterized by changes in all three layers of the vascular wall (Rabinovitch 2012) and is frequently accompanied by loss of pre-capillary arteries. Current therapies target the vasoconstrictive component of the disease with limited long-term success as the underlying remodeling processes remain unchecked (Lau et al. 2017).

Basic Mechanisms

PAH is attributed to excessive pulmonary vasoconstriction and vascular obstruction occurring in distal muscular-type arteries ranging from 500μm down to 70μm in diameter, in addition to stiffening of large elastic main, lobar, and segmental pulmonary arteries (Humbert et al. 2019). Key features are medial hypertrophy/hyperplasia, intimal and adventitial fibrosis, adventitial immune cell infiltration, and plexiform lesions (Schermuly et al. 2011). Affected pulmonary arteries can be differentiated from pulmonary veins through their topography within the lung – since arteries are always neighbored by an airway (bronchiole) – and through their microscopic anatomy, which includes a neatly defined tunica media that is delimited by the *elastic laminae interna* and *externa*. In addition, small pre-capillary pulmonary arteries ranging from 70μm down to 20μm in diameter (arterioles) are involved in all forms of human PAH through processes of loss and obliteration, abnormal muscularization, and perivascular inflammation (Rabinovitch 2012). In contrast to the muscular-type arteries, arterioles can only be indirectly distinguished from small post-capillary venules of the same size by vessel tracing and reconstruction of the vascular tree. The capillary compartment that arises from the arteriolar microvasculature is involved in vessel obliteration, and there is accumulating evidence supporting the involvement of the post-capillary pulmonary venous vasculature in all PAH groups with varying degrees of intensity. Pulmonary veins running within interlobular septa normally comprise a thinner medial layer as compared with similar sized arteries and contain only a single *elastic lamina*, allowing histological distinction. However, remodeled pulmonary veins may histologically appear "arteriolized," mimicking the exact microscopic anatomy of muscular-type pulmonary arteries (Humbert et al. 2019). On a cellular level, unlike the intima and media, which are composed of single, although heterogeneous, cell types, the adventitia contains, in addition to fibroblasts, leukocytes (including macrophages, dendritic cells, and mast cells), progenitor cells, nerves (sympathetic and parasympathetic), lymphatics, as well as an extra blood supply, the vasa vasorum (Rabinovitch 2008) (Fig. 1).

Immunohistological staining of small pulmonary artery and vein cross sections from human donor and idiopathic PAH (IPAH) patients demonstrates a characteristic decrease in arterial vascular luminal diameter through prominent changes in the endothelium of the vessel wall, thickening of the vascular smooth muscle cell layer (brown) that is delimited by the elastic laminae *interna* and *externa* (dark violet), and accumulation of cells in the adventitial cell layer (pink). Pulmonary veins appear with a thickened smooth muscle cell layer and a single elastic lamina. Scale bar: 20μm.



Pulmonary Arterial Hypertension, Fig. 1 Van Gieson staining of human donor and IPAH lung tissue

Vasoreactivity

Patients with PAH present with a reduced capacity to generate vasodilatory mediators, such as prostaglandin, nitric oxide, cyclic guanosine monophosphate, or vasoactive intestinal peptide along with an increased presence of vasoconstrictive molecules, such as endothelin-1, serotonin, or thromboxane. It is conceivable that the beneficial effects of the currently clinically available treatments for PAH patients, such as prostacyclin, NO pathway modulators, and endothelin antagonists, are – at least in part – a result of rebalancing the signaling of these vasoactive mediators.

In addition, abnormalities in K^+ and Ca^{2+} channel expression and gating characteristics have been linked to a pathologic pulmonary vascular tone, particularly in vascular smooth muscle cells; and selective downregulation of voltage-gated K^+ channels causes an altered potassium flux into the cell, membrane depolarization, opening of voltage-gated Ca^{2+} channels, and induction of smooth muscle cell contraction via a Ca^{2+}-calmodulin mechanism that involves myosin light-chain kinase. In addition, Ca^{2+} signaling through transient receptor potential (TRP) ion channels is an important mechanism that contributes to vascular tone as, for example, the acute hypoxic vasoconstrictor response relies on TRPC6 function.

Pulmonary Vascular Remodeling

The pathogenesis of PAH involves complex and multifactorial processes as evidenced by the notion that PAH arises as a consequence of many different etiologies, including heritable mutations, congenital heart disease, systemic inflammatory conditions, viral infection, and drug and toxin exposure (Simonneau et al. 2019). Common to all forms of PAH are histological changes in all three layers of the vessel wall, suggesting that diverse upstream triggers converge on a core arterial remodeling program that may represent a clinically relevant therapeutic target to halt or reverse PAH disease progression.

Genetic Mutations

Genetic mutations in the gene encoding the bone morphogenetic protein type 2 receptor (BMPR2), a receptor of the transforming growth factor-beta

(TGFb) superfamily, account for over 80% of familial PAH and approximately 20% of sporadic cases. However, disease penetrance in patients harboring a BMPR2 mutation is often incomplete (20–30%) (Southgate et al. 2020), implying that other (epi-) genetic and environmental factors equally contribute to PAH disease pathogenesis. Yet, PAH-causing genetic mutations in other components of the TGFb/BMP signaling pathway have been identified (such as ACVRL and ENG), and case reports of rare sequence variation in the BMP signaling components, SMAD1, SMAD4, and SMAD9 provide further evidence for deregulated TGFb/BMP signaling as a causal driver of PAH pathogenesis. However, genetic mutations in other pathways than the TGFb/BMP pathway have been similarly linked to PAH susceptibility, as, for example, deleterious genetic variants in potassium channels (KCNK3 and ABCC8) or transcription factors (TBX4 and SOX17) are causal for PAH disease development (Gräf et al. 2018). A pathologically distinct form of PAH that predominantly affects the pulmonary venous circulation is caused by EIF2AK4 mutations that lead to pulmonary veno-occlusive disease (PVOD) (Eyries et al. 2014).

Signaling Abnormalities

On a cellular level, a deregulation in cell proliferation and survival accounts for the accumulation of vascular cells within the vessel wall. Medial smooth muscle and intimal endothelial cells in the pulmonary vasculature express G-protein-coupled receptors (GPCRs) that – depending on the upstream trigger – mediate complex downstream intercellular signaling events. In this regard, vasoactive mediators such as endothelin, angiotensin II, serotonin, and vasoactive intestinal peptide are extracellular ligands that upon GPCR binding induce a conformational switch in the intracellular docking domain of heterotrimeric G-proteins (composed of Gα and Gβγ subunits). This switch leads to dissociation of the heterotrimeric complex and thereby activation of the respective G-proteins with subsequent downstream signaling. While Gαs signaling can cause an elevation in intracellular cAMP levels, subsequent PKA activation, and reduced intracellular Ca^{2+} concentrations along with vasorelaxation, Gαq activates PLCβ and IP_3 signaling and initiates Ca^{2+} release from internal stores, such as the sarcoplasmatic reticulum, which promotes vasoconstriction through a Ca^{2+}-calmodulin-dependent mechanism that involves myosin light-chain phosphorylation, actin-myosin cross-bridging, and smooth muscle cell contraction (Strassheim et al. 2018). RhoA and Rho-kinase (ROCK), for example, are additional downstream targets of GPCR-mediated Gα12/13 activation in response to vasoactive ligands such as serotonin.

Reduced BMPR2 expression and dysfunctional BMP signaling recapitulate many of the cellular derangements attributed to PAH pathogenesis; and even patients with PAH without a BMPR2 mutation have reduced BMPR2 expression and BMP signaling in lung and blood cells. Given the high attrition rates of PAH studies, substantial development costs, and the slow pace of new drug development, repositioning of "old" drugs is increasingly recognized as an attractive path to identify novel PAH treatment options. Therefore, deregulated signaling pathways in PAH patients are frequently targeted with repurposed drugs such as FK506 (tacrolimus), enzastaurin, or (multi-) kinase inhibitors and show promise in pre-clinical PAH models, with some early encouraging results in selected patients. Many of these drugs are cancer therapeutics, and resident pulmonary vascular cells isolated from PAH patients' lungs share a number of cancer-cell characteristics, such as sustained proliferative capacity, resistance to cell death, de-regulated cellular energetics, and avoidance of immune cell destruction (Cool et al. 2020). Therefore, besides vasoactive mediators and dysbalanced BMP/TGFb signaling, growth factors such as platelet-derived growth factors (PDGFs), fibroblast growth factor (FGFs), or epidermal growth factors (EGFs), all similarly deregulated in cancer pathologies, are critically involved in PAH pathogenesis. These factors act as ligands and signal through their respective receptor kinases to over-activate downstream kinase-dependent phosphorylation cascades, which in turn cause aberrant cell behavior, such as increased proliferation, increased resistance to

apoptosis, or metabolic abnormalities. One critical signaling hub in PAH disease is signaling through PDGFRb in pulmonary artery smooth muscle cells and pericytes, which drives thickening of the medial pulmonary artery layer and hypoxia-dependent distal muscularization of previously non-/partially muscularized pulmonary arterioles (Sheikh et al. 2015). PDGFRb activates a variety of downstream signaling pathways, including Ras/MAPK, PI3K, and PLCγ, that further modulate transcriptional programs and – depending on the upstream trigger and cell type – cause changes in cell behavior. In a clinical phase III trial, pharmacological treatment of PAH patients with imatinib, a multi-kinase inhibitor that inhibits PDGFRb signaling among other pathways, improved pulmonary vascular remodeling, at the cost of serious adverse events, such as peripheral edema and subdural hematoma (Hoeper et al. 2013). Along this line, overactivation of various pro-proliferative signaling pathways has been reported in the context of PAH, and several multi-targeted tyrosine-kinase inhibitors approved as promising cancer therapeutics were subsequently linked to PAH pathogenesis and tested in therapeutic settings. For example, sorafenib, a multi-kinase inhibitor that inhibits PDGFRs, VEGFRs, Flt3, c-Kit, c-RAF, and b-RAF, has been studied in a phase Ib safety trial in PAH patients (Gomberg-Maitland et al. 2010). Based on promising pre-clinical results, a phase II trial to study the effects of nilotinib (Bcr-Abl, c-Kit, PDGFR-α/β inhibitor) was initiated. The latter was terminated due to unwanted side effects; and limited therapeutic use in PAH patients was similarly recognized for nintedanib and dasatinib (Bcr-Abl, c-Kit, PDGFR-α/β, and Src inhibitor).

To narrow down the broad spectrum of kinase signaling pathways that are frequently activated through mitogens and growth factors, more specific targeting of intracellular kinases that mediate the pro-proliferative signaling responses was studied. Dysregulation of p38 MAPK signaling is directly linked to PAH disease development, and inhibition of p38-MAPK signaling has emerged as a potential therapeutic option in PAH. Inhibition of the p38-MAPK pathway improved pulmonary hypertension in pre-clinical models and was linked to blockage of IL-6 induced pro-inflammatory signaling. Along the same line, inhibition of ASK1 – a p38 upstream kinase that is activated in response to oxidative stress – halted disease progression in pre-clinical models. Another mediator pathway is characterized by a JAK/STAT signaling hub that directly links upstream trigger of cell immunity, proliferation, and survival with transcription of specific gene sets under STAT control. JAK/STAT deregulation has been recognized in PAH pathobiology and has more recently been associated with fibrotic disorders such as myelofibrosis. As an example, Jak2 is a major mediator and regulator of growth factor-induced signaling through downstream activation of Stat3 that causes non-canonical PI3K signaling and MAPK activation (Paulin et al. 2012). Ruxolitinib, a small-molecule inhibitor of JAK/STAT signaling, improved pulmonary vascular remodeling in pre-clinical PAH models and in patients with myelofibrosis through inhibition of smooth muscle cell proliferation and rebalancing of vasoreactivity. A similar signaling cascade involves mammalian target of rapamycin (mTOR), a cellular regulator of metabolism, proliferation, and survival, that functions through two distinct multi-protein complexes (mTOR complex 1 (mTORC1) and mTORC2). mTORC1 activates its downstream targets p70 S6 kinase 1 (S6K1) and 4E-binding protein 1 (4E-BP1) through phosphorylation that in turn increases protein synthesis and cell proliferation. mTOR inhibition with rapamycin reduces pulmonary vascular remodeling in pre-clinical PAH models, and in a clinical safety and efficacy trial, everolimus (structurally related to rapamycin) reduced pulmonary vascular resistance without severe side effects in patients.

On a subcellular level, hyper-proliferation of pulmonary artery smooth muscle cells is linked to aberrant cell cycle progression which is tightly controlled through cyclin-dependent kinase (CDK) activity. CDKs are increasingly active in pulmonary artery smooth muscle cells from IPAH patients contributing to their pro-proliferative phenotype. CDKs regulate cell cycle progression in a Rb (retinoblastoma protein)-dependent

manner to control E2F proliferative target genes and cell growth. Thereby, CDKs drive the pro-proliferative phenotype of these cells, and pharmacological targeting of CDK4/CDK6 causes cell cycle arrest, reduces proliferation through modulation of CDK-Rb-E2F signaling, and reduces disease pathogenesis in pre-clinical PAH models. However, whether direct cell cycle inhibition in a long-term treatment approach in PAH patients is effective, well-tolerated, and safe is currently unknown (Weiss et al. 2019).

Metabolism and Hypoxia

In an attempt to meet their increased energetic demand within the diseased environment, pulmonary vascular cells undergo metabolic reprogramming with an energetic shift from oxidative phosphorylation towards glycolysis (Warburg effect). This shift is coupled with multiple abnormalities in mitochondrial function, including suppressed activity of pyruvate dehydrogenase and glucose oxidation, superoxide dismutase 2 deficiency, as well as mitochondrial hyperpolarization and fragmentation (Archer 2016). Increased glutaminolysis and fatty acid oxidation further contribute to energetic adaptation. The change in ATP energy production comes along with an increased resistance towards mitochondria-dependent cell death. This apoptosis resistance is critically regulated by pyruvate dehydrogenase kinase (PDK) which balances mitochondrial enzyme pyruvate dehydrogenase (PDH) function. Therapy with dichloroacetate (DCA), a PDH inhibitor, improved hemodynamics in some PAH patients, while others remained unresponsive. This heterogeneity in treatment responsiveness was *post hoc* linked to single nucleotide polymorphisms in sirtuin 3 (Sirt3) and uncoupling protein 2 (Ucp2), both upstream regulators of PDH. This provides further evidence for significant molecular heterogeneity in PAH patients suffering from a similar clinical and histological pathology. It is worth to mention, that it is unclear, whether the increased energy demand is caused by the vascular remodeling processes or vice versa. Malnutrition and cellular oxygen homeostasis, however, are similarly controlled by a key transcription factor, hypoxia-inducible factor (HIF), a master regulator of oxygen homeostasis that acts as a heterodimeric complex composed of an oxygen-sensitive α-subunit and an oxygen-insensitive β-subunit (Pullamsetti et al. 2020). In oxygenized cells, HIFα is inactivated via hydroxylation by prolyl hydroxylases and HIF-inhibiting factors, leading to HIFα ubiquitination and proteasomal degradation. Cellular hypoxia results in stabilization and nuclear translocation of HIFα, whereas activated HIF acts as a transcriptional regulator of a broad range of genes associated with vascular tone, cellular metabolism, proliferation, survival, and autophagy. In the lung, abnormal HIF-1 and HIF-2 activation due to cellular hypoxia have been linked to PAH disease pathogenesis with cell type-specific roles for both transcription factors. This is important as the mechanisms responsible for endothelial activation likely differ from the mechanisms responsible for smooth muscle cell activation. However, a number of stimuli, including shear stress from increased pulmonary blood flow, viral infection (HIV), and alveolar hypoxia, may potentially activate such a response in (genetically) predisposed individuals. The nature of the initial stimulus that precedes the vasoconstrictive/vasoproliferative events is unknown. However, an attractive hypothesis is that injury to the endothelium leads to apoptosis of the usually quiescent cells; destabilization of the pulmonary vascular intima, preferably at branching points; and uncontrolled proliferation of endothelial cells. Most of the physiological consequences of PH would then emanate from the resultant narrowing of the pulmonary vessels.

Inflammation

The inflammatory processes underlying PAH pathogenesis are complex, vary in their degree and form, and are often spatially confined with a strict underlying temporal sequelae of events. Pre-clinical work suggests that aberrant reparative immunity and loss of self-tolerance result in exuberant inflammation, which seems to perpetuate vascular injury and remodeling. In PAH patients, inflammatory cells localize around remodeled lung vessels, and levels of multiple cytokines are abnormally elevated in peripheral blood. The

levels of circulating cytokine heterogeneity among different PAH populations are unknown up this point; however, first machine learning approaches using unsupervised computer algorithms for inflammatory phenotyping begin to uncover specific cytokine profiles that are more and more linked to clinical phenotypes (Sweatt et al. 2019). Substantial evidence indicates a role for elevated pro-inflammatory circulating cytokines MCP-1, TNFα, IL-1β, and IL-6 in PAH patients. Further, chemokine-dependent mechanisms lead to inflammatory cell recruitment towards PAH lungs. FKN (also known as C-X3-C motif chemokine 1), for example, is a soluble chemotactic protein that is found as a membrane-anchored cell-adhesion molecule on endothelial cells. The actions of FKN are mediated by CX3C chemokine receptor 1 (CX3CR1), a transmembrane receptor expressed by monocytes, microglial cells, neurons, natural killer cells, mast cells, and subpopulations of T lymphocytes. Unlike other chemokines, FKN can mediate the rapid-capture, integrin-independent adhesion, and activation of circulating CX3CR1+ leukocytes under high blood flow (Fong et al. 1998). FKN expression is increased in circulating CD4+ and CD8+ T lymphocytes from patients with PAH, in inflammatory cells surrounding plexiform lesions, and in pulmonary arterial smooth muscle cells of remodeled arteries. Other cytokines overproduced in patients with PAH are RANTES and chemokine ligand 2 (CCL-2). CCL-2 overproduction is a feature of the abnormal pulmonary endothelial cell phenotype in IPAH, and, compared with healthy controls, pulmonary artery SMCs from patients with IPAH exhibited stronger migratory and proliferative responses to CCL-2.

In addition, infectious organisms can affect the lung circulation either directly by obliterating lung vessels or indirectly via release of inflammatory cells, pro-inflammatory mediators, or activation of the immune system (Knafl et al. 2020). Some of these cells include monocytes, macrophages, T lymphocytes, and dendritic cells that are also found in plexiform lesions and other vascular lesions of PAH-affected human lungs.

PAH is directly associated with latent viral infections (e.g., HIV, human herpes virus [HHV], Epstein-Barr virus, and cytomegalovirus) (Cool et al. 2011), whereas the causative role of viruses in PAH induction is not well understood. PAH disease is further associated with schistosomiasis, a parasitic infection that causes substantial inflammation and pulmonary vascular disease. Over 200 million people worldwide are currently infected with schistosomiasis, and up to 20% of those with long-term infection develop PAH. Lung tissue parasitic egg burden induces inflammatory and immune processes and may underlie PAH pathogenesis in infected patients.

Summary

Genetic, signaling, metabolic, and environmental abnormalities that involve various deregulated signaling molecules underlie PAH pathogenesis and lead to intimal changes, including endothelial injury, endothelial cell proliferation, non-endothelial cell invasion, and enhanced matrix deposition; medial changes with abnormal vascular smooth muscle cell behavior (pro-proliferative, pro-migratory, anti-apoptotic); and chronic inflammatory conditions through adventitial immune cell infiltration, elevated circulating cytokines, and recruitment of circulating cell types to the lung. On a molecular level, deregulation of many signaling molecules contribute to an imbalance in vasoactive mediators, increased cell proliferation and resistance to apoptosis, chronic inflammation, and metabolic abnormalities. The ongoing efforts in deciphering cellular heterogeneity and cause-consequence relations will pave the way for precise pharmacological strategies tailored to individuals to tackle the progressive pulmonary vasculopathy seen in PAH patients.

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Pulmonary Emphysema

▶ Chronic Obstructive Pulmonary Disease

Pulmonary Vascular Disease

▶ Pulmonary Arterial Hypertension

Pulmonary Vascular Remodeling

▶ Pulmonary Arterial Hypertension

Purinergic System

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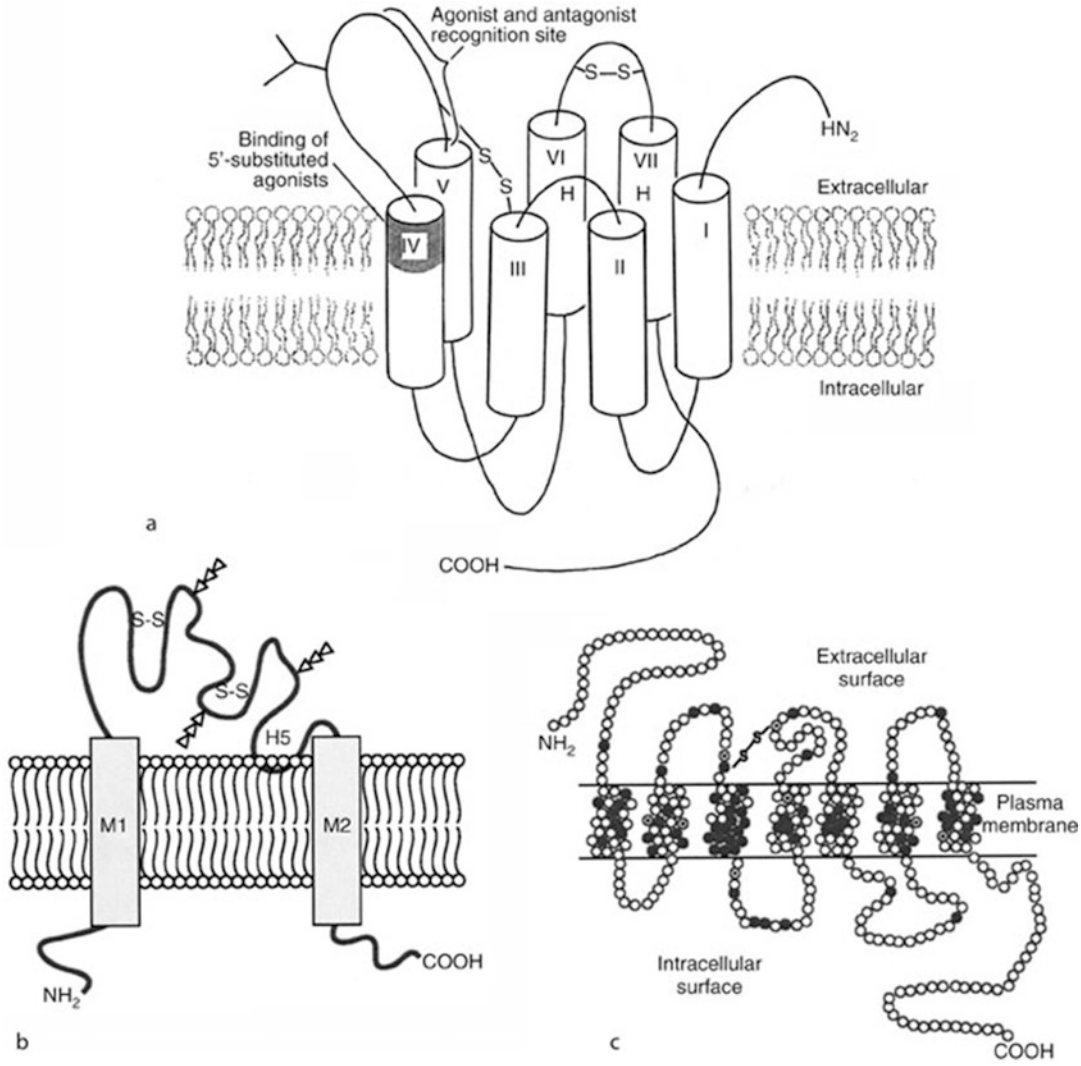
Definition

The purinergic system is a signaling system, where the purine nucleotides, **ATP** and **ADP**, and the nucleoside, **adenosine**, act as extracellular messengers. This concept, which was first proposed over 30 years ago (Burnstock 1997), met with considerable resistance for many years, because ATP had been established as an intracellular energy source involved in various metabolic cycles and it was thought that such a ubiquitous molecule was unlikely to be involved in selective extracellular signaling. However, ATP was one of the first molecules to appear in biological evolution so that it is not really surprising that it should have been utilized early for extracellular, as well as intracellular, purposes. The existence of potent extracellular enzymes that regulate the amount of ATP and adenosine available for signaling also provides support that ATP has extracellular actions.

Basic Characteristics

Purinoceptor Subtypes

Implicit in purinergic signaling is the presence of receptors for ATP (Ralevic and Burnstock 1998). A basis for distinguishing adenosine receptors (P1), from ATP/ADP receptors (P2), was proposed in 1978. This helped resolve some of the earlier ambiguous reports, which were complicated by the breakdown of ATP to adenosine by ectoenzymes, so that some of the actions of ATP were directly on P2 receptors, while others were due to indirect action via P1 receptors. Four subtypes of P1 receptors have been cloned, namely, A_1, A_{2A}, A_{2B}, and A_3 (Fig. 1a). P2 receptors belong to two families based on molecular structure and second messenger systems, namely P2X ionotropic ligand-gated ion channel receptors and P2Y metabotropic G protein-coupled receptors. This framework allows for a logical expansion as new receptors are identified. There are currently seven subtypes of P2X receptors and eight subtypes of P2Y receptors identified and characterized in mammals. P2X receptors are characterized by two transmembrane domains, short intracellular N- and C-termini, and an extensive extracellular loop with conservation of 10 cysteines (Fig. 1b). Broadly, P2X1 receptors are strongly represented in smooth muscle, P2X2, P2X4, and P2X6 receptors in the central nervous system, P2X3 receptors on sensory neurones, P2X5 receptors associated with cell proliferation, and differentiation and P2X7 receptors with cell death. The ion pores appear to consist of three subunits forming homomultimers or heteromultimers, including P2X1/2, P2X1/4, P2X1/5,



Purinergic System, Fig. 1 Membrane receptors for extracellular ATP and adenosine. P1 family of receptors for extracellular adenosine are G protein-coupled receptors signaling by inhibiting or activating adenylate cyclase (**a**). The P2 family of receptors bind extracellular ATP or ADP and are comprised of two types of receptors (P2X and P2Y). The P2X family of receptors are ligand-gated ion channels (**b**), and the P2Y family are G protein-coupled receptors (**c**). (**a,** reproduced from 2 with permission from the American Society for Pharmacology and Experimental Therapeutics; (**b**), from Brake, A. J., Wagenbach, M. J. and Julius, D. (1994). New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor. *Nature* **371**, 519–523, reproduced with permission from Nature; (**c**) Modified from Barnard, E. A., Burnstock, G. and Webb, T.E. (1994). G protein-coupled receptors for ATP and other nucleotides: a new receptor family. *Trends in Pharmacological Sciences* **15**, 67–70, reproduced with permission from Elsevier)

P2X2/3, P2X2/6, and P2X4/6. P2Y receptors, in common with other protein-coupled receptors, have seven transmembrane domains, an extracellular N, and intracellular C-terminus (Fig. 1c). $P2Y_1$ receptors are ADP-selective in mammals and 2-methylthioADP and MRS 2179 are selective agonists and antagonists, respectively. At $P2Y_2$ and $P2Y_4$ receptors in the rat, ATP and UTP are equipotent, but the two receptors can be distinguished with antagonists. $P2Y_6$ is UDP-selective. $P2Y_{11}$ is unusual in that there are two transduction pathways, adenylate cyclase

as well as inositol trisphosphate, which is the second messenger system used by the majority of the P2Y receptors. The $P2Y_{12}$ receptor is found on platelets. $P2Y_{13}$ is ADP selective and acts via adenylate cyclase, while $P2Y_{14}$ receptors are selective for UDP-glucose and UDP-galactose.

Physiology

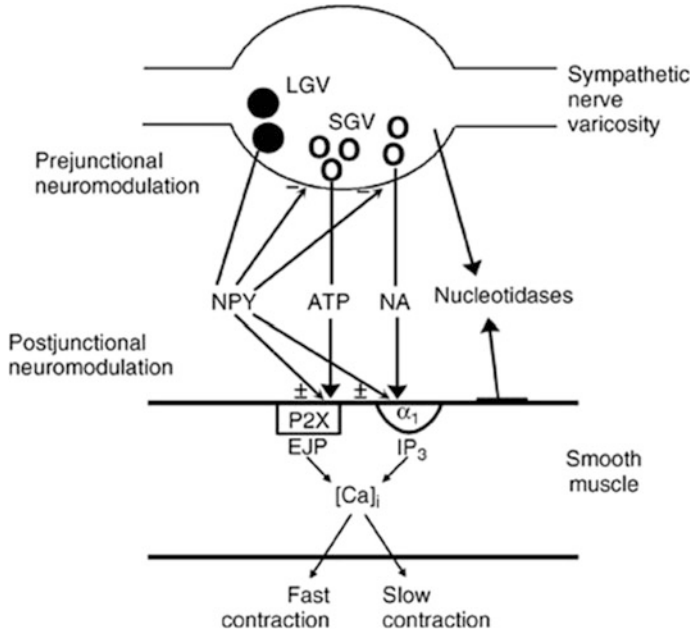
Short-Term Neuronal Signaling

There was early evidence that ATP was a **neurotransmitter** in nonadrenergic, noncholinergic (NANC) nerves supplying the gut and bladder. There is now supporting evidence that ATP is a cotransmitter in many nerve types (Burnstock 1997), probably reflecting the primitive nature of purinergic signaling. Thus, there is now evidence for ATP as a cotransmitter with:

- Noradrenaline (NA) and neuropeptide Y in sympathetic nerves
- ATP with acetylcholine and vasoactive intestinal peptide in some parasympathetic nerves
- ATP with nitric oxide and vasoactive intestinal peptide in enteric NANC inhibitory nerves
- ATP with calcitonin gene-related peptide and substance P in sensory-motor nerves

There is also evidence for ATP as a cotransmitter with γ-aminobutyric acid or with glutamate, serotonin, NA, or dopamine in nerves in the central nervous system.

In sympathetically innervated tissues, such as vas deferens or blood vessels, ATP produces fast responses mediated by P2X receptors followed by a slower component mediated by G protein-coupled α-adrenoceptors (Fig. 2); neuropeptide



Purinergic System, Fig. 2 Schematic of sympathetic cotransmission. ATP and NA released from small granular vesicles (SGV) act on P2X and α_1 receptors on smooth muscle, respectively. ATP acting on inotropic P2X receptors evokes excitatory junction potentials (EJPs) and increases in intracellular calcium ($[Ca^{2+}]_i$) and fast contraction, while occupation of metabotropic α_1 adrenoceptors leads to production of inositol triphosphate (IP_3), increases in $[Ca^{2+}]_i$, and slows contraction. Neuropeptide Y (NPY) stored in large granular vesicles (LGV) acts after release both as a prejunctional inhibitory modulator of release of ATP and NA and as a postjunctional modulatory potentiator of the actions of ATP and NA. Soluble nucleotidases are released from nerve varicosities and are also present as ectonucleotidases. (Reproduced from Burnstock, G. (2007). Neurotransmission, Neuromodulation: Cotransmission. In: *New Encyclopaedia of Neuroscience,* Elsevier, The Netherlands (In Press), with permission from Elsevier)

Y usually acts as a pre- or postjunctional modulator of the release and/or action of noradrenaline and ATP. Similarly, for parasympathetic nerves supplying the urinary bladder, ATP provokes a fast, short-lasting twitch response via P2X receptors, whereas the slower component is mediated by G protein-coupled muscarinic receptors. In the gut, ATP released from NANC inhibitory nerves produces the fastest response, nitric oxide gives a less rapid response, and vasoactive intestinal peptide produces slow tonic relaxations. In all cases of **cotransmission**, there are considerable differences in the proportion of the cotransmitters in nerves supplying different regions of the gut or vasculature, in different developmental or pathophysiological conditions and between species.

The first clear evidence for nerve-nerve purinergic **synaptic transmission** was in 1992, when it was shown that excitatory postsynaptic potentials in the celiac ganglion and in the medial habenula in the brain were reversibly antagonized by suramin, a P2 receptor antagonist. Since then, there have been many articles describing either the distribution of various P2 receptor subtypes in the brain and spinal cord or electrophysiological studies of the effects of purines in brain slices, isolated nerves, and glial cells. Synaptic transmission has also been found in the myenteric plexus and in various sensory, sympathetic, and pelvic ganglia.

Short-Term Nonneuronal Signaling

There are many examples of purinoceptor-mediated responses in nonneuronal cell types (Abbracchio and Williams 2001). **Endothelial cells**, which express $P2Y_1$, $P2Y_2$, and probably $P2Y_6$ and P2X4 receptors, when occupied, release nitric oxide leading to vasodilatation (Fig. 3). The discovery of P2X receptors in endothelial cells suggests a role regulating gap and tight junctions involved in permeability and in cell adhesion. $P2Y_1$ receptors in pancreatic **β**-cells have been shown to be involved in insulin secretion, and $P2Y_2$ receptors are present on hepatocytes. $P2Y_{12}$, P2X1, and $P2Y_1$ receptors are expressed in platelets and $P2Y_1$ and $P2Y_2$ receptors on nonmyelinating and myelinating Schwann cells, respectively. Purinergic receptors are also involved in signaling to endocrine cells, leading to hormone secretion and in neuron-glial cell interactions.

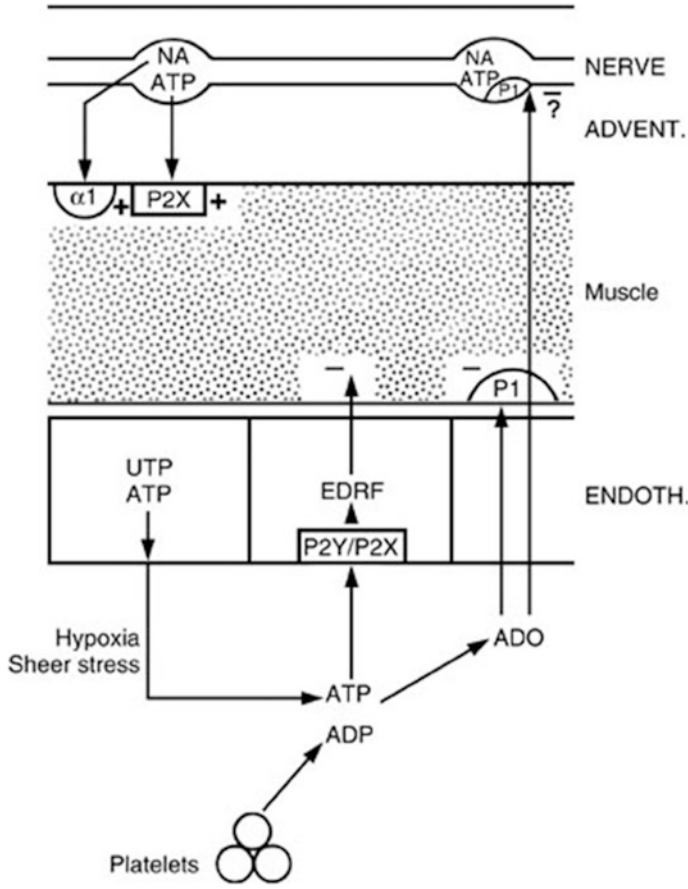
Long-Term (Trophic) Signaling

Purinergic signaling is also concerned with long-term events, such as cell proliferation, migration, differentiation, and death associated with development and regeneration (Burnstock 1997; Abbracchio and Williams 2001; Burnstock 2006). For example, **α**,**β**-methylene ATP produces proliferation of glial cells, whereas adenosine inhibits proliferation. A p2y8 receptor was cloned from the frog embryo, which appears to be involved in the development of the neural plate. $P2Y_1$ receptors seem to have a role in cartilage development in limb buds and in development of the mesonephros. P2X5 and P2X6 receptors have been implicated in the development of chick skeletal muscle. In studies of purinoceptor expression in developing mouse myotubes, there was progressive expression of P2X5, P2X6, and P2X2 receptors. The P2X1 receptor is prominent in contractile smooth muscle phenotype, but is absent in the synthetic smooth muscle phenotype grown in culture, while P2Y receptor expression is substantially increased. There are several reports showing that P2X and P2Y receptors in **osteoclasts** and **osteoblasts** are involved in bone development and remodeling.

Pathophysiology and Therapeutic Potential

There is increasing interest in the therapeutic potential of purinergic compounds in relation to both P1 and P2 receptors (Burnstock 2006). A number of purine-related compounds have been patented.

It is well established that the **autonomic nervous system** shows marked plasticity. The expression of cotransmitters and receptors shows dramatic changes during development and ageing, in nerves that remain after trauma or surgery and in disease conditions. There are now a number of examples where the purinergic component of cotransmission is increased in pathological conditions(Burnstock 2006). Purinergic nerve-



Purinergic System, Fig. 3 A schematic representation of the interactions of ATP released from perivascular nerves and from the endothelium (ENDOTH). ATP and UTP are released from endothelial cells during changes in blood flow (sheer stress) and hypoxia to act on endothelial P2Y and P2X receptors leading to production of EDRF (nitric oxide) and subsequent vasodilatation (−). In contrast, ATP released as a cotransmitter with noradrenaline (NA) from perivascular sympathetic nerves at the adventitia (ADVENT)/muscle border produces vasoconstriction (+) via P2X receptors on the muscle cells. Adenosine (ADO), resulting from rapid breakdown of ATP by ecto-enzymes, produces vasodilatation by direct action on the muscle via P1 receptors and acts on the perivascular nerve terminal varicosities to inhibit transmitter release. (Modified from Burnstock G, (1987). Local control of blood pressure by purines. *Blood Vessels* **24**, 156–160, reproduced with permission from Karger AG)

mediated contractions of the human bladder are increased up to 40% in pathophysiological conditions such as interstitial cystitis, outflow obstruction, and probably also neurogenic bladder. ATP plays a significantly greater cotransmitter role in sympathetic nerves supplying hypertensive blood vessels. Upregulation of P2X1 and $P2Y_2$ receptor mRNA in hearts of rats with congestive heart failure has been reported. Adenosine modulates long-term synaptic plasticity in the hippocampus; it attenuates long-term potentiation (LTP); P1 receptor antagonists facilitate LTP. It is suggested that adenosine-related compounds might prove helpful in the treatment of memory disorders and intellectual performance related to caffeine intake.

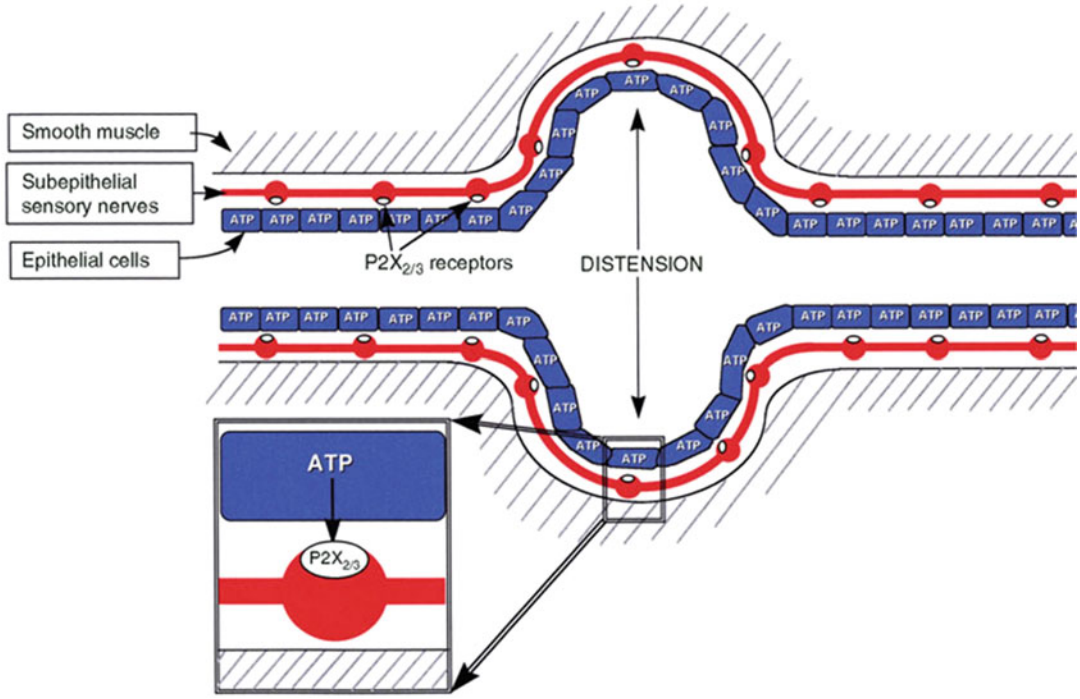
A new hypothesis for purinergic **mechanosensory transduction** in visceral organs involved in the initiation of pain has been proposed. It is suggested that distension of tubes (such as the ureter, salivary duct, and gut), and sacs (such as urinary and gall bladder), leads to the release of ATP from the lining epithelial cells that diffuses to subepithelial sensory nerves expressing P2X3 and/or P2X2/3 nociceptive receptors, which

mediate messages to pain centers in the central nervous system (Burnstock 2001) (Fig. 4). Recording in P2X3 knockout mice has shown that the micturition reflex is impaired and that responses of sensory fibers to P2X3 agonists are gone, suggesting that P2X3 receptors on sensory nerves in the bladder have a physiological as well as a nociceptive role.

P1 (adenosine) receptors were explored as therapeutic targets before P2 receptors. Adenosine was identified early and is in current use to treat **supraventricular tachycardia**. A_{2A} receptor antagonists are being investigated for the treatment of **Parkinson's disease** and patents have been lodged for the application of P1 receptor subtype agonists and antagonists for myocardial ischemia and reperfusion injury, cerebral ischemia, stroke, intermittent claudication, and renal insufficiency.

Purinergic receptors have a strong presence in bone cells. P2X and P2Y receptors are present on osteoclasts, with P2Y receptors only being present on osteoblasts. ATP, but not adenosine, stimulates the formation of osteoclasts and their resorptive actions *in vitro* and can inhibit osteoblast-dependent bone formation. A study has shown that very low (nM) concentrations of ADP acting through $P2Y_1$ receptors turn on osteoclast activity. Modulation of P2 receptor function may have potential in the treatment of osteoporosis. The anticancer activity of adenine nucleotides was first described in 1983 and since then, intraperitoneal injection of ATP into tumor-bearing mice has resulted in significant anticancer activity against several fast-growing aggressive carcinomas.

Purinergic signaling is important in the special senses. For example, $P2Y_2$ receptor activation increases salt, water, and mucus secretion in the



Purinergic System, Fig. 4 Schematic representation of the hypothesis for purinergic mechanosensory transduction in tubes (e.g., ureter, vagina, salivary and bile duct and gut) and sacs (e.g., urinary and gall bladders, and lung). It is proposed that distension leads to release of ATP from the epithelium lining the tube or sac, which then acts on $P2X_{2/3}$ receptors on subepithelial sensory nerves to convey sensory (nociceptive) information to the CNS. (From Burnstock, G. (1999) Release of vasoactive substances from endothelial cells by shear stress and purinergic mechanosensory transduction. *Journal of Anatomy*, **194**, 335–342, with permission from Blackwell Publishing)

eye and thus represents a potential treatment for dry eye disease. P2 receptor agonists have greater efficacy in reducing intraocular pressure than cholinergic and adrenergic agents, raising possibilities for novel treatment of glaucoma. It has been suggested that ATP may regulate fluid homeostasis, cochlear blood flow, hearing sensitivity and development, and thus may be useful in the treatment of Ménières disease, tinnitus, and sensorineural deafness.

There have been very promising developments concerning purinergic drugs aimed at treating **thrombosis**. Clopidogrel and ticlopidine are antagonists to the $P2Y_{12}$ receptor and appear to reduce the risks of recurrent strokes and heart attacks, especially when combined with aspirin. Further therapeutic targets include: chronic renal failure, congestive heart failure, hypertension, stroke, angina, asthma, chronic obstructive pulmonary disease, epilepsy, sleep apnea, diabetes, inflammation, erectile function, and wound healing.

Recent advances have been made about the pathophysiology and therapeutic potential of purinergic signalling (Burnstock 2017).

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Q

Quantification of Drug Effect

► Drug–Receptor Interaction

S. Offermanns, W. Rosenthal (eds.), *Encyclopedia of Molecular Pharmacology*,
https://doi.org/10.1007/978-3-030-57401-7

R

RAAS

► Renin–Angiotensin–Aldosterone System

RAD24

► 14-3-3 Proteins

RAD25

► 14-3-3 Proteins

Regulation of Ingestive Behavior

► Appetite Control

Regulation of Smooth Muscle Contractility

► Smooth Muscle Tone Regulation

Rel Proteins

► NF-κB, Molecular Target

Relaxin

► Relaxin Family Peptides and Their Receptors

Relaxin Family Peptides and Their Receptors

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Synonyms

Relaxin (human relaxin-2), INSL3 (insulin-like peptide 3; formerly Leydig insulin-like peptide); Relaxin-3 (mammalian relaxin-3), INSL5 (insulin-like peptide 5); RXFP1 (relaxin family peptide receptor 1; LGR7; RXFPR1; RX1); RXFP2 (relaxin family peptide receptor 2; LGR8; GPR106; RXFPR2); RXFP3 (relaxin family peptide receptor 3; SALPR; RLN3R1; RXFPR3;

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https://doi.org/10.1007/978-3-030-57401-7

GPCR135); RXFP4 (relaxin family peptide receptor 4; RLN3R2; RXFPR4; GPCR142; GPR100) (Alexander et al. 2019).

Definition

The relaxin family peptides relaxin, insulin-like peptide (INSL)3, relaxin-3, and INSL5 mediate hormonal and neuropeptide actions by acting on a group of four relaxin family peptide (RXFP) receptors (Bathgate et al. 2013; Halls et al. 2015; Alexander et al. 2019; Valkovic et al. 2019). The receptor systems have roles in the cardiovascular system, formation of connective tissue and bone, and reproduction and act in the brain as neuropeptides to modulate stress, anxiety and mood, arousal, and behaviors such as feeding and drug seeking. The antifibrotic effects of relaxin at RXFP1 have therapeutic potential given the role of fibrosis in progressive cardiovascular and renal disease and asthma.

Basic Characteristics

The relaxin family peptides are two-chain peptides closely resembling insulin in structure. All have an A-chain linked to a B-chain by two interchain disulfide bonds and an intrachain disulfide bond in the A-chain. Relaxin, INSL3, relaxin-3, and INSL5 are the cognate ligands for the relaxin family peptide receptors RXFP1–4, respectively (Bathgate et al. 2013; Halls et al. 2015). Relaxin was discovered as a factor in pregnant guinea-pig serum that relaxed the birth canal, and the study of its biological actions, structure, and molecular biology led to the identification of the genes encoding the related INSL3, relaxin-3, and INSL5 (Bathgate et al. 2013; Halls et al. 2015). While there is evidence that relaxin acting at RXFP1 has widespread functions in the reproductive system, brain, and cardiovascular system as well as general metabolic and antifibrotic actions, INSL3 activation of RXFP2 has specific and limited actions mainly in the reproductive system. INSL3 is found in the Leydig cells of the testis and plays a critical role in gubernaculum development and testis descent and in females has a role in maintaining ovarian function. In contrast to the other relaxins, relaxin-3 acting at RXFP3 is well conserved across species and is believed to be the ancestral peptide that has well-defined roles as a neuropeptide in mammals. The cognate ligand for RXFP4, INSL5, is more widely distributed with particularly high concentrations found in the L-cells of the gastrointestinal tract where it likely has metabolic functions (Bathgate et al. 2013; Halls et al. 2015).

While the relaxin family peptide receptors are all GPCRs with the typical seven transmembrane-spanning regions, RXFP1 and RXFP2 differ structurally quite markedly from RXFP3 and RXFP4. RXFP1 and 2 have a large extracellular domain comprising ten leucine-rich repeats (LRR) and an N-terminal low-density lipoprotein receptor type A module (LDLa). Binding of relaxin to RXFP1 involves a high-affinity component between the B-chain of the peptide and the LRR and lower-affinity binding to receptor ECLs followed by an essential interaction involving the LDLa module. Receptor activation increases cellular cAMP levels and activates ERK1/2, tyrosine kinases, NO signaling, as well as changing gene transcription and glucocorticoid receptor signaling. RXFP2 signaling is simpler and usually but not always involves increases in cAMP generation. In contrast to RXFP1 and 2, RXFP3 and 4 are classical family A GPCRs with small N-terminal domains, and activation leads to a decrease in intracellular cAMP levels (Bathgate et al. 2013; Halls et al. 2015; Valkovic et al. 2019).

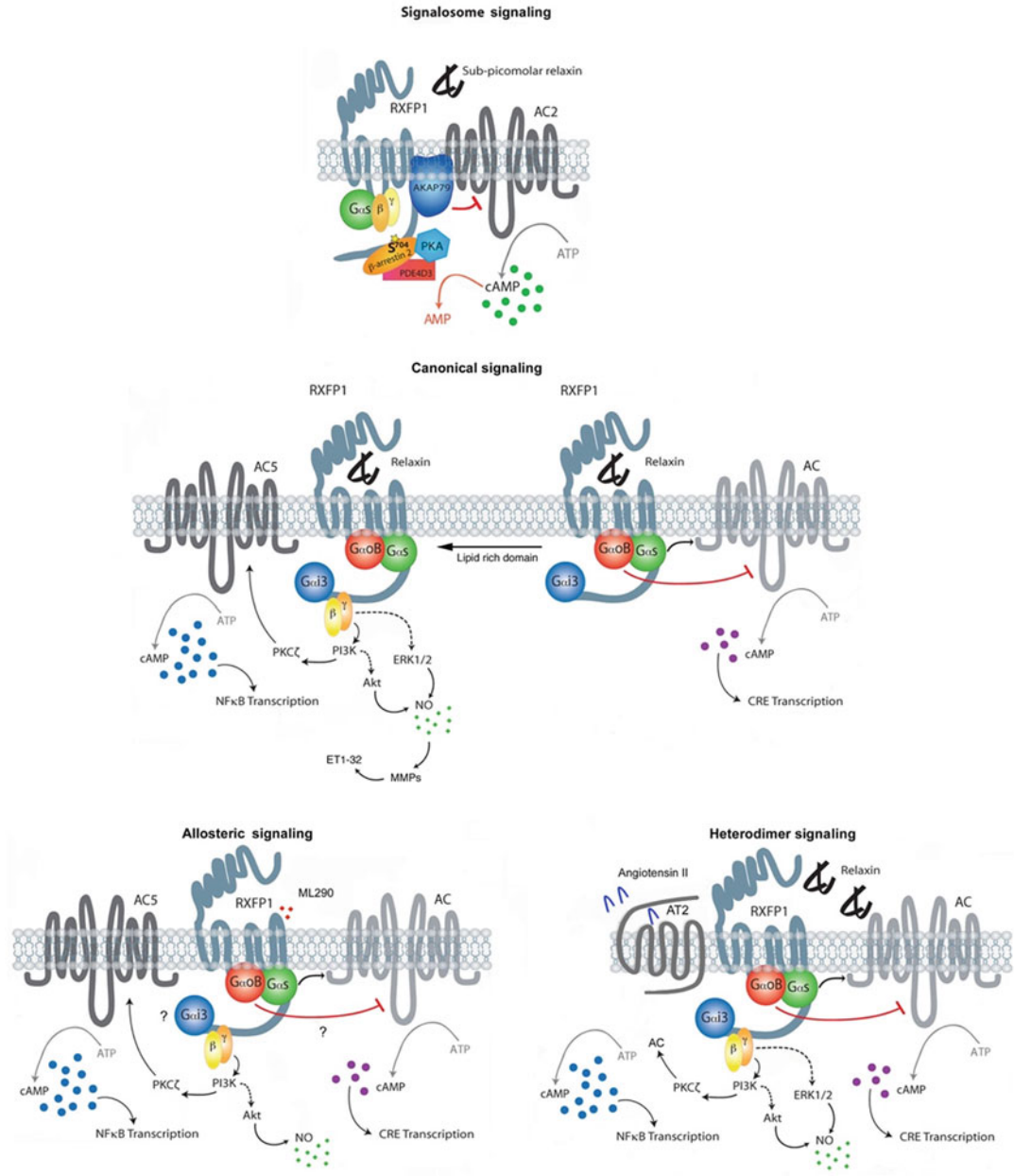
RXFP1 and 2 share 60% amino acid identity and 80% homology. Many splice variants of the receptors have been identified with many being nonfunctional. A secreted variant RXFP1-truncate is increased in pregnancy and appears to act as a functional antagonist of RXFP1 (Alexander et al. 2019). RXFP3 and 4 are encoded by a single exon in human, but RXFP4 is a pseudogene in rat that does not encode a functional protein.

RXFP1 and 2 are unique among GPCRs in containing the LDLa module at the N-terminus that is believed to act as a tethered ligand. Receptor variants that lack the LDLa module bind their cognate ligands normally but cannot signal.

Studies with chimeric receptors suggest that interaction between the LDLa module and TM regions is important for producing a receptor conformation necessary for signaling. The LRR region contains glycosylation sites that are important for receptor translocation and efficacy of signaling but not binding. The key residues whereby relaxin interacts with RXFP1 form the RxxxRxxI motif in the B-chain with the arginines interacting with acidic residues in LRR8 and LRR6 and the isoleucine interacting hydrophobically with LRR4 and LRR5 (Halls et al. 2015). The activation of RXFP2 by INSL3 utilizes different residues in the B-chain and also different interactions with the receptor TM region. Although relaxin can bind to RXFP2 as well as RXFP1, it does so in a different manner that also differs from the binding of INSL3 at RXFP2. For both RXFP1 and 2, the primary high-affinity binding site is located in the LRR, but there is also evidence for a lower-affinity binding site in the TM region (Valkovic et al. 2019). It has been suggested that this interaction directs the LDLa to bind to the TM region of a homodimer partner to drive receptor activation. As in many GPCRs, the ICL3 region of RXFP1 and 2 is important for interaction with the G proteins Gαs and GαoB. For RXFP1 but not RXFP2, the C-terminal tail is essential for cAMP generation mediated by an unusual mechanism involving Gαi3 (Halls et al. 2015). The C-terminal tail of RXFP1 also contains other consensus sequences for phosphorylation and protein interactions not shared by RXFP2 perhaps reflecting the more complex signaling and wider physiological role of the relaxin RXFP1 system. In contrast, activation of RXFP3 and 4 does not appear to involve the N-terminus of the receptors. Arginines in relaxin-3 interact with acidic residues in ECL1 and 2 of RXFP3 and allow the C-terminus of the peptide to insert into a classical GPCR-binding pocket and activate the receptor. Studies using RXFP3/4 chimeras suggest that for both receptors TM2, 3, and 5 and ECL2 are involved in binding and activation (Halls et al. 2015).

RXFP1 signaling is complex and involves cAMP, ERK1/2, tyrosine kinases, and NO (Fig. 1). RXFP1 couples to Gαs to increase cAMP levels, modulated by GαoB, but it also couples to Gαi3 to produce a delayed surge in cAMP via a Gβγ-PI3K-PKCζ pathway that activates AC5 (Bathgate et al. 2013; Halls et al. 2015; Valkovic et al. 2019). The latter mechanism is responsible for the pertussis toxin-sensitive positive inotropic and chronotropic effects of relaxin in the heart (Bathgate et al. 2013). cAMP accumulation in response to relaxin may also occur by G protein-independent mechanisms such as activation of tyrosine kinases, but these responses are very cell-type dependent. Many cardiovascular responses involve NO, and relaxin acting via RXFP1 has been shown to activate eNOS and nNOS and increase iNOS expression. Many cell types respond to relaxin by transient activation of ERK1/2, including human endometrial, coronary artery, and pulmonary arterial cells. A more sustained response is observed in HeLa and HUVECs. The antifibrotic actions of relaxin are associated with inhibition of TGF-β_1 signaling involving PI3K and NOS-NO-cGMP. Relaxin also activates a number of noncanonical signaling pathways such as the glucocorticoid receptor (GR). Relaxin coprecipitates with the GR and can compete for glucocorticoid binding (Halls et al. 2015). Many of the inhibitory actions of TNF-α on vasodilatation can be reversed by relaxin acting at GR. The actions of relaxin on gene transcription likely involve in part its actions at the GR. Another unusual aspect of RXFP1 signaling is the relaxin signalosome (Summers et al. 2018). While relaxin in the nM range activates RXFP1, the circulating peptide concentrations are usually lower. However, studies using highly sensitive FRET-based biosensors illustrate that a RXFP1-dependent cAMP response can be produced by attomolar concentrations of relaxin acting at signalosomes (Fig. 1). The signalosome comprises RXFP1 in a protein complex with AC2 and AKAP79, thus facilitating interaction with Gαs and Gβγ subunits and regulated by PDE4D3 that is scaffolded to the receptor C-terminus by β-arrestin. cAMP produced by the signalosome is also compartmentalized suggesting specialized physiological roles (Summers et al. 2018).

There is evidence that RXFP1 receptors form homo- and heterodimers and that these are responsible for signal transduction and some of the



Relaxin Family Peptides and Their Receptors, Fig. 1 Major signaling pathways for relaxin acting at RXFP1. Signalosomes are signaling complexes activated by sub-pM relaxin that respond to the amounts of relaxin in the circulation. Canonical signaling in many cell types involves interaction of RXFP1 with at least 3 G proteins and the generation of cAMP and cGMP. Signaling is compartmentalized with RXFP1-Gα_{i3} interactions occurring in lipid domains in the cell membrane. The agonist ML290 interacts at an allosteric site on RXFP1 to produce a biased signaling profile that preferentially activates cGMP over cAMP. There is also evidence that suggests that the anti-fibrotic effects of relaxin involve the formation of heterodimers between RXFP1 and the angiotensin AT2 receptor

therapeutically relevant actions of relaxin. Although it has been suggested that RXFP1 signaling involves binding to one homodimer partner and interaction of the LDLa module with the other partner receptor, there is no convincing evidence currently available to support this concept (Halls

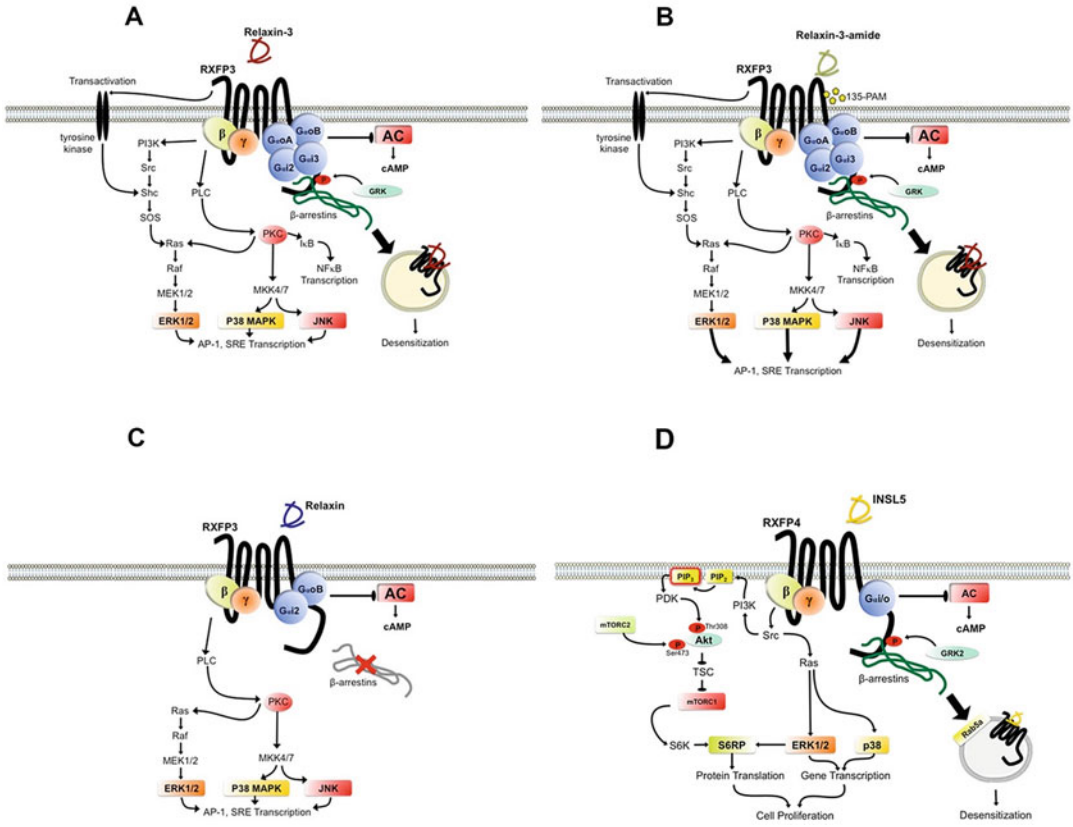
et al. 2015; Summers et al. 2018). Bell-shaped concentration-response curves are also a feature of many studies of relaxin-RXFP1 pharmacology and have been reported for in vitro and in vivo assays and in clinical trials. In vitro studies suggest that the bell-shaped curves are the result of sequential coupling of RXFP1 to Gαs and GαoB at different levels of receptor occupation (Sarwar et al. 2015). Bell-shaped curves become sigmoid after treatment of cells with a selective Gi/o inhibitor (Sarwar et al. 2015; Summers et al. 2018; Valkovic et al. 2019). Some actions of relaxin such as its antifibrotic actions are dependent on the formation of heterodimers between RXFP1 and other GPCRs (Fig. 1). Thus the antifibrotic actions of relaxin are absent in angiotensin AT_2 receptor knockout mice or in animals treated with the AT_2R antagonist PD123319. Likewise, recent studies have shown that the antifibrotic actions of relaxin are also blocked by the AT_1R antagonists irbesartan or candesartan (Halls et al. 2015; Summers et al. 2018; Chow et al. 2019). BRET studies demonstrate a constitutive interaction between RXFP1 and the AT_2 and AT_1R. Although AT_1 or AT_2R antagonists do not directly interact with relaxin binding to RXFP1, blockade of either receptor abolishes the antifibrotic effects of relaxin (Summers et al. 2018; Chow et al. 2019). These studies may provide an explanation for the observation that the antifibrotic actions of relaxin are only seen in pathological states where AT receptor expression is increased and why relaxin has failed in clinical trials for conditions where a proportion of patients were undergoing treatment with drugs acting on the renin-angiotensin system.

The small-molecule allosteric-biased agonist ML290 does not require the LDLa module to interact with RXFP1 and stimulate signal transduction. In addition, it does not utilize the binding sites used by relaxin and does not compete for relaxin binding (Kocan et al. 2017). The pattern of G protein activation by ML290 differs from that of relaxin, and while ML290 resembles relaxin in producing cAMP and cGMP accumulation and p38MAPK activation, although with lower potency, it differs from relaxin in that it does not acutely activate ERK1/2 in any system studied (Fig. 1) (Kocan et al. 2017). Like relaxin, ML290 promotes MMP2 expression and inhibits TGF-β_1-induced Smad2 and 3 phosphorylation suggesting that it has antifibrotic properties (Valkovic et al. 2019).

RXFP2 signaling pathways are a subset of those activated by RXFP1. INSL3 activation increases cAMP involving Gαs and GαoB, but there is no subsequent involvement of Gαi3 or a high-sensitivity signalosome response. In some cell types such as testicular germ cells or oocytes, INSL3 causes RXFP2-mediated inhibition of cAMP accumulation (Halls et al. 2015; Summers et al. 2018).

RXFP3 signaling follows a classical pattern of a Gi/o-coupled GPCR with inhibition of cAMP accumulation and activation of ERK1/2 and other MAP kinases (Fig. 2a). The particular Gi/o subtypes involved may vary with cell type, but the response pattern tends to be consistent. Ligand-directed signaling bias has been observed for relaxin-3, relaxin, and the RXFP3 antagonist R3(BΔ23-27)R/I5 (Fig. 2c). RXFP3 signaling was first observed for relaxin using microphysiometry but followed up by weak inhibition of cAMP generation by relaxin and INSL3 and AP-1 reporter gene activation by relaxin. The RXFP3 antagonist R3(BΔ23-27) R/I5 effectively blocked relaxin-3 but not relaxin-mediated AP-1 activation or relaxin-3 NFκB activation. BRET studies revealed a different pattern of G protein activation by relaxin-3 and relaxin. RXFP3 can also be allosterically modulated by 135PAM1, but the actions are limited by probe dependence (Fig. 2b) (Kocan et al. 2018).

RXFP4 like RXFP3 is a Gi/o-coupled receptor that when activated increases GTPγs binding and inhibits forskolin-stimulated cAMP accumulation but also increases phosphorylation of ERK1/2, p38MAPK, Akt-Ser473, Akt-Thr308, and S6 ribosomal protein (S6RP) (Ang et al. 2017) (Fig. 2d). Rescue studies with PTX-resistant G proteins suggest that $G\alpha_{oA}$, $G\alpha_{oB}$, and $G\alpha_{i2}$ and to a lesser extent $G\alpha_{i1}$ and $G\alpha_{i3}$ are involved. INSL5 exposure results in interaction with β-arrestins, internalization, and desensitization (Kocan et al. 2018).



Relaxin Family Peptides and Their Receptors, Fig. 2 Signaling pathways activated by RXFP3 and RXFP4 following activation. (**a**) RXFP3 canonical signaling following activation by relaxin-3; (**b**) allosteric modulation of relaxin-3 amide signaling by 135PAM1; (**c**) biased signaling by relaxin; (**d**) RXFP4 canonical signaling in response to INSL5. RXFP3 and RXFP4 inhibit cAMP production by coupling to Gi/o proteins and activate extracellular signal-regulated kinase (ERK)1/2 and phosphatidylinositol 3-kinase (PI3K). Both receptors undergo phosphorylation by GRK2 leading to recruitment of β-arrestins, desensitization, and internalization

RXFP1 is widely distributed in female reproductive tissues including the pubic symphysis, cervix, uterus, nipples, and mammary glands, and there is also evidence that relaxin has important roles in the cardiovascular adaptive changes associated with pregnancy including increases in plasma volume, cardiac output, and heart rate, together with decreased blood pressure and vascular resistance. In male and female brain, RXFP1 is localized to discrete regions of the olfactory system, neocortex, hypothalamus, hippocampus, thalamus, amygdala, midbrain, and medulla (Bathgate et al. 2013; Halls et al. 2015; Summers et al. 2018). Relaxin inhibits collagen biosynthesis and promotes collagen breakdown in many tissues as well as reproductive tissues, which has led to an interest in its use as an antifibrotic agent. However, the antifibrotic actions of relaxin are dependent on the presence not only of RXFP1 but also the angiotensin AT_2 receptor and likely also the AT_1 receptor, since the actions are blocked by either AT_1 or AT_2 receptor antagonists (Chow et al. 2019). RXFP2 has a much more restricted distribution being found in the uterus and in testis in Leydig cells, spermatocytes, spermatids, and the epididymal epithelium. Deficits in INSL3/RXFP2 signaling are correlated with reduced bone mass, and RXFP2 mutations may be linked with osteoporosis in men. RXFP3 is predominantly expressed in the brain with high RNA levels in olfactory bulb, paraventricular and supraoptic nuclei, and preoptic and posterior areas

of the hypothalamus, hippocampus, septum, and amygdala with lower levels in the cortex, periaqueductal gray, nucleus incertus, and areas of brainstem. There is low expression of RXFP3 in the adrenal gland, testis, salivary gland, and pancreas but little evidence of physiological function. Early studies of RXFP4 distribution showed protein expression in the heart, skeletal muscle, salivary gland, bladder, kidney, liver, placenta, stomach, jejunum, thyroid, ovary, and bone marrow with the highest expression in the pancreas. Subsequent RNA expression studies show expression in human colon but also in the placenta, testis, thymus, prostate, kidney, and brain with expression in the colon closely matching expression of INSL5 (Bathgate et al. 2013; Halls et al. 2015; Kocan et al. 2018; Summers et al. 2018).

The role of the relaxin/RXFP1 system in reproduction varies enormously across species. In humans, relaxin plasma levels are greatest in the first semester of pregnancy suggesting a role in implantation, but it may also be involved in the cardiovascular adaptations later in pregnancy and in the growth and development of mammary glands. Relaxin is found in seminal plasma and may have a role in preparing the uterine endometrium for implantation. In the brain, RXFP1 is found in the SFO and OVLT where it acts to reduce plasma osmolality. Relaxin increases cardiac output and heart rate and decreases vascular resistance – all adaptive changes to pregnancy but also present in nonpregnant animals. These effects contribute to increases in renal plasma flow and GFR that are clear in animals but require confirmation in humans (Bathgate et al. 2013; Halls et al. 2015). The vasodilator actions of relaxin are complex and involve NOS activation, cAMP generation, VEGF, placental growth factor, MMPs, ET_B receptors, and antifibrotic effects in blood vessel walls (Sarwar et al. 2017). NOS activation and cAMP generation are acute responses, whereas the other mechanisms are longer term. Relaxin acting at RXFP1 is a potent inotropic and chronotropic agent in atria, and the effects are preserved in failing hearts. The peptide has cardioprotective actions in rat models of ischemia and reperfusion and has anti-arrhythmic properties. In chronic heart failure, circulating levels of relaxin increase and are correlated with the degree of failure. This led to clinical trials to treat acute heart failure that although successful up to Phase III in limited trials eventually failed in a large-scale extended Phase III trial. It is possible that concurrent treatment with drugs acting on the renin-angiotensin system may have affected the outcome of these trials (see above). Attempts have also been made to harness the antifibrotic actions of relaxin that have been demonstrated in numerous animal models. Relaxin failed Phase III clinical trials for the treatment of scleroderma possibly due to an incomplete understanding of the disease (Bathgate et al. 2013; Halls et al. 2015). Dermal fibrosis in the relaxin knockout mouse can be reversed by relaxin treatment in the early but not the late stages of the condition. The expression of relaxin is increased in a variety of tumors, and high levels are associated with a poor prognosis and increased tumor growth, metastasis, and angiogenesis. The INSL3 RXFP2 system has specialized roles in reproductive function such as the development of the gubernaculum and in Leydig cell function and in females in the maturation of ovarian follicles. There is also evidence that it may be involved in the CNS, in tumor metastasis, in bone function, and in the kidney (Bathgate et al. 2013; Halls et al. 2015).

The relaxin-3 RXFP3 system is almost exclusively confined to the brain, and although relaxin-3 has been shown to be active at RXFP1 and RXFP4, the localization patterns of peptide and receptor suggest that this has no physiological importance. The colocalization of relaxin-3 and RXFP3 in the hypothalamus, stria terminalis, lateral septum, periaqueductal gray, and dorsal raphe suggests roles in stress and in feeding and metabolism. There is also evidence for roles in behavioral activation and arousal and that modulation of the system could be useful in the treatment of addiction. The evidence that the INSL4 RXFP4 system has important metabolic roles is becoming more compelling. INSL5 is synthesized and released from enteroendocrine L cells and circulates to act on RXFP4 receptors located in the hypothalamus, pituitary, testis, epididymis, ovary, uterus, pancreas, and liver. INSL5

knockout mice have impaired glucose homeostasis, and humans with RXFP4 polymorphisms have a high BMI and a tendency to obesity. Although INSL5 plasma levels are associated with feeding, the precise mechanisms involved have yet to be identified.

Drugs

Although the cognate relaxin family peptide ligands for the relaxin receptors RXFP1–4 have been defined, some interactions have been identified in in vitro systems. Human relaxin activates RXFP2 in addition to RXFP1 and acts as a biased agonist at RXFP3. Relaxin-3 is active at RXFP1 and RXFP4 as well as at its cognate receptor RXFP3. INSL5 is a weak antagonist at RXFP3 and is the cognate agonist for RXFP4.

Relaxin peptides show considerable variation across species, but all have the cysteines necessary for formation of disulfide bonds between the A- and B-chains and the conserved RxxxRxxI/V motif in the B-chain. Replacement of arginine, isoleucine, or valine in the conserved region leads to loss of activity. In the A-chain, G14 is necessary for chain flexibility and structure. Variants of relaxin such as H2:A(4-24)(F23A) retain potency at RXFP1 but have no activity at RXFP2. Others such as H2:(B7-24) or H2:(A4-24)(B7-24) have reduced binding and potency for cAMP generation but retain antifibrotic activity (Halls et al. 2015; Valkovic et al. 2019). C1q-tumor necrosis factor-related protein 8 and similar short linear peptides appear to activate many of the RXFP1 signaling pathways associated with the antifibrotic effect. Although studies indicate an interaction between CTRP8 and RXFP1, it is not clear if this reflects a physiological or pathological process (Halls et al. 2015; Summers et al. 2018; Valkovic et al. 2019). An HTS strategy designed to identify small molecular weight compounds active at RXFP1 identified ML290 that acts as an allosteric-biased agonist at RXFP1. Unlike relaxin, ML290 activates RXFP1 by interacting with TM7 and ECL3 (Kocan et al. 2017). ML290 activates human, macaque, and pig but not mouse RXFP1 leading to the development of a "humanized" mouse to enable studies in cellular and animal disease models (Summers et al. 2018). In contrast to relaxin, INSL3 is quite selective for RXFP2. Truncation of the A-chain of relaxin peptides improves selectivity for RXFP1, whereas similar modifications to INSL3 abolish activation of RXFP2 but not binding emphasizing the different modes of interaction. Unlike RXFP1, it has proved relatively easy to produce antagonists of RXFP2. Deletion of residues from the N-terminus of the A- or the B-chain or disruption of an intrachain disulfide bond of INSL3 produces competitive peptide antagonists of RXFP2. B-chain variants can be produced that are antagonists with relatively high affinity (Halls et al. 2015; Valkovic et al. 2019).

Relaxin-3 although similar in structure to other relaxin peptides interacts with RXFP3 and 4 in a different manner than it interacts with RXFP1. Alterations to the A-chain have little effect on RXFP3 binding but reduce activity at RXFP1. In the B-chain, R8, R16, I5, and F20 are required for relaxin-3 binding to RXFP3 and 4 with R12 also required for RXFP3 (Halls et al. 2015; Valkovic et al. 2019). Antagonists have been developed by modification of the B-chain combined with the INSL5 A-chain (R3(BΔ23-27)R/I5) or (H3: B1-22R). Relaxin activates RXFP3 with a signaling profile different from that of relaxin-3. A positive allosteric modulator 135PAM1 has been described that displays probe selectivity with C-terminal amidated relaxin-3 or R3/I5 (Halls et al. 2015; Kocan et al. 2018). INSL5 differs from relaxin-3 that activates both RXFP3 and 4, in that it activates RXFP4 but acts as a weak antagonist at RXFP3. INSL5 A- and B-chains are also inactive at RXFP4. F20 is necessary for affinity and the B-chain C-terminus for activity without affecting affinity. Synthesis of human INSL5 is challenging leading to the development of simpler analogues that retain the C-terminus of the B-chain and interchain disulfide bonds necessary for activity. Some antagonists have been synthesized including minimized relaxin-3 analogue 3 and R3(BΔ23-27)R/I5 (Kocan et al. 2018). Small-molecule agonists active at RXFP3 and 4 are now starting to emerge that have many of the properties of the endogenous peptides (DeChristopher et al. 2019).

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Relaxin-3

▶ Relaxin Family Peptides and Their Receptors

Renin–Angiotensin System (RAS)

▶ Renin–Angiotensin–Aldosterone System

Renin–Angiotensin–Aldosterone System

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Synonyms

RAAS; Renin–angiotensin system (RAS)

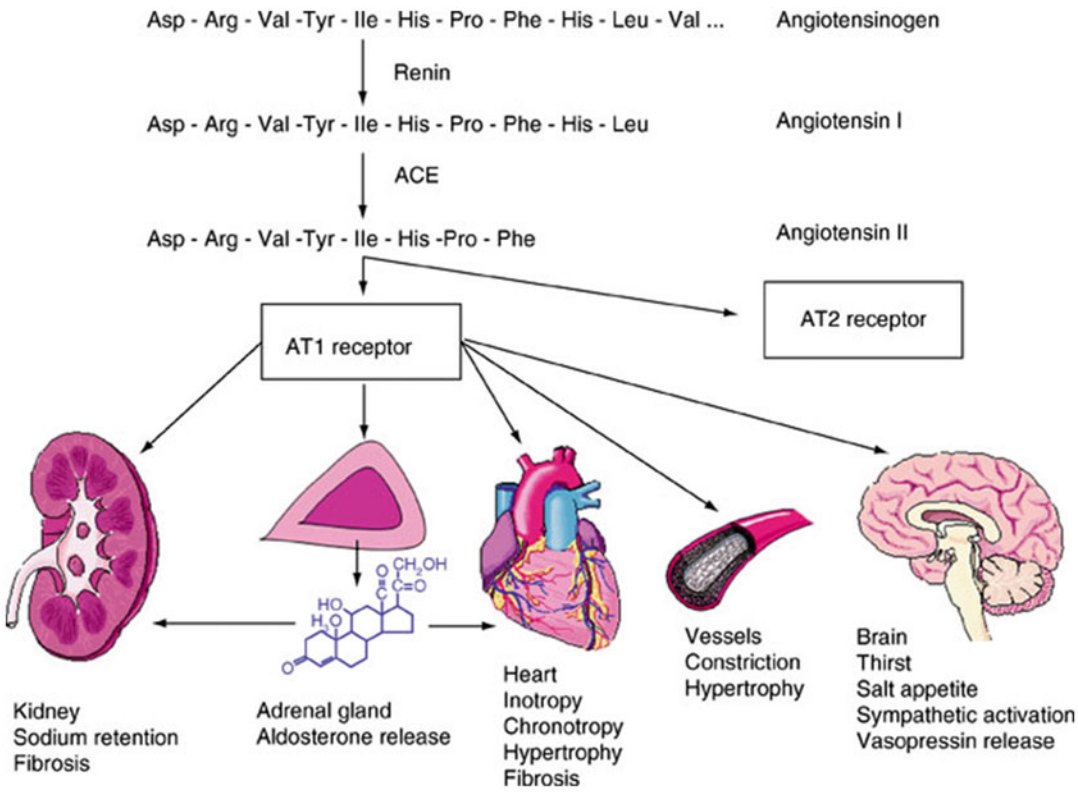
Definition

The renin–angiotensin–aldosterone system (RAAS) generates the peptide hormone angiotensin II and subsequently the mineralocorticoid aldosterone, which both exert considerable impact on blood pressure (blood pressure control) and fluid homeostasis and have prime etiologic and therapeutic significance for cardiovascular diseases.

Basic Characteristics

General

Renin is a circulating enzyme formed in specialized smooth muscle cells in the kidney that cleaves its only, liver-born substrate, angiotensinogen, in the plasma to form the inactive decapeptide angiotensin I (Bader 2010). Angiotensin I is then metabolized further into the octapeptide angiotensin II via the endothelium-bound angiotensin converting enzyme (ACE). Angiotensin II elicits an increase in blood volume and blood pressure by stimulating vasoconstriction, sodium retention, thirst, the sympathetic nervous system, and aldosterone secretion from the adrenal gland (Fig. 1). Aldosterone is a steroid hormone that binds to the mineralocorticoid receptor and amplifies the sodium-retaining effect. Physiologically this makes sense, since the RAAS is activated under conditions of acute volume loss by the induction of the rate-limiting enzyme renin. Major renin-inducing stimuli include a fall in renal perfusion pressure, a decrease in salt content in the distal tubule sensed and transmitted via the macula



Renin–Angiotensin–Aldosterone System, Fig. 1 Renin–Angiotensin–Aldosterone System

densa, an increase in the renal sympathetic tone, and a reduction in angiotensin II concentration employing a negative feedback mechanism. In addition to their hemodynamic actions, angiotensin II and aldosterone also induce growth- and fibrosis-related processes in several organs, such as vessels, heart, and kidney.

The renin–angiotensin–aldosterone system. Angiotensin II is generated in a two-step enzymatic process from the liver-borne protein angiotensinogen by the kidney-derived enzyme renin and the endothelium-bound angiotensin converting enzyme (ACE). The octapeptide interacts with two receptors, AT1 and AT2. The AT1 receptor confers most of the known actions of angiotensin II such as the liberation of aldosterone from the adrenal gland. Aldosterone via the mineralocorticoid receptor (MR) and angiotensin II together induce sodium retention in the kidney and fibrotic processes in kidney and heart. Moreover, angiotensin II elicits constriction of vessels, has positive inotropic and chronotropic actions on the heart, promotes growth in vessels and heart, and induces thirst, salt appetite, vasopressin release, and the activation of the sympathetic nervous system in the brain. Some of these effects are also mediated by locally produced angiotensin II in the respective tissues.

To elicit these effects, angiotensin II binds to two main receptors, AT1 and AT2, which both belong to the ▶ G-protein-coupled receptor family. Most of the abovementioned effects of angiotensin II are, however, mediated by the AT1 receptor, while the physiological function of the AT2 receptor is enigmatic. In most studied cases, the AT2 receptor counteracts the AT1 effects by exerting growth-inhibiting and vasodilatating actions partly by the stimulation of kinin generation. Therefore, AT2 agonists are developed for the therapy of multiple diseases.

Angiotensin II binding to the AT1 receptor stimulates via the G-protein, G_q, the activity of phospholipase C to generate the second messengers inositol phosphate (IP3) and diacylglycerol, and inhibits via G_i the activity of adenylyl cyclase to reduce the synthesis of cyclic AMP. Diacylglycerol activates protein kinase C and can be converted to arachidonic acid and Eicosanoids. Furthermore, angiotensin II induces the generation of reactive oxygen species by the stimulation of membrane-bound NAD(P)H oxidase. One of the immediate consequences of these early signals is activation of tyrosine kinases that include PYK2, c-Src, JAK2, platelet-derived growth factor (PDGF) receptor, and the epidermal growth factor (EGF) receptor, as well as of the serine/threonine kinases, ERK, Akt/protein kinase B and S6 kinase, and subsequent induction of immediate early genes and protein synthesis.

Besides the plasma renin–angiotensin system (RAS), intrinsic tissue RAS exist (Bader 2010). Angiotensin II is generated not only in the circulation but also locally in organs from precursors and enzymes either locally synthesized or imported from the plasma. These systems are autonomously regulated and have physiological functions inside the respective organs. Local RAS have been described for organs involved in cardiovascular control such as kidney, vessels, heart, adrenal gland, and brain.

Adrenal Gland

In the zona glomerulosa of the adrenal gland, renin is locally synthesized, and together with angiotensinogen and ACE generates angiotensin II, which acts as paracrine or intracrine hormone on adrenocortical cells. Circulating as well as this locally produced angiotensin II stimulates aldosterone release by increasing the expression of aldosterone synthase, the rate-limiting enzyme of aldosterone synthesis.

Kidney

The kidney contains the major site of renin synthesis, the juxtaglomerular cells in the wall of the afferent arteriole. From these cells, renin is secreted not only into the circulation but also into the renal interstitium. Proximal tubular cells synthesize angiotensinogen and ACE. The RAS proteins interact in the renal interstitium and in the proximal tubular lumen to synthesize angiotensin II. In the proximal tubule, angiotensin II activates the sodium/hydrogen exchanger (NHE) that increases sodium reabsorption. Aldosterone elicits the same effect in the distal tubule by activating epithelial sodium channels (ENaC) and the sodium-potassium-ATPase. Thereby, it also induces water reabsorption and potassium secretion.

In the interstitium, angiotensin II induces proliferation of mesangial cells and fibroblasts and the synthesis of collagen and other matrix molecules by these cells via the AT1 receptor. Moreover, by the concomitant stimulation of chemoattractant cytokines, inflammation is induced. These processes are mediated by endothelin, transforming growth factor β, and reactive oxygen species and finally lead to interstitial fibrosis and glomerulosclerosis observed in hypertension and diabetes.

Heart and Vessels

In heart and vessels, angiotensin II is generated mostly by renin and angiotensinogen imported from the plasma and locally synthesized ACE. Additionally in the human heart, mast cells contain the enzyme chymase that also metabolizes angiotensin I to angiotensin II. The physiological relevance of this enzyme is controversial. Circulating as well as locally generated angiotensin II induces vasoconstriction and exerts direct inotropic and chronotropic actions on the heart. These effects are enhanced by a facilitation of noradrenaline release from sympathetic nerve endings.

Alike in the kidney, angiotensin II also in the heart induces inflammation and fibrosis by increasing endothelin, transforming growth factor β, reactive oxygen species, and proinflammatory cytokines. Furthermore, angiotensin II induces hypertrophy of cardiomyocytes and smooth muscle cells in the heart and vessels, respectively, partially employing the same mediators. This is aggravated by increased circulating aldosterone levels that also elicit fibrotic processes in the heart by a yet unresolved mechanism.

Brain

Circulating angiotensin II can only reach the circumventricular organs of the brain, which express AT1 receptors and lack a blood-brain barrier normally limiting the access of peptides to brain receptor sites. However, areas beyond the blood-brain barrier have been shown to be responsive to angiotensin II and to express AT1 receptors. These sites are affected by locally synthesized RAS components. Possibly, other enzymes than renin may also be involved in angiotensin II generation in the brain, for example, cathepsins. Circulating and locally synthesized angiotensin II induces thirst, salt appetite, and vasopressin release, stimulates the sympathetic nervous system and moderates the baroreceptor reflex, and thereby increases blood volume and blood pressure.

Alternative RAS

Angiotensins can be further metabolized by ACE2 or neutral endopeptidase 24.11 (neprilysin, NEP) to angiotensin-(1–7) lacking the C-terminal Phe residue (Santos et al. 2018). This peptide interacts with its receptor Mas and has been shown to exert protective effects in cardiovascular tissues mostly opposing the actions of angiotensin II. Strategies to pharmacologically activate this system are pursued and may lead to novel drugs in the future.

Drugs

ACE Inhibitors

Pharmacological intervention in RAS began in the late 1960s with the discovery that the venome of the Brazilian snake *Bothrops jararaca* contains a substance that inhibits ACE. In first clinical trials, this substance proved to be a potent antihypertensive agent but it had the disadvantage that it could only be taken by injection. By modeling the active site of ACE and designing drugs potentially binding to this site, the first orally available ACE inhibitor, captopril, was discovered. In the meantime, at least a dozen compounds have been developed and marketed: captopril, enalapril, lisinopril, perindopril, cilazapril, benazepril, quinapril, fosinopril, ramipril, moexipril, and trandolapril. ACE inhibitors are first choice antihypertensive drugs. Furthermore, a multitude of large-scale clinical studies have proven a strong beneficial effect of these drugs on morbidity and mortality in congestive heart failure, for example, after myocardial infarction, and chronic renal diseases, for example, caused by diabetes or hypertension. In the heart, ACE inhibitors exert their beneficial actions by reducing preload and afterload and inhibiting myocardial fibrosis and remodeling processes. In the kidney, a reduction in glomerular pressure as well as antifibrotic and anti-inflammatory actions contribute to the efficiency of the drugs.

ACE not only activates angiotensin but is also involved in the metabolism of other peptides, for example, it is a major kinin-degrading enzyme. Therefore, ACE inhibitors also increase kinin concentrations. Furthermore, it has recently been shown that these drugs potentiate kinin effects by modulating a direct interaction between the ACE protein and the kinin B2 receptor, which is independent from the enzymatic activity of ACE. Kinin potentiation may be involved in the beneficial action of ACE inhibition since kinins are known to exert cardio- and renoprotective actions. However, it is also the major reason for the adverse side effects of ACE inhibitors, namely cough and angioedema. Another observed side effect, first-dose orthostatic hypotension, is probably due to both angiotensin inhibition and kinin potentiation.

Since angiotensin II is important for kidney development in mammals, ACE inhibitors and other drugs interfering with RAS should not be given during pregnancy.

AT1 Antagonists

A second group of drugs interfering with RAS are specific antagonists for the AT1 receptor. The first example of this class was losartan which was followed by at least seven other sartans (telmisartan, candesartan, valsartan, eprosartan, irbesartan, olmesartan, azilsartan). These drugs exert a more complete angiotensin blockade, since alternative pathways of angiotensin generation not affected by ACE inhibitors and employing cathepsins or chymase become ineffective by AT1 antagonism. They are also more

specific for the RAS than ACE inhibitors, since other peptide systems should not be affected. However, the compensatory increase in renin concentration after AT1 blockade leads to an accumulation of angiotensin II, which activates the AT2 receptor. It is yet unknown whether this AT2 stimulation often followed by kinin generation is involved in the action of AT1 antagonists.

In clinical trials, AT1 antagonists have proven to be as effective as ACE inhibitors in hypertension, congestive heart failure, and renal failure, with a more favorable side effect profile.

Renin Inhibitors

The most logical point to interfere pharmacologically with the RAS is the rate-limiting enzyme renin. Intervention at this step is more specific than ACE inhibition and AT1 antagonism, since hardly any angiotensin peptide can be generated and no other peptide system is directly affected. Therefore, fewer compensatory and unwanted effects are expected, and thus, renin inhibitors should be safe drugs. However, the development of such drugs faced several problems: First, since the human renin protein is different from the rodent enzymes and only interacts with primate or human angiotensinogen, the testing of such drugs cannot be performed in classical animal models. Second, the first renin inhibitors were difficult to synthesize and exhibited a low bioavailability. However, with the availability of a transgenic rat model expressing the human renin and angiotensinogen genes as test system novel substances could be developed. A first renin inhibitor, aliskiren, has been approved for clinical use after clinical trials which have proven its safety and efficacy (Musini et al. 2017).

Vasopeptidase Inhibitors

(Vasopeptidase inhibitors) Two new classes of drugs have been developed that inhibit NEP and one RAS component, either ACE or AT1. Besides generating angiotensin-(1–7) (see above), NEP also degrades vasodilatory peptides such as kinins, natriuretic peptides, and adrenomedullin, and therefore, its inhibition should complement the vasodilatory action of ACE or AT1 inhibition. Dual inhibitors for ACE and NEP, such as omapatrilat, sampatrilat, and gemopatrilat, were more effective than ACE inhibitors in blood pressure reduction in clinical trials, but also caused more side effects and therefore these drugs have been withdrawn. The most important side effects were partially life-threatening angioedema probably elicited by an overproduction of kinins which are degraded by ACE and NEP. Recently a combination drug, LCZ696, containing the NEP inhibitor sacubitril and the AT1 antagonist valsartan was approved for the treatment of heart failure after a successful clinical trial (McMurray et al. 2014). The side effect profile of this drug seems to be acceptable.

Aldosterone Antagonists

ACE inhibitors do not completely block aldosterone synthesis. Since this steroid hormone is a potent inducer of fibrosis in the heart, specific antagonists, such as spironolactone and eplerenone, have recently been very successfully used in clinical trials in addition to ACE inhibitors to treat congestive heart failure (Kapelios et al. 2019). Formerly, these drugs have only been applied as potassium-saving diuretics in edematous diseases, hypertension, and hypokalemia as well as in primary hyperaldosteronism. Possible side effects of aldosterone antagonists include hyperkalemia and, in case of spironolactone, which is less specific for the mineralocorticoid receptor than eplerenone, also antiandrogenic and progestational actions.

Cross-References

- ▶ ACE Inhibitors
- ▶ Blood Pressure Control

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Reslizumab

► Interleukin-5

Retinoic Acid

► Retinoids

Retinoids

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Synonyms

Retinoic acid; Vitamin A metabolites

Definition

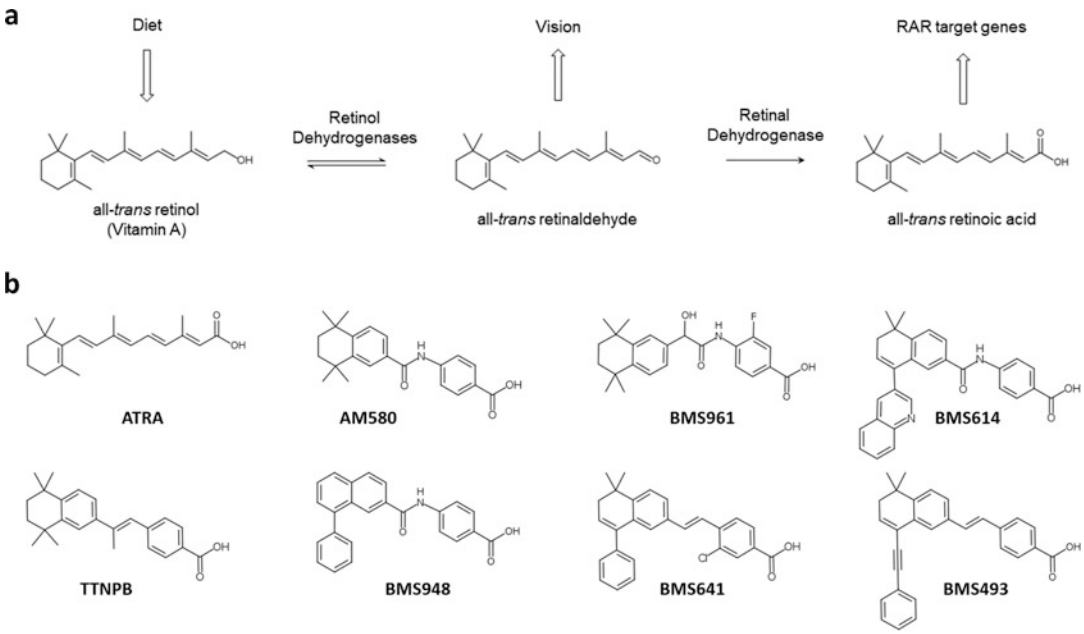
Retinoid is a generic term covering compounds that include both vitamin A (all-*trans* retinol) and its naturally occurring metabolites and synthetic analogs. These molecules are critical agents in a wide variety of essential biological processes, such as embryogenesis and organogenesis, apoptosis, reproduction, vision, and regulation of growth and differentiation of normal and neoplastic cells in vertebrates. The ability of these molecules to regulate expression of several hundred genes through binding to nuclear transcription factors (all-*trans* retinoic acid receptors (RARs)) is considered to mediate most of these functions (Chambon 1996; Mark et al. 2006).

Humans are unable to synthesize vitamin A *de novo* and must get it from the diet. Major sources of natural retinoids (retinol, retinyl ester, or beta-carotene) are animal fats, fish liver oil, and vegetables. All-*trans* retinoic acid (ATRA) is considered to be the main and the most potent biologically active metabolite of vitamin A (Blomhoff and Blomhoff 2006). The large group of synthetic retinoids covers a panel of modulators with activities ranging from agonists to inverse agonists. All these compounds are fat-soluble unsaturated isoprenoids, hydrophobic, and of small size, so that they can easily cross the lipid bi-layer of cell membranes. The basic structure of a retinoid is composed of three parts: a trimethylated cyclohexene ring that is a bulky hydrophobic group, a conjugated tetraene side chain that serves as a linker unit, and a polar carbon–oxygen functional group (Fig. 1). For instance, ATRA is composed of 20 carbon atoms arranged as a beta-ionone ring with a conjugated isoprene tail that terminates with a carboxylate functional group.

Basic Mechanisms

Transport, Biosynthesis, Metabolism

Vitamin A serves as a parent compound for active derivatives that impart two very different physiologic effects (Fig. 1a). All-*trans* retinaldehyde, an aldehyde derivative, is the active chromophore of



Retinoids, Fig. 1 Metabolic flow from vitamin A (all-*trans* retinol) to all-*trans* retinoic acid (ATRA). (**a**) In the cytosol, vitamin A is reversibly oxidized to all-*trans* retinaldehyde by members of the retinol dehydrogenase family. All-*trans* retinaldehyde, the active chromophore of vision, is irreversibly oxidized to the transcriptionally active ATRA by the retinal dehydrogenase (RALDH). (**b**) Chemical structures of a number of synthetic retinoids ranging from pan and subtype-selective RAR agonists (ATRA, TTNPB, AM580 (RARα), BMS948 (RARβ), BMS641 (RARβ), BMS961 (RARγ)) to the selective RARα antagonist (BMS614) and the pan RAR inverse agonist (BMS493)

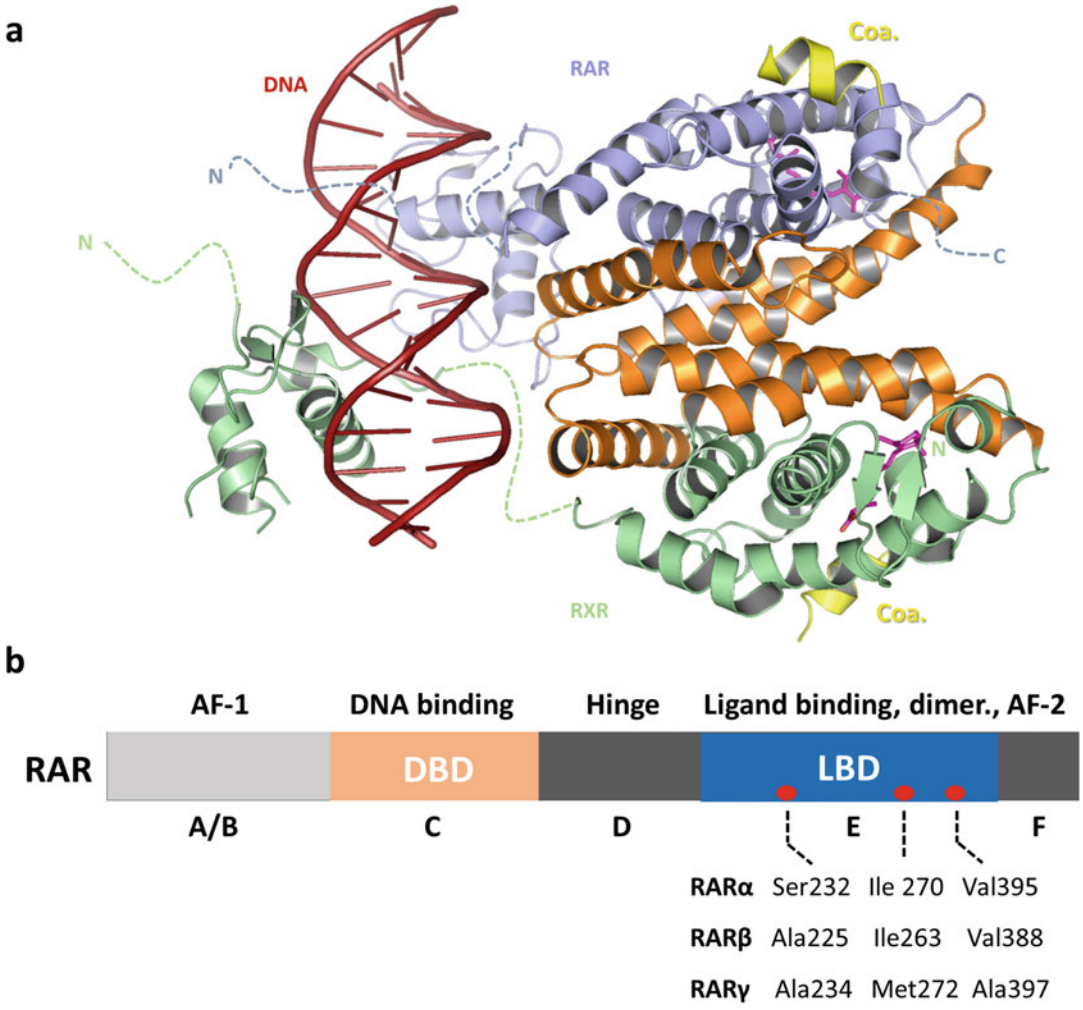
vision while ATRA plays a key role in genomic regulation and has the ability to reverse developmental defects in vitamin A-deficient (VAD) animals. In the body, retinoids need to be solubilized, protected, and detoxified (Blomhoff and Blomhoff 2006). To this end, various specific and aspecific binding proteins carry retinoids in both extracellular and intracellular environments. In the extracellular compartments, retinol-binding protein (RBP) is the most relevant retinoid binding protein and binds preferentially vitamin A. Other extracellular binding proteins show different binding preferences. Inside the cells, the two isoforms of the cellular retinoid acid-binding protein (CRBP1 and CRBP2) bind only ATRA, both CRBP3 and CRBP4 preferentially bind vitamin A, while the cellular retinal-binding protein (CRALBP) displays a marked preference for all-*trans* retinal. These CRBPs are not absolutely essential under circumstances of high dietary vitamin A supply. However, their importance becomes more evident when the dietary retinoid uptake is low and insufficient.

Active retinoids are produced *in vivo* from the oxidation of vitamin A (Fig. 1a). Synthesis of ATRA is a two-step enzymatic process in which the cytosolic alcohol dehydrogenase performs the oxidation of all-*trans* retinol to all-*trans* retinaldehyde, followed by the irreversible oxidation of the latter into ATRA by the retinaldehyde dehydrogenase (Blomhoff and Blomhoff 2006). This subsequent oxidation of all-*trans* retinaldehyde is the rate-limiting step in the ATRA production. On the other hand, to keep the level of ATRA within a narrow range, its concentration is maintained by its degradation into hydroxylated metabolites carried out by the cytochrome P450 CYP26 enzyme.

Nuclear and Nongenomic Signaling Pathways of RARs

Retinoids exert their pleiotropic effects through the three RAR paralogs RARα (NR1B1), RARβ

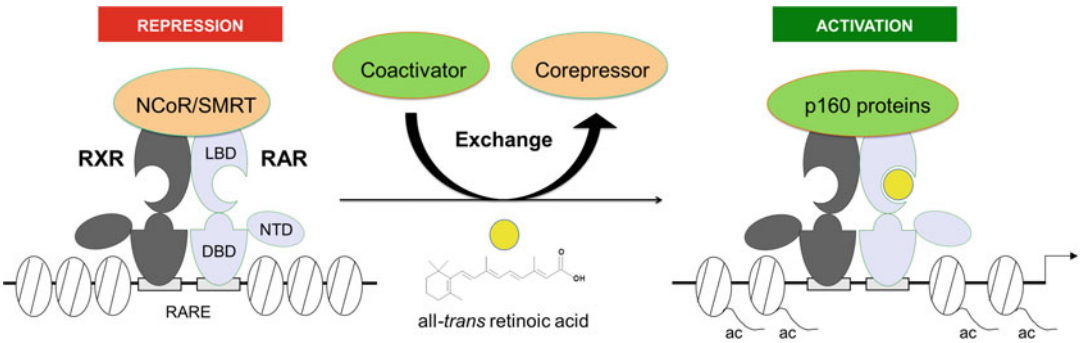
(NR1B2), and RARγ (NR1B3) that originate from three distinct genes and belong to the nuclear hormone receptor superfamily (Germain et al. 2006a, b; Khorasanizadeh and Rastinejad 2016). For each RAR subtype, several isoforms exist. RARs are modular proteins composed of six regions of homology (designated from A to F, from the N- to the C-terminal end) harboring specific functions (Fig. 2) (Chandra et al. 2017; Germain et al. 2006a, b). Regions C and E, which encompass the DNA binding domain (DBD) and the ligand binding domain (LBD), respectively,



Retinoids, Fig. 2 Structural and functional organization of RARs. (**a**) Crystal structure of RARβ/RXRα heterodimer bound to DNA (Chandra et al. 2017). LBDs (dark and light blue) and DBDs (dark and light green) are represented as ribbons. Helix H12 (AF-2) of each monomer is highlighted in purple. The coactivator peptides (Coa.) interacting with the heterodimer are drawn in yellow and the agonist ligand (9-*cis*-RA) in each subunit are drawn as pink sticks. Dotted lines denote regions with unresolved structures. (**b**) Functional organization of RARs. RARs consist of six functional domains (A–F) based on regions of conserved sequence and function. The DBD (C) mediates sequence-specific DNA recognition. The LBD (E) mediates ligand binding, dimerization, and a ligand-dependent transactivation function (AF-2). The N-terminal A/B region contains a ligand-independent activation function (AF-1). The region D corresponds to a linker allowing the proper orientation of DBD and LBD within DNA-bound dimers. The function of the F region is poorly understood. Residues of the ligand binding pocket specific of each RAR subtype are indicated

are the most conserved and important domains that govern the classical model of RAR transcriptional activity. In contrast, the A/B, D, and F regions are poorly conserved. The D region is considered to serve as a hinge between the DBD and the LBD, allowing different positions of the two domains. It might allow the DBD and the LBD to adopt different conformations without creating steric hindrance problems. D region also contains nuclear localization signals. The A and B regions correspond to the N-terminal domain (NTD) and includes the activation function AF-1 which plays a role in the control of transcription of retinoid target genes. It is interesting to note that within the NTD, the A region differs between the different subtypes and between isoforms. In contrast, the B region is rather conserved and depicts a proline-rich motif, which contains phosphorylation sites. Finally, not much information has been provided on the function of the C-terminal F domain.

In vitro studies demonstrated that RARs function essentially as heterodimers with the retinoid X receptors (RXRs) which are the functional entities and act as ligand-dependent transcriptional regulators by binding to the specific retinoic acid response element (RARE) DNA sequences found into the promoter region of retinoid target genes (Piskunov et al. 2014). RXR-RAR heterodimers control transcription *via* several distinct mechanisms, including both repression and activation (Fig. 3). In the absence of RAR agonist, the RXR-RAR heterodimer recruits the corepressor proteins NCOR1 (former N-CoR) or NCOR2 (former SMRT). These two major corepressors have been shown to mediate the active repression associated with unliganded (apo-) RARs and with many other transcription factors. Note in this respect that the three RAR subtypes display graded repression function, with RARα having the highest affinity for corepressors and RARβ exhibiting the weakest. Corepressors do not appear to possess intrinsic repressive properties but are rather large multidomain proteins that can be considered as docking platforms to tether the repression machinery to transcription factors. Indeed, NCOR1 and NCOR2, which share similar domain organizations, interact directly with DNA-bound unliganded RXR-RAR heterodimers through their nuclear receptor interaction domain (NRID) and allow the recruitment of various silencing factors such as histone deacetylases (HDACs) or DNA-methyl transferases (DNMTs) that may lead to an inactive condensed chromatin



Retinoids, Fig. 3 ATRA facilitates the exchange between corepressor and coactivator complexes that are central to the control of target gene transcription. RXR-RAR heterodimers bind to the specific retinoic acid response element (RARE) DNA sequences found in the promoter region of retinoid target genes. In the absence of ATRA, the RXR-RAR heterodimer recruits the corepressor proteins NCoR or SMRT and associated enzymatic factors that may lead to an inactive condensed chromatin structure, preventing transcription. Upon ATRA binding to the RAR ligand binding domain (LBD), corepressors are released, and coactivators (p160 proteins) are recruited. The subsequent recruitment of epigenetically active and/or chromatin-modifying complexes such as histone acetyltransferases or histone arginine methyltransferases leads to chromatin alterations that facilitate activation of target gene expression by the basal transcriptional machinery. *NTD* N-terminal domain, *DBD* DNA binding domain, *ac* acetyl

structure over the target promoter preventing transcription. Identification of HDAC3 as the catalytic component of corepressor complexes has provided a mechanistic link between transcriptional repression by RARs and histone deacetylation. Upon ATRA binding, RAR LBDs undergo a conformational change inducing the release of corepressor complexes and allowing the recruitment of a series of coactivator complexes. The p160 coactivator family includes SRC-1 (also known as NCoA1), TIF-2 (SRC-2 and GRIP1), and RAC3 (SCR-3, ACTR, TRAM-1, pCIP) that serve as adaptors recruiting other complexes with different enzymatic activities such as the histone acetyltransferases (HATs), CBP/p300 (CREB binding protein) and p/CAF (p300/CBP-associated factor), the histone methyl transferases (HMTs) CARM1 (coactivator-associated arginine methyltransferase 1) and PRMT1 (protein arginine methyl transferase 1), ubiquitinases/deubiquitinases or nucleosome remodeling complexes such as SWI/SNF (switch/sucrose nonfermenting). All these complexes alter the chromatin structure surrounding the promoter of target genes to allow their activation.

Beside their pivotal role in the genomic pathways of RARs, retinoids have also been shown to interact with a membrane-associated fraction of these receptors (Piskunov et al. 2014). Indeed, it is now well accepted that a few percent of RARs are associated with membrane lipid rafts in a number of cell types. Retinoid binding to membrane RARs rapidly (within minutes) induces cascades of transient interaction and activation of several types of kinases, thereby spreading signals to downstream cytosolic or nuclear machineries. These ATRA-activated kinases are able to phosphorylate several nuclear proteins involved in the transcription of retinoid target genes such as histones, RAR coregulators, and RARs themselves, thus indicating crosstalk between the genomic and nongenomic RAR signaling. Accordingly, RAR LBDs contain phosphorylation sites that serve as substrate for kinases including the cyclic AMP-dependent protein kinase (PKA) and stress-activated protein kinase (MSK1), while the NTD contains a proline-rich motif used by cyclin-dependent kinases (CDKs) and mitogen-activated protein kinases (MAPKs).

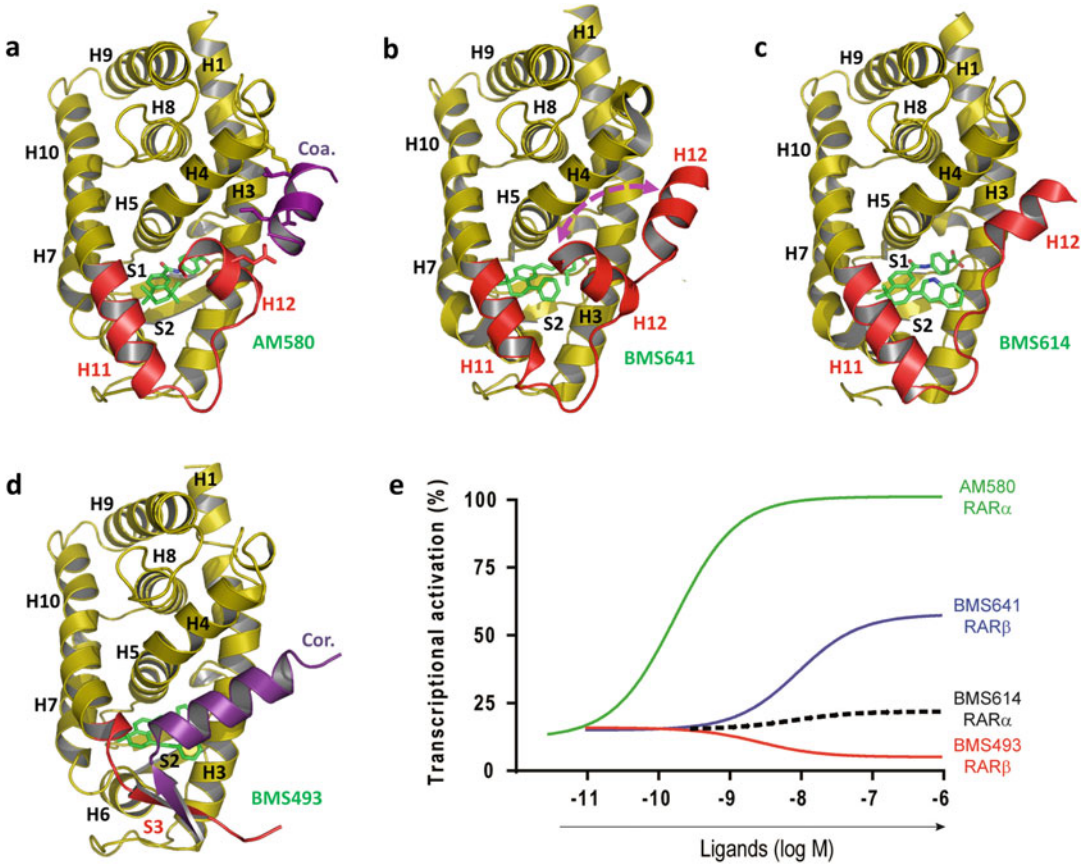
Various Classes of RAR Modulators

Whereas natural retinoids such as ATRA act as RARs activators and are therefore classified as "agonists," a plethora of synthetic ligands with a wide range of activities have been developed for potential therapeutic use (Fig. 1b) (Altucci et al. 2007). These derivatives have been shown to modulate the transcriptional activity of RARs in a positive or negative fashion and in a cell-specific manner. The spectrum of RAR modulators ranges from full agonists to pure antagonists through partial- and inverse agonists. As aforementioned, agonist ligands allow the recruitment of coactivators by RARs and the subsequent transcription of RAR-dependent genes. In contrast, by a mechanism described below, full or "neutral" antagonists prevent the association of RARs with any type of coregulatory proteins thus generating "necked" receptors characterized by very weak transcriptional activation or repression levels. Partial agonists lie between these two types of ligands so that they can act as cell-selective modulators with agonist or antagonist properties depending on the cellular context, in particular the ratio between coactivators and corepressor levels. The fourth pharmacological class of RAR ligands corresponds to that of inverse agonists which strengthen the interaction of the receptor with corepressors, thereby further increasing the basal repression function of RARs.

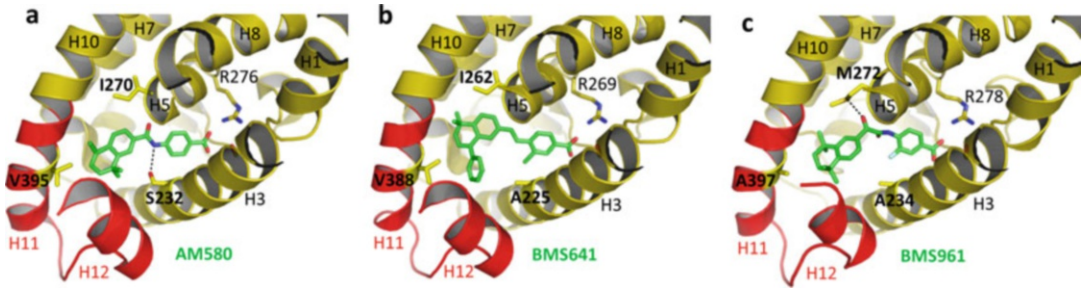
Structural Basis of Ligand Activity

Crystallographic studies of RAR LBDs in complex with all types of ligands have provided a deep insight into the molecular basis of ligand action, revealing how different pharmacological classes of ligands remodel the receptor surface to increase or reduce interaction with a given type of coregulator (le Maire and Bourguet 2014). The LBD of RARs is organized as an antiparallel α-helical sandwich with 12 conserved helices arranged in three layers with a β-turn (S1 and S2) located between helices H5 and H6. Helices H4, H5, H8, H9, and H11 are sandwiched between H1,

H2, and H3 on one side and H6, H7, and H10 on the other side (Fig. 4). In contrast, the C-terminal helix H12 (also termed activation or AF-2 helix) is flexible and can adopt various conformations depending on the type of bound ligand. This LBD architecture generates a ligand-binding pocket (LBP) primarily made of hydrophobic residues from helices H3, H5, H7, H11, and the β-sheet segment. Structural studies have revealed that ligands are stabilized in the RAR LBPs through extensive van der Waals contacts and a network of ionic and hydrogen bonds between their carboxylate moieties, a conserved arginine in H5, and water molecules (Fig. 5).



Retinoids, Fig. 4 Structural basis of retinoid receptor modulation by different classes of ligands. (**a**) Overall structure of the agonist (AM580, green sticks)-bound RARα LBD in complex with a coactivator fragment (Coa., purple). The C-terminal portion of the LBD that is subjected to important conformational changes is highlighted in red. (**b**) Overall structure of the partial agonist (BMS641, green sticks)-bound RARβ LBD. The pink harrows indicate that the activation helix remains dynamics in the presence of this type of ligand. (**c**) Overall structure of the antagonist (BMS614, green sticks)-bound RARα LBD. The antagonist extension of BMS614 prevents H12 from adopting the active conformation. (**d**) Overall structure of the inverse agonist (BMS493, green sticks)-bound RARα LBD in complex with a corepressor fragment. The CoRNR1 peptide (Cor., purple) is composed of a short β-strand and of a four-turn α-helix. Residues that belong to H11 in the other structures adopt a β-sheet (S3) conformation in this structure. H1-H12: α-helices 1 to 12, S1-S3: β-strand 1 to 3. (**e**) Functional classification of retinoids. Upon binding to RARs, retinoids control the expression of RAR target genes. AM580 fully activates transcription through RARα (green line) and is classified as full agonist. BMS641 exhibits a weaker efficacy than full agonists and is considered as a so-called partial agonist to RARβ (blue line). By preventing the interaction with both corepressors and coactivators, BMS614 is defined as neutral antagonist to RARα (dotted line). The so-called inverse agonist BMS493 increases the repression function of RARβ in keeping with its ability to strengthen the interaction of RARs with corepressors (red line)



Retinoids, Fig. 5 Structural basis for RAR-subtype selectivity. The ligand-binding pockets of RARα (**a**), RARβ (**b**), and RARγ (**c**) bound to their selective agonist AM580, BMS641, and BMS961, respectively, are shown. Amino acid side chains that differ between the different isotypes are depicted (yellow), together with the conserved arginine (H5) and serine (H3) (olive) that serve as anchoring points for the ligands. Dotted lines indicate key hydrogen bonds for ligand selectivity. H1-H12: α-helices 1 to 12

Agonist (*e.g.,* AM580, Fig. 1b) binding stabilizes a LBD conformation where the AF-2 helix seals the LBP (Fig. 4a). This particular position defines a hydrophobic surface comprising the C-terminal part of helix H3, helix H4, the L3–4 loop linking H3 and H4, and H12. This recognition surface accommodates the short LXXLL-containing helical motif of coactivator proteins. The coactivator helix is held in place by interactions of the leucine residues with the hydrophobic groove and by hydrogen bonds with a lysine at the C-terminus of H3 and a glutamate in H12 which together form a "charge clamp." It is noteworthy that this conformation is not specific to RAR as it can be observed in all agonist-bound NRs and thus, corresponds to the canonical transcriptionally active form of nuclear receptors (Fig. 4e).

In contrast to full agonists, partial agonists (*e.g.,* BMS641, Fig. 1b) are able to bind to RARs with high affinity, but even so, they incompletely stabilize the active form (Fig. 4b). The partial antagonist activity of these ligands is a consequence of the low interaction strength between H12 in the active position and the H3/H11 surface that renders the activation helix more dynamic. In this case, the association with coactivators is weaker than in the full agonist-bound situation. Nevertheless, the presence of coactivators helps stabilize the active conformation so the transcriptional outcome of partial agonist binding greatly depends on the intracellular concentration of coregulators (Fig. 4e).

The crystal structures of RAR LBD in complex with the antagonist BMS614 (Figs. 1b and 4c) or the inverse agonist BMS493 (Figs. 1b and 4d) show that these compounds encompass bulky extensions protruding between helices H3 and H11 which are too long to be accommodated within the LBP with H12 in the active conformation. A consequence of this steric clash is that the AF-2 helix either adopts an alternative conformation by docking to the coregulator binding groove, as seen in the BMS614 complex, or has no defined position and appears highly mobile, as in the presence of BMS493. By relocating the AF-2 helix to the coregulator groove, the antagonist BMS614 prevents any interaction with both coregulator types and renders the receptor transcriptionally silent (Fig. 4e). In contrast, the inverse agonist BMS493 favors the recruitment of corepressors by generating an interaction surface for the CoRNR1 motif present in NCOR1/2 and therefore increases the repression function of RARs (Fig. 4e). Unlike the AM580, BMS641, and BMS614 complexes in which residues C-terminal of helix H10 adopt a helical structure (H11), these residues assume an extended β-strand (S3) conformation in the BMS493 complex, allowing for the formation of an antiparallel β-sheet with N-terminal residues of CoRNR1 (compare Fig. 4a–d). The remainder of CoRNR1 folds as a four-turn α-helix that docks into the coregulator groove of RAR through the conserved LXXXIXX(I/V)IXXX(Y/F) motif reminiscent of

the short LXXLL sequence of coactivators (le Maire et al. 2010).

RAR-Subtype Selective Retinoids

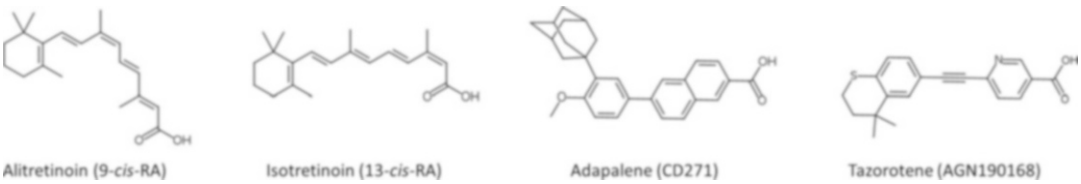
While topologically very similar, the LBPs of RARα, β, and γ differ by three residues located in helices H3, H5, and H11, and these amino acid divergences have been exploited for the design of synthetic retinoids with RAR-subtype selectivity that represent unique pharmacological tools for the study of RARs-specific functions (le Maire et al. 2012, 2019). In their LBPs, RARα (Ser232, Ile270, Val395) and RARβ (Ala225, Ile262, Val388) differ just by one amino acid in helix H3, whereas RARβ and RARγ (Ala234, Met272, Ala397) differ by two residues located in helices H5 and H11 (Fig. 2). RARα selectivity is favored by the formation of a specific hydrogen bond between serine residue S232 in H3 and a nitrogen atom of an amide bond as observed with AM580 (Fig. 5a), whereas the presence of an hydrogen bond between methionine M272 in helix H5 and an hydroxyl moiety present in the central region of the ligand is a characteristic feature of selective RARγ ligands, as exemplified by BMS961 (Fig. 5c). Finally, RARβ selectivity exploits the presence of a somewhat larger LBP subpocket that is able to accommodate bulkier groups attached to the ligand as, for example, in the case of BMS641 (Fig. 5b).

Pharmacological Relevance

Retinoids in Medical Use

RARs have been associated with several diseases such as cancer or skin disorders. Accordingly retinoids are used in a variety of chemopreventive and chemotherapeutic settings (di Masi et al. 2015; Uray et al. 2016). Three generations of retinoid acid analogs have been generated for systemic and topical treatment of various skin disorders (Fig. 6). The first generation includes nonaromatic retinoids such as vitamin A, ATRA (tretinoin), 13-*cis*-RA (isotretinoin), and 9-*cis*-RA (alitretinoin). The second generation of molecules includes monoaromatic retinoid analogs and the third generation of compounds corresponds to polyaromatic derivatives such as adapalene (CD271) and tazorotene (AGN190168). It is noteworthy that thousands of synthetic retinoids have been developed up to now. The recognized potential of retinoids in skin disorders was demonstrated by the clinical efficacy of ATRA in keratinizing disorders. However, ATRA was considered too toxic and minor structural changes of this molecule yielding *cis*-isomers resulted in oral retinoids with improved pharmacological properties. In particular, 9-*cis*-RA and 13-*cis*-RA are used for dermatological indications including acne, psoriasis, antiaging, or photo-damage. In addition to these ATRA isomers, the two synthetic retinoids adapalene and tazarotene are also efficient for the treatment of acne and stable plaque psoriasis, respectively.

Retinoids are mostly involved in the regulation of processes like differentiation and cell death which play critical roles in the outcome of malignant transformation of tissues. The most direct implication of RAR in human cancer is given by acute promyelocytic leukemia (APL), which is caused by a somatic translocation between chromosomes 15 and 17 that fuses RARα and promyelocyte leukemia protein (PML) human genes, leading to the alteration of the signaling of both RARα and PML proteins (de The et al. 2017). The resulting fusion protein PML-RARα contains large parts of both proteins, including the LBD



Retinoids, Fig. 6 Retinoids in clinical use. For each molecule, the name of the commercial drug is given as well as the chemical name in brackets

and the DBD of RARα, and displays increased binding efficiency to transcriptional corepressors which do not dissociate in the presence of physiological concentrations of ATRA. The subsequent silencing of RAR target genes arrests myelopoiesis at the promyelocyte stage and prevents the differentiation of APL cells. However, the use of supraphysiological doses of ATRA induces dissociation of the silencing complex, postmaturation apoptosis, and remission in patients with APL. Therefore, ATRA-based differentiation therapy of APL restores normality by guiding the cancer cell back into the regular cycle of a differentiated cell and forces it into death. In addition to this successful retinoid-based differentiation therapy, retinoids are used as chemopreventive agents and to treat various cancers such as refractory cutaneous Kaposi's sarcoma (9-*cis*-RA), cutaneous T-cell lymphoma (CTCL) (tazarotene), and neuroblastoma (13-*cis*-RA).

Retinoids as Pharmacological Tools

RARs are attractive drug targets because they are master regulators of a large variety of major (patho)physiological processes and their cognate ligands are small molecules that are convenient for chemical synthesis. Moreover, the success of the cancer differentiation therapy in the ATRA-treated APL has stimulated considerable interest in fields of retinoid research. However, despite their chemotherapeutical potential, retinoic acids can be both beneficial and detrimental, and their pharmacological use has been restricted because of their pleiotropic activities and their teratogenic effects. Accordingly, a major goal of drug development is the generation of RAR modulators that display increased target selectivity and/or functional specificity in order to overcome the limitations of current drugs. In addition, even if a great number of gene ablation studies have partially revealed the global impact of RARs in physiological processes, there is still a need for pharmacological agents to address the specific function of RAR subtypes *in vitro* and in animal models. Overall, the challenge is to integrate the structural and molecular biological information accumulated over the years into studies on the (patho) physiological relevance of RAR signaling, to provide novel tools for research and therapy. Then major synthetic chemistry efforts have been directed to the identification of potent compounds leading to the generation of thousands of configurationally and/or conformationally restricted analogs of ATRA. Importantly, subtle changes in the chemical structure of these synthetic retinoids can direct the activity toward various types of functional specifications, such as agonism/antagonism/inverse agonism, subtype selectivity, or cell/pathway selectivity, and translate into very selective effects on the transcriptional potential of the receptor. Of note, it is of utmost importance to accurately characterize these modulators for an appropriate use avoiding data misinterpretations. Accordingly, the molecular details by which synthetic retinoids direct specificity and functionality of RARs have to be precisely uncovered (le Maire et al. 2019). It has been possible to generate entirely RAR subtype-selective agonists, such as AM580 (RARα), BMS948 (RARβ), and BMS961 (RARγ) (Fig. 1b). Note that to be considered as selective a given ligand (agonist and antagonist) has to exhibit an affinity difference greater than 100-fold between its primary target and other receptors (see International Committee of Pharmacology Committee on Receptor Nomenclature and Classification). Under 100-fold difference, the ligand may be defined as a compound that shows a preference for a given receptor. Hence, the potency (amount of a drug that is needed to produce a defined effect) of the compound has to be considered. Another property to consider is the transcriptional efficacy of the compound, namely maximum effect that a drug can produce regardless of dose, and consequently independent of its binding affinity. The differential interaction with coregulators is the basis of the diverse functionality of retinoids and allows their functional classification as agonists (*e.g.,* AM580), partial agonists (*e.g.,* BMS641), neutral antagonists (*e.g.,* BMS614), and inverse agonists (*e.g.,* BMS493), as described above. As coregulators are platforms for complexes with epigenetic activities, such as histone methylation/acetylation and deacetylation, such different classes of ligands can be used to regulate RAR-mediated gene programs in very different directions.

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Rheumatoid Arthritis

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Synonyms

Anti-TNF; Biologics; Bio-originators; Biosimilars; Cytokines; Disease-modifying anti-rheumatic drugs; Genetics; JAK inhibitors; Pathogenesis; Small molecules; Therapy

Definition

Rheumatoid arthritis (RA) is best thought of as an inflammatory syndrome with autoimmune features with its predominant expression in synovial joints. It is the most common form of inflammatory polyarthritis. Current thinking favors the hypothesis that interplay between genetic factors, sex hormones, and possibly an infectious agent or another immune-activating agent initiates an autoimmune pathogenic mechanism that culminates in a disease with inflammatory and destructive features.

To date there are no laboratory tests that are pathognomonic for RA, but the presence of anti-cyclic citrullinated protein antibody (ACPA) and/or IgM rheumatoid factor is relatively specific for this condition. However, neither of these tests are sufficiently specific to establish the classification of RA, and prognosis varies widely within seropositive and seronegative patient populations, respectively. Because of the heterogeneity in presentation and disease course, in order to recruit relatively homogeneous populations into clinical trials, the American College of Rheumatology formulated the 1987 revised classification criteria for the diagnosis of RA (Arnett et al. 1988) based on a hospital population of patients with established, active disease. These criteria combined a constellation of clinical, serological, and

radiological features that help to differentiate RA from other forms of inflammatory arthritis with a diagnostic sensitivity and specificity of about 90% for active disease. However, these requirements have a much poorer sensitivity for a diagnosis of RA in the early stages of presentation at which time there may be an insufficient number of features required to satisfy the classification criteria. Recognizing that best outcomes are achievable when treatment intervention is as early as feasible in the evolution of the illness, updated classification criteria were published in 2010 by the American College of Rheumatology (ACR) and European League Against Rheumatism (EULAR) and designed to identify patients with unexplained synovitis in at least one peripheral joint and a short duration of symptoms who would benefit from early therapeutic intervention (Aletaha et al. 2010). In this system, a score-based algorithm is employed for the classification of RA based on four domains: joint involvement; serological test results; acute-phase reactant test results; and patient self-reporting of the duration of signs and symptoms. The maximum number of possible points is 10, and classification of definitive RA requires a score of 6/10 or higher. Patients with a score lower than 6/10 should be reassessed over time. If patients already have erosive changes characteristic of RA, they meet the definition of RA, and application of this diagnostic algorithm is unnecessary. The score is calculated as shown in Table 1.

Rheumatoid Arthritis, Table 1 The 2010 ACR/EULAR classification criteria for RA

Joints Distribution (0–5)	
1 Large joint	0
2–10 Large joints	1
1–3 Small joints (large joints not counted)	2
4–10 Small joints (large joints not counted)	3
>10 joints (at least one small joint)	5
Serology (0–3)	
Negative RF *and* negative ACPA	0
Low-positive RF *or* low-positive ACPA	2
High-positive RF *or* high-positive ACPA	3
Symptom Duration (0–1)	
<6 weeks	0
≥6 weeks	1
Acute Phase Reactants (0–1)	
Normal CRP *and* normal ESR	0
Abnormal CRP *or* abnormal ESR	1

Basic Mechanisms

Characteristics

Once established, RA is characterized by deforming symmetrical polyarthritis associated with synovitis of joint and tendon sheaths, articular cartilage loss, and erosion of juxta-articular bone of varying extent and severity (Taylor 2006). IgM rheumatoid factor is detectable in the blood in a majority of patients. It is an autoantibody whose autoantigen is the Fc portion of IgG. The prevalence of rheumatoid factor increases with duration of disease in rheumatoid arthritis: at 3 months the prevalence is 33%, while at 1 year it is 75%. Up to 20% of RA patients remain negative for rheumatoid factor (also known as "seronegative rheumatoid arthritis") throughout the course of their disease. Antibodies to cyclic-citrullinated peptides (anti-CCP) have a similar sensitivity to rheumatoid factor, but higher specificity (Taylor 2019b). As in the case of high-titer rheumatoid factor, anti-CCP antibodies are associated with persistence and destructiveness of rheumatoid arthritis and may precede the onset of clinical disease.

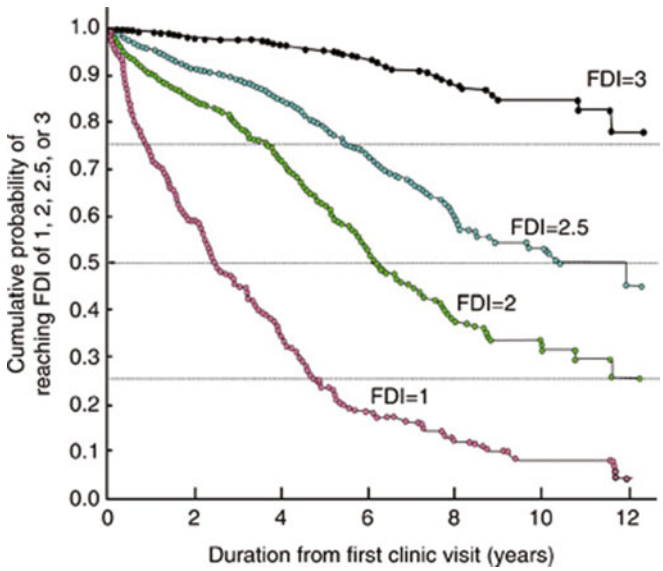
In a proportion of patients, systemic and extra-articular features may be observed during the course of the disease (and rarely prior to joint disease). These include anemia; weight loss; vasculitis; serositis; nodules in subcutaneous, pulmonary, and sclera tissues; mono-neuritis multiplex; and interstitial inflammation in lungs as well as in exocrine salivary and lacrimal tissue. However, these systemic manifestations occur relatively late in the disease progression.

The clinical presentation of RA is heterogeneous with a wide spectrum of age of onset, degree of joint involvement, and severity. Similarly, the disease course of RA is variable. This ranges from a brief, mild, self-limiting oligoarticular illness with minimal joint damage to a sustained polyarticular, synovial inflammation resulting in relentlessly

progressive cartilage destruction, erosion of bone, and ultimately changes in joint integrity with corresponding functional impairment. Up to 90% of patients with marked synovitis have radiographic evidence of bone erosion within 2 years of diagnosis, despite treatment with conventional synthetic disease-modifying anti-rheumatic drugs, the most commonly prescribed being MTX, leflunomide, sulfasalazine, and hydroxychloroquine. If disease remains uncontrolled, without successful intervention with a targeted therapy, the majority of patients with a more aggressive disease evolution become clinically disabled within 20 years (Fig. 1). For those with severe disease or extra-articular features, the mortality is equivalent to that of patients with three vessel coronary artery disease or stage IV Hodgkin's lymphoma.

Cellular Pathology

RA is characterized by chronic inflammation of synovial joints with synovial proliferation and infiltration by blood derived cells, in particular, memory T cells, macrophages, and plasma cells, all of which show signs of activation. Prominent vasculature is a feature of RA synovitis that is observed as a fine network of vessels over the rheumatoid synovium at arthroscopic inspection of RA joints. Angiogenesis is evident on microscopic examination of synovial biopsies from the earliest stages of disease development. Formation of new blood vessels permits a supply of nutrients and oxygen to the augmented inflammatory cell mass and so contributes to the perpetuation of synovitis. In the chronic phase of disease, capillaries and post-capillary venules are particularly evident in the synovial sublining region. In histological sections mononuclear and polymorphonuclear leukocytes can sometimes be found in close apposition to vascular endothelium, probably in the process of margination and adhesion prior to migration into the inflamed tissue. The synovial tissue becomes markedly hyperplastic and locally invasive at the interface of cartilage and bone with progressive destruction of these tissues in the majority of cases. This invasive tissue is referred to as "pannus," comprising mainly lining cells with the appearance of proliferating mesenchymal cells with very little sublining lymphocytic infiltration. The accompanying destruction of bone and cartilage is thought to be mediated by cytokine-induced degradative enzymes, notably the matrix metalloproteinases. Although RA has its principal manifestation in joints, there is also evidence of systemic involvement, for example, the upregulation of acute phase proteins, and a wide variety of extra-articular features may develop. These occur predominantly in patients who are rheumatoid factor positive and carry the HLA-DR4 gene.



Rheumatoid Arthritis, Fig. 1 The development of disability over time in a group of RA patients studied prior to the era of targeted therapies. *FDI* functional disease index where 1 = moderate disability, 2 = more marked disability, 3 = severe disability, and 4 = very severe disability. (From Wolfe and Cathey 1991)

Genetic Factors

Genetic factors were originally implicated in the etiopathogenesis of RA following the discovery that in population studies, there is a slight increase in the frequency of RA in first-degree relatives of patients with this disease, especially if seropositive for rheumatoid factor. In hospital-based population studies of identical twins, concordance rates of disease are around 30%, compared with 5% in nonidentical twins. The figures are lower in community-based studies and, although still supportive of the concept of a genetic contribution, argue against the proposition that RA is the result of a dominant single-gene disorder. These and other epidemiological studies have led to the conclusion that RA is a polygenic disease and that non-inherited factors are also of great importance (Kurkó et al. 2013).

Genes encoding particular class II human leukocyte antigens (HLA) are among candidates for involvement in predisposition to RA. This discovery came about with the observation that 60–70% of Caucasian patients with RA are HLA-DR4 positive by cellular or serological techniques compared with 20–25% of control populations. Furthermore, patients with more severe RA, especially those with extra-articular complications such as vasculitis and Felty's syndrome, are even more likely to be HLA-DR4 positive than patients with less severe disease confined to joints.

Class II HLA molecules are expressed on the surface of antigen-presenting cells. They play a key role in presentation of processed linear peptide antigens of at least nine amino acids to T cells. Antigen is bound to the HLA-binding cleft formed by the α and β chains of the HLA class II molecule. This trimolecular HLA–antigen complex binds in turn to the variable portion of the T-cell receptor.

Nucleotide sequencing of HLA-DR β *1* exons coding amino acid residues 70–74 has revealed that HLA-DR4 subtypes Dw4, Dw14, and Dw15 share similarities with each other (with a conservative substitution of glutamine with lysine at position 71 in Dw4) and with HLA-DR1. The sequence predicts susceptibility to RA and is associated with RA in 83% of Caucasian patients in the United Kingdom. In contrast, negative associations are observed in individuals who are DR4w10, in whom the charged basic amino acids glutamine and arginine in positions 70 and 71 are replaced by the acidic amino acids aspartic and glutamic acid. In Dw13 individuals, in whom a negative association is also observed, arginine is substituted for glutamic acid in position 74. Molecular modelling studies suggest that amino acid residues 70–74 are located in the α-helix forming the wall of the peptide-binding groove and thus likely to be involved in antigen binding and subsequent interaction with T-cell receptors (Kurkó et al. 2013). Acidic substitutions could profoundly alter protein structures and thereby alter affinity for peptide antigens. However, molecular mechanisms accounting for susceptibility to RA remain to be elucidated. Possibilities include permissive binding of specific peptides such as those on autoantigens or on environmental antigens, initiation of disease by specific binding of superantigens to HLA molecules, or modulation of the T-cell repertoire by selection or tolerance. HLA and some non-HLA genes have been linked to autoimmunity to citrullinated proteins (anti-CCP) as well as smoking. Smoking, oral microbiota, and possibly other environmental and lifestyle-related factors may trigger the development of anti-CCP in some patients. It has been hypothesized that the severity of disease and extra-articular complications are related to homozygosity and the density of disease-associated MHC molecules that critically influence the selection of the T-cell repertoire and tolerance to antigens.

Cytokines

Cytokines are small, short-lived proteins and important mediators of local intercellular communication. Cytokines mediate both innate and adaptive immune cellular responses and play a key role in integrating responses to a variety of stimuli that contribute to inflammation and joint injury that characterize RA pathology. Much of the activity of key cytokines in RA pathology is paracrine in nature. By binding their cognate receptors on target cells in their immediate vicinity, these molecules participate in many important biological

activities including cell proliferation, activation, death, and differentiation. In experimental systems, some cytokines are proinflammatory, such as interleukin-1 (IL-1) and tumor necrosis factor (TNF); others, such as interleukin-10 (IL-10) and transforming growth factor β (TGFβ), exert predominantly anti-inflammatory effects. However, it is now known that many cytokines, for example, interferon γ (IFNγ), with chiefly proinflammatory activity can also in some instances have anti-inflammatory properties. Similarly, IL-10 and TGFβ may also exhibit proinflammatory properties under certain experimental conditions. Interleukin-6 (IL-6) does not have a classical pro-inflammatory action but has been implicated in the process of erosion of bone in inflammatory arthritis. Paracrine or autocrine pathways involving cytokines with either pro- or anti-inflammatory activity form complex networks determining whether chronic inflammation results. Following engagement with receptors on the surface of responder cells, cellular responses are mediated by an intracellular cascade of phosphorylation steps through a number of distinct signaling pathways. As understanding of cytokine signaling has advanced, various intracellular signaling molecules have been proposed as potential therapeutic targets. The Type I/II cytokines, which include IL-6, signal through the Janus kinase (JAK) enzymes comprising a family of four intracellular tyrosine kinases. The only targeted small molecule therapies to be approved for RA to date are JAK inhibitors.

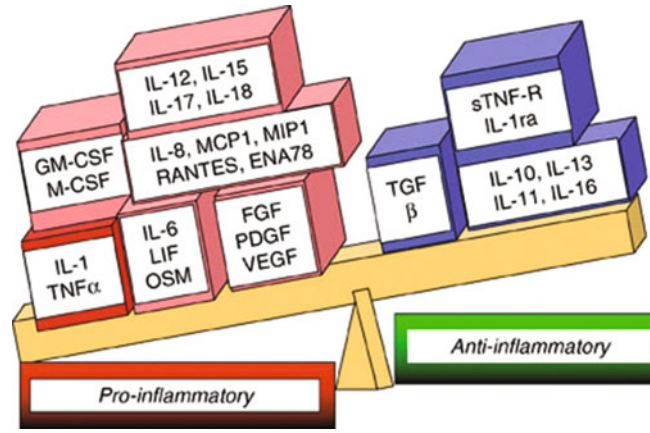
Role of Cytokines in the Pathogenesis of RA

Cytokines derived from macrophages and fibroblasts are abundant in the rheumatoid synovium. These include IL-1, TNF, granulocyte macrophage colony-stimulating factor (GM-CSF), IL-6, and numerous chemo-attractant cytokines known as chemokines. Many of these factors are of importance in regulating inflammatory cell migration and activation. By contrast, given the extent of synovial inflammation and lymphocytic infiltration, factors produced by T cells, for example, IFNγ, interleukin-2 (IL-2), and interleukin-4 (IL-4), are surprisingly sparsely expressed. However, there are a number of cytokines that cause co-stimulation of T helper cells including interleukin (IL)-7, IL-12, IL-15, and IL-18. There is a predominance of Th1 cell activity, as defined by IFNγ production, and low Th2 cell activity as defined by IL-4 production (Firestein and McInness 2017).

An extensive range of proinflammatory cytokines can be detected in RA synovial samples, regardless of differences in donor disease duration, severity, or even conventional (non-biologic) drug therapy. Proinflammatory cytokines are spontaneously produced over several days in dissociated RA synovial membrane cell cultures comprising a heterogeneous population of cells. This occurs in the absence of extrinsic stimulation, suggesting that the cultures produce one or more soluble factors regulating prolonged cytokine synthesis. Addition of anti-TNF antibodies to these cell cultures was observed to strikingly reduce the production of other proinflammatory cytokines, including IL-1, GM-CSF, IL-6, and IL-8. In contrast, blockade of IL-1 by means of the IL-1 receptor antagonist results in reduced production of IL-6 and IL-8 but not that of TNF. These observations led to the concept that TNF occupies a dominant position at the apex of a proinflammatory cytokine network.

In support of the concept of a cytokine disequilibrium within the chronic inflammatory situation in rheumatoid synovium is the observation that multiple anti-inflammatory mediators are also upregulated, but at a level insufficient to suppress synovitis. Examples include the abundant expression of IL-10, IL-13, and TGFβ both in latent and active form. Naturally occurring cytokine inhibitors, such as interleukin-1 receptor antagonists (IL-1ra) and soluble TNF receptors, the specific inhibitors of IL-1 and TNF, respectively, are also upregulated in the rheumatoid joint (Fig. 2).

TNF is a pleiotropic cytokine with biological properties that include enhanced synovial proliferation, production of prostaglandins and metalloproteinases, as well as regulation of other proinflammatory cytokines. The predicted clinical success of anti-TNF therapy in RA was based on the demonstration of RA synovial tissue expression of TNF and its receptors, in vitro experiments employing dissociated synovial cell cultures, and



Rheumatoid Arthritis, Fig. 2 The concept of a cytokine disequilibrium. Many cytokines are detectable in rheumatoid synovial tissues, including those with predominantly anti-inflammatory properties. But the net effect is a dominance of proinflammatory activity

preclinical in vivo studies. A number of independent in vivo studies demonstrated that antibody therapies blocking bioactivity of TNF, administered either during the induction phase of murine collagen-induced arthritis or, more importantly, after the onset of disease, were able to ameliorate clinical symptoms and prevent joint destruction. Furthermore, in a murine model, the overexpression of a human TNF transgene modified at its 3′ end to prevent degradation of its mRNA was associated with the development of a destructive form of polyarthritis 4–6 weeks after birth. This could be prevented by administration of a human TNF-specific mAb.

Pharmacological Intervention

A major emphasis in the management of RA over recent years has been on early diagnosis and treatment, before the onset of erosions, in order to best realize opportunities to achieve and sustain the ideal goal of remission and to prevent joint damage and disability. The heterogeneity of presentation of RA and subsequent disease course is such that pharmacological treatment will need to be adapted to the needs of the individual. Optimum suppression of inflammation is considered the most effective means to achieve optimal outcomes. In early RA, treatment is initiated with a csDMARD, most commonly methotrexate, unless contraindicated. Methotrexate can be administered either orally or parenterally and has a large dose-titratable range. Because of these features, as well as its efficacy, safety, and cost-effectiveness, methotrexate holds a unique place in the management of RA (Taylor et al. 2019). Methotrexate monotherapy is recommended as an initial pharmacological strategy, but it can also be used as an "anchor drug" in combination with another csDMARDs or targeted therapies. Symptomatic benefit following initiation of csDMARDs tends to be relatively slow in onset, often taking many weeks. Short-term glucocorticoids are often used as a "bridging" therapy when a more slowly acting oral csDMARD is initiated so that rapid suppression of synovitis can be achieved before the slower benefits of the csDMARD are expressed. Corticosteroids can be used effectively in a range of dose regimens and routes of administration but should be tapered as rapidly as clinically feasible.

There have been unprecedented advances in our understanding of the pathophysiology of RA over the last few decades which have been translated into a broad range of efficacious, so-called targeted, therapies directed against relevant cells and molecules contributing to disease pathogenesis. These include parenterally administered biologic DMARDs (bDMARDs) and orally available targeted synthetic DMARDs (tsDMARDs). bDMARDs are protein-based drugs derived from living organisms that are designed to either inhibit or augment specific component of the immune system.

The first bDMARDs to be approved were TNF inhibitors. The first generation of bDMARDs, referred to as bio-originators, was approved with finite patent life. Following patent expiry of the

earliest bio-originator bDMARDs, biosimilars have emerged. A biosimilar is a biological medicinal product that is highly similar to an already authorized original biological medicinal product (reference medicinal product or bio-originator) in terms of quality, safety, and efficacy, based on a comprehensive comparability exercise. Following their introduction to the clinic, in many healthcare economies high procurement costs of bio-originator bDMARDs limited their access to patients meeting eligibility criteria. In the case of the class of anti-TNF bDMARDs, bio-originators included three monoclonal antibodies (infliximab, adalimumab, and golimumab), a TNF receptor fusion protein (etanercept), and a pegylated antibody-binding fragment (certolizumab). There are now several biosimilars of infliximab, etanercept, and adalimumab which are introducing cost competition and may potentially widen patient access to the anti-TNF bDMARD class. Another class of bDMARDs directed against cytokines comprise antibodies directed against interleukin-6 receptor (IL6R). Two bio-originator anti-IL6R mAbs have been approved, tocilizumab and sarilumab.

An entirely different treatment approach to the blockade of pro-inflammatory cytokines is the targeting of cells implicated in the persistence of RA. At present there are two other classes of bDMARD with specificity for cellular targets, namely, cell surface molecules associated with B cell subsets, most notably CD20, and co-stimulation molecules expressed on antigen-presenting cells that recognize cognate ligands on T cells. There is one approved bio-originators directed against the CD20 antigen expressed on a B cell subset, rituximab, and there are now approved biosimilars. The bio-originator abatacept is the only representative of the final class of currently approved bDMARD. It is an inhibitor of the CD28-CD80/86 co-stimulatory signal necessary for T cell activation comprising a fusion protein composed of the Fc region of the human IgG1 fused to the extracellular domain of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4).

In contrast to bDMARDs, which are large molecular weight proteins that must be injected and are incapable of penetrating the lipid bilayer of cell membranes, tsDMARDs are low molecular weight, orally available, "small molecules." The only tsDMARDs currently available for the treatment of RA are JAK inhibitors or "jakinibs," multi-cytokine inhibitors that can cross the cell membrane to block activity of one or more cytoplasmic JAKs. Tofacitinib selectively inhibits JAK1 and JAK3 and was the first JAK inhibitor to be approved, initially with twice daily dosing. A modified release formulation for once daily use has since been developed. Baricitinib selectively inhibits JAK1 and JAK2 and is dosed once daily. Both drugs have undergone extensive clinical trials and demonstrated rapid improvements in symptoms and signs when used in combination with concomitant MTX, other conventional disease-modifying anti-rheumatic drugs (cDMARDs), or as monotherapy (Taylor 2019a) with benefits reported as early as 2 weeks. Both agents inhibit structural damage progression. Remarkably, in MTX inadequate responders, the combination of MTX and baricitinib 4 mg od demonstrated superiority for ACR20 responders and DAS28-CRP reduction over the standard of care biologic adalimumab used with background MTX. Other JAK inhibitors in development include upadacitinib and filgotinib, both with selectivity for JAK1. Both drugs have demonstrated efficacy in combination with MTX and as monotherapy in phase III trials.

In summary, the introduction to the clinic in the late 1990s of bDMARDs directed against TNF heralded the beginning of the era of targeted therapies. Other bio-originator bDMARDs with distinct mechanisms of action followed, and most recently, JAKs have been validated as a therapeutic target with the introduction of orally available small molecular therapies. This expansion of therapeutic options has contributed to a greatly improved outlook for people living with RA and set a high standard for symptom control and prevention of joint destruction.

Cross-References

- ▶ Chemokine Receptors
- ▶ Cyclooxygenases
- ▶ Glucocorticoids

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RNA-Sequencing

▶ Gene Expression Analysis and Next-Generation Sequencing

RXFP1

▶ Relaxin Family Peptides and Their Receptors

RXFP2

▶ Relaxin Family Peptides and Their Receptors

RXFP3

▶ Relaxin Family Peptides and Their Receptors

RXFP4

▶ Relaxin Family Peptides and Their Receptors

Ryanodine Receptor

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Synonyms

Ca^{2+}-induced Ca^{2+} release (CICR) channel; Foot

Definition

Ryanodine receptor (RyR) is an intracellular Ca^{2+} release channel in the **sarcoplasmic reticulum** (SR) or the endoplasmic reticulum (ER). RyR binds ryanodine (a plant alkaloid, see Drugs) with a high affinity, after which it is named.

Basic Characteristics

Occurrence and Role

There are three genetically distinct isoforms of RyR in mammals: RyR1, RyR2, and RyR3

(Table 1). RyR1 is predominantly expressed in the skeletal muscles, and RyR2 is the major isoform in the heart (Ogawa 1994; Sutko and Airey 1996). RyR3 is ubiquitously expressed in a minute amount over the various tissues. All the three isoforms are expressed in the central nervous system (CNS), but their localizations are different (Sutko and Airey 1996). RyR1 and RyR2 play a pivotal role in **excitation-contraction coupling** (E-C coupling) of skeletal and cardiac muscles, respectively.

Structure

RyR forms a large homotetrameric channel composed of four identical monomers of ~5,000 amino acid residues. Three-dimensional architectures obtained by cryo-electron microscopy at near-atomic resolution demonstrate a large, four-fold symmetric cytoplasmic assembly and a small transmembrane assembly forming an ion channel (Ogawa et al. 2020; Zalk and Marks 2017) (Fig. 1). A cytoplasmic assembly consists of 12 domains, including 10 N-terminal domains (NTD, SPRY1, P1, SPRY2, SPRY3, HANDLE, HD1, P2, HD2, and CENTRAL), the S2S3 domain in the channel region, and the C-terminal domain (CTD) (Fig. 1a–c). The cytoplasmic assembly has been identified as "foot" in the electron microscopic observation of the skeletal muscle triads (Franzini-Armstrong and Protasi 1997) (Fig. 2). All the three isoforms show very similar structures and 65% amino acid residues are identical between any two of the RyR isoforms (Ogawa 1994; Sutko and Airey 1996). However, there are three major regions of diversity, designated DR1, DR2, and DR3 (Fig. 1a). DR2 is characteristically missing in RyR3. These divergent regions could account for functional difference between these isoforms. RyR forms multiprotein complex with several associated proteins, such as calmodulin (CaM) and 12 kDa FK506-binding protein (**FKBP12**) (Fill and Copello 2002; Meissner 2017) (Fig. 1a–c).

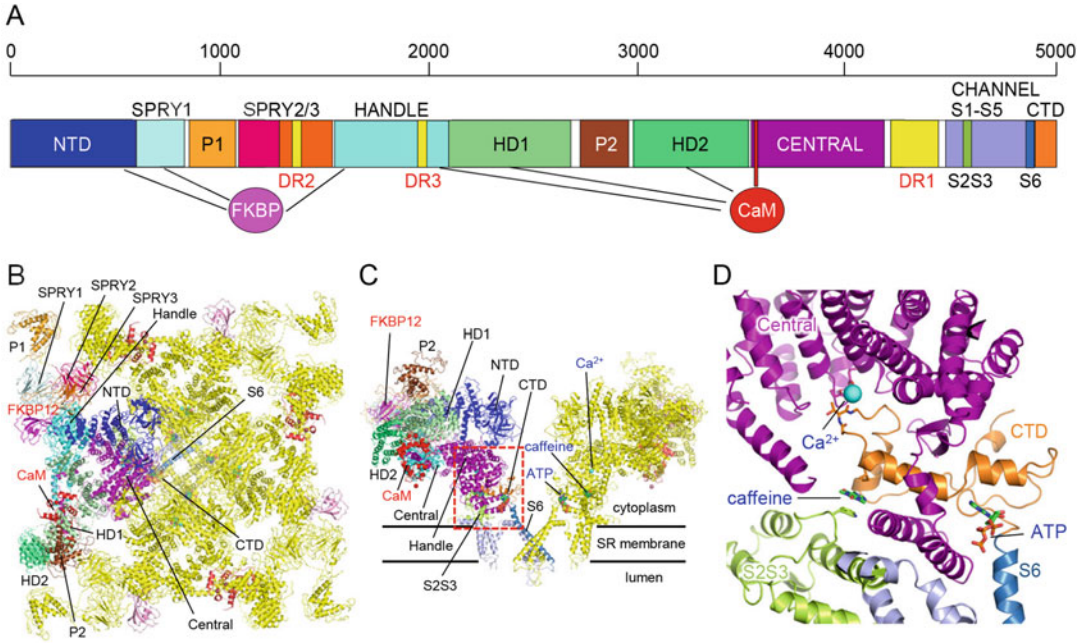
A C-terminal ~1/10 part constitutes the ion channel formed by six transmembrane segments (Fig. 1a, c). Like many other ion channels, the two most C-terminal transmembrane segments constitute the ion-conducting pore, and a loop between them forms a "P-loop" containing a short pore helix and the selective filter (Ogawa et al. 2020; Zalk and Marks 2017). The RyR forms the cation channel characteristic of a large conductance (~700 pS with Na^+ or K^+ and ~100 pS with Ca^{2+}) (Fill and Copello 2002). The RyR channel is selective for Ca^{2+} over monovalent cations (pCa/pNa = 6-7). The selectivity, however, is

Ryanodine Receptor, Table 1 Three mammalian RyR isoforms

	RyR1	RyR2	RyR3
Gene locus (human)	19q13.1	1q42.1-43	15q14-15
Amino acid residues	5,037	4,976	4,872
Occurrence			
Peripheral	Skeletal muscle	Heart	Ubiquitous (minuscule)
			Skeletal muscle[a]
CNS	Purkinje cells	Ubiquitous	Hippocampus
			Corpus striatum
Trigger for Ca^{2+} release			
Ca^{2+} (CICR)	Yes	Yes	Yes
$Ca_V1.1$ (DICR)	Yes	No	No
Knockout mice	Perinatally lethal	Embryonic lethal	No gross abnormalities[b]
Related diseases	MH, CCD, MmD	CPVT, ARVC2, IVF, LQTS, LVNC	Schizophrenia
			Hyperinsulinism

[a]RyR3 is temporarily expressed in mammalian skeletal muscle during development and downregulated to a negligible amount in adult except for diaphragm and soleus muscles. In some nonmammalian vertebrates (e.g., fish, frogs, and birds), nearly equivalent amounts of RyR1 and RyR3 are expressed in adult skeletal muscles (Ogawa 1994; Sutko and Airey 1996)

[b]Some alterations in CNS function (e.g., enhanced locomotor activity or alterations in synaptic plasticity) are reported (Fill and Copello 2002; Kushnir and Marks 2010)



Ryanodine Receptor, Fig. 1 Three-dimensional architecture and domain organization of the RyR. A, Schematic illustration of domain organization in one protomer. A cytoplasmic assembly consists of 12 domains, including 10 N-terminal domains (NTD, SPRY1, P1, SPRY2, SPRY3, HANDLE, HD1, P2, HD2, and CENTRAL), the S2S3 domain in the channel region, and the C-terminal domain (CTD) and a C-terminal transmembrane assembly forms an ion channel. DR1, DR2, and DR3 represent divergent regions between the isoforms. DR2 is characteristically missing in RyR3. B-D, Overall structure of RyR2 in the open state in complex with FKBP12, calmodulin, Ca^{2+}, ATP, and caffeine (PDB accession code: 6JIY). Colors follow the schematic illustration shown in A. Ca^{2+} is shown as a cyan ball; ATP and caffeine are shown in sphere representation. B, top view (from the T-tubule); C, side view (parallel to the SR membrane), two of the four protomers facing each other are shown; D, magnified view of the red dotted square shown in C

less than that of the voltage-dependent Ca^{2+} channel (VDCC) showing pCa/pNa > 1,000.

Ca^{2+} Release Mechanisms

All the RyR channels are gated by cytoplasmic Ca^{2+}, known as Ca^{2+}-induced Ca^{2+} release (CICR) (Endo 2009; Fabiato 1983; Rios 2018) (Table 1). CICR functions as an amplifier of small Ca^{2+} signals in various excitable and nonexcitable tissues and well documented in E-C coupling in the heart. In addition, RyR1 also mediates depolarization-induced Ca^{2+} release (DICR), which is gated through some protein-protein interactions. DICR is the principal Ca^{2+} release mode in E-C coupling in vertebrate skeletal muscle.

CICR and Its Endogenous Modulators

In CICR, the RyR channel is activated by μM Ca^{2+} and inhibited by sub-mM or higher Ca^{2+} (Endo 2009; Meissner 2017; Murayama and Kurebayashi 2011; Rios 2018). This biphasic Ca^{2+} dependence can be explained by two distinct Ca^{2+} binding sites: high-affinity Ca^{2+} site for activation (A-site) and low-affinity Ca^{2+} site for inactivation (I-site). The A-site is located at the interface between Central and CTD (Ogawa et al. 2020; Zalk and Marks 2017) (Fig. 1c, d). The I-site remains to be unidentified. In addition to the occupation with Ca^{2+} of the A- and I-sites, the CICR activity of RyR is also determined by a third parameter independent of Ca^{2+} which sets the maximal attainable level.

CICR is modulated by several endogenous ligands. Mg^{2+} inhibits CICR by acting as a competitive antagonist in the A-site and as an agonist in the I-site (Meissner 2017; Murayama and Kurebayashi 2011). Adenine nucleotides such as ATP greatly stimulate CICR by increasing the maximal attainable level without changing Ca^{2+} dependence (Endo 2009). The binding site for ATP is located at the interface between central domain, S6, and CTD (Ogawa et al. 2020; Zalk and Marks 2017) (Fig. 1c, d). Posttranslational modifications of specific residues (phosphorylation, oxidation, or S-nitrosylation) modulate the CICR activity (Fill and Copello 2002; Meissner 2017).

CICR is modulated by a large number of associated proteins (Fill and Copello 2002; Kushnir and Marks 2010; Meissner 2017). Among them, CaM and FKBP12/12.6 tightly bind to RyR and are well investigated. Calmodulin binding site is an elongated cleft formed by Handle, HD1, HD2, and Central domains (Ogawa et al. 2020) (Fig. 1b, c). The action of CaM is isoform-dependent: it activates RyR1 and RyR3 at a low (submicromolar) Ca^{2+} and inhibits at a higher Ca^{2+}, whereas it inhibits RyR2 irrespective of Ca^{2+} concentrations (Meissner 2017). FKBP12 and FKBP12.6 bind to RyR1 and RyR2, respectively (Fill and Copello 2002). They bind to the cytoplasmic region surrounded by the NTD-C, SPRY1, SPRY3, and Handle domains (Ogawa et al. 2020; Zalk and Marks 2017) (Fig. 1b, c). FKBP12 stabilizes the RyR1 channel activity, whereas effect of FKBP12/12.6 on RyR2 is controversial. RyR3 could bind FKBP12 with unchanged activity.

The RyR channels seem to be regulated by luminal Ca^{2+}. Luminal Ca^{2+} may activate the RyR2 channels in the heart (Bers 2008; Priori and Chen 2011). The association of **calsequestrin** with RyR2 via triadin or junctin is proposed as a possible regulatory mechanism (Beard et al. 2004; Fill and Copello 2002). Such activation by luminal Ca^{2+} remains controversial in the skeletal muscle.

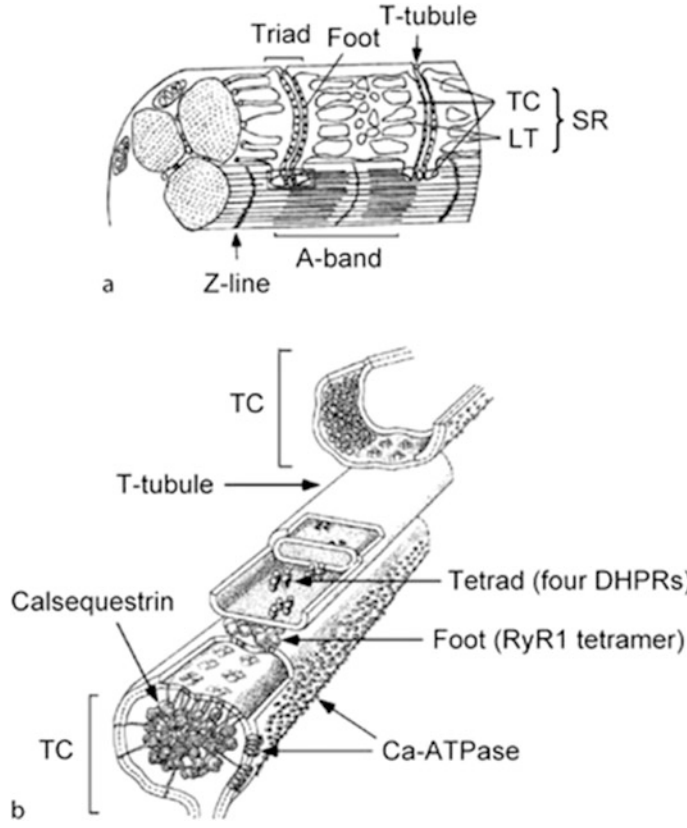
E-C Coupling in Skeletal Muscle

In E-C coupling in vertebrate skeletal muscle, the RyR1 channel releases Ca^{2+} from the SR on depolarization of the T-tubules. The process is known as depolarization-induced Ca^{2+} release (DICR) (Schneider 1994). In skeletal muscle, T-tubule invaginates at the specific sites to form "triad" structure with the terminal cisternae (TC) of the SR on the both sides (Franzini-Armstrong and Protasi 1997) (Fig. 2a). At the triad, four skeletal muscle-type DHPRs (containing $Ca_v1.1$ as the pore-forming subunit) assemble into "tetrad" and are closely apposed in precise register to the alternate RyR1 tetramers (feet) which are aligned in two rows on each junctional face of the TC (Fig. 2b). The current view of DICR is that depolarization of T-tubule induces the conformation change in the **dihydropyridine receptor** (DHPR) containing $Ca_v1.1$, which, in turn, activates the coupled RyR1 channels through the protein-protein interaction (Tanabe et al. 1990). Ca^{2+} influx through DHPR is not necessary in DICR. Thus, DHPR serves as the voltage sensor instead of the Ca^{2+} channel. The RyR1-$Ca_v1.1$ interaction cannot be replaced by any other isoforms of RyR or DHPR and are supported at least three proteins: DHPR β1a, stac3, and junctophillin2 (Perni et al. 2017). Studies with chimeric RyRs have proposed that multiple regions within the cytoplasmic assembly may be involved in this interaction.

In the skeletal muscle, RyR1 can also mediate CICR. Therefore, it may reasonably be hypothesized that Ca^{2+} released from the RyR1 coupled with DHPR might induce CICR via the uncoupled RyR1 in physiological **twitch** or **tetanus**. The rate of CICR estimated under physiological condition, however, is much slower than the rate of DICR, suggesting only minor contribution of CICR to the physiological muscle **contraction** (Murayama and Kurebayashi 2011). Enhanced CICR is the pathogenesis of some muscle diseases (see Disease).

E-C Coupling in Heart

In E-C coupling in the heart, the RyR2 channel releases Ca^{2+} from the SR on depolarization of the plasma membrane or T-tubules by CICR mechanisms (Bers 2008). The cardiac junctional SR forms dyad structure (similar to triad structure in the skeletal muscle) with the plasma membrane or T-tubule, where a cluster of L-type voltage-dependent Ca^{2+} channel (cardiac **dihydropyridine**



Ryanodine Receptor, Fig. 2 Schematic drawings of mammalian skeletal muscle (a) and characteristic configuration of the components essential for E-C coupling in the triad (b). A, the T-tubule invaginates at the A-I junction to form triad structure with terminal cisternae (TC) of the SR. LT, longitudinal tubule of the SR. Modified from Fawcett (1994) Bloom and Fawcett a "Textbook of Histology" (12th ed., Chapman & Hall, New York and London), p. 278. B, at the triad, two rows of feet (RyR1 tetramers) are aligned in the junctional face of the TC, and tetrads (four DHPRs) in T-tubules are located in precise register with opposite alternate feet. Modified from Block et al. (1988) J Cell Biol 107: 2587–2600

receptor (DHPR) containing $Ca_v1.2$) is closely apposed to a cluster of RyR2 in the SR, although there is no direct association between individual RyR2 and DHPR molecules (Franzini-Armstrong and Protasi 1997). RyR2 is activated by Ca^{2+} which enters via the Ca^{2+} channel on depolarization. "**Ca^{2+} sparks**" are thought to be an elementary event of the Ca^{2+} release in which multiple RyR2 channels in a cluster open synchronously (Lederer et al. 2004). In the Ca^{2+} overloaded muscles, Ca^{2+} release via RyR2 may occur spontaneously without Ca^{2+} influx and propagate in the cell as **Ca^{2+} waves** (Bers 2008).

Similar Ca^{2+} release mechanism operates also in smooth muscles, neurons, and some peripheral tissues.

Diseases

Genetic mutations in RyR1 are linked to some cases of **malignant hyperthermia** (MH), **central core disease** (CCD), and multiminicore disease (MmD) (Dirksen and Avila 2002; Riazi et al. 2018), whereas mutations in RyR2 are associated with arrhythmogenic diseases such as **catecholaminergic polymorphic ventricular tachycardia** (CPVT1), arrhythmogenic right ventricular cardiomyopathy (ARVC2), left ventricular noncompaction (LVNC), long QT syndrome (LQTS), and idiopathic ventricular fibrillation (IVF) (Priori and Chen 2011). More than 300 mutations have been identified for each of RyR1 and RyR2. Several mutations in RyR3 are reported to be linked with schizophrenia and hyperinsulinism.

These mutations may alter the Ca^{2+} release channel functions. MH and some CCD mutations in RyR1 cause gain-of-function effect, that is, increased CICR activity, whereas most CCD mutations lead to loss-of-function of the channel (referred to as E-C uncoupling) (Dirksen and Avila 2002). CPVT and LVNC mutations in RyR2 cause gain-of-function effects, whereas

RyR2 mutations linked to LQTS and IVF include both gain-of-function and loss-of-function mutations (Priori and Chen 2011).

In addition to genetic mutations, post-tlanslational modifications of RyRs such as phosphorylation or redox modifications have been reported to destabilize the channel and result in a pathological Ca^{2+} leak from the SR in skeletal and cardiac muscles (Bers 2014; Denniss et al. 2018; Kushnir and Marks 2010).

Drugs

Ryanodine

Ryanodine is a neutral plant alkaloid from *Ryania speciosa*. Ryanodine binds specifically to the pore of open RyR channel with a high affinity (K_D ~nM) and locks the channel to a subconductance open state (Meissner 2017; Ogawa 1994). The binding of [^{3}H]ryanodine is used as a quantitative measure for the RyR channel activity (Murayama and Kurebayashi 2011).

Caffeine

Caffeine is a strong activator of CICR: it greatly sensitizes RyR to activating Ca^{2+} and increases the maximum attainable level at mM range (Meissner 2017; Ogawa 1994). Caffeine binds at the interface between central, S2S3, and CTD (Ogawa et al. 2020; Zalk and Marks 2017) (Fig. 1c and d). Caffeine-binding site normally reduces the Ca^{2+} sensitivity through interaction between tryptophan in S2S3 and isoleucine in CTD. Caffeine binds to the tryptophan to break the interaction, resulting in increased Ca^{2+} sensitivity (Ogawa et al. 2020). Caffeine is frequently used for experimental evaluation of functional occurrence of RyRs. It is also used for diagnosis of MH: muscles biopsied from MH patients contract with a lower dose of caffeine than normal subjects, due to an enhanced CICR activity (Riazi et al. 2018) (see Disease).

4-Chloro-m-cresol (4-CmC)

4-CmC, a preservative in commercial preparations of some intravenous drugs, activates CICR in a way similar to that of caffeine (Meissner 2017). 4-CmC is more potent than caffeine and shows isoform-dependent activation profiles: it is much less effective in RyR3 than RyR1 or RyR2.

Inhalational Anesthetics

Inhalational anesthetics, such as halothane and isoflurane, stimulate CICR in RyR1 (Endo 2009). Administration of these agents to carriers of MH mutations in the *RYR1* gene will cause the MH episodes (Riazi et al. 2018).

FK506 and Rapamycin

These immunosuppressants increase the activity of RyR1 by deprivation of FKBP12 (Fill and Copello 2002). Their effect on RyR2 is controversial.

Dantrolene

Dantrolene is an antidote for MH which inhibits the CICR activity (Endo 2009). Dantrolene strongly inhibits RyR1 but is much less effective on RyR2. Action of dantrolene is temperature-dependent (more effective at 37 °C than at 25 °C).

Procaine and Tetracaine

Procaine and tetracaine, local anesthetics that block sodium channels, strongly inhibit all the RyR isoforms at mM range (Endo 2009; Meissner 2017; Ogawa 1994). Tetracaine is more potent than procaine.

Ruthenium Red

Ruthenium red inhibits all the RyR isoforms at μM range (Meissner 2017; Ogawa 1994). The site of ruthenium red action seems to be near or within ion channels.

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S

S100 Proteins

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Synonyms

Allergy; Biomarkers; Calcium-binding; Cancer; Cardiomyopathy; Clinical chemistry; Diagnostics; Drug targets; EF-hand; Head injury; Inflammation; Laboratory medicine; Zinc-binding

Definition

S100 proteins are small acidic proteins and are characterized by two distinct EF-hand calcium-binding motifs with different affinities. They also bind zinc, copper, and iron at positions distinct from the Ca^{2+}-binding sites resulting in modulation of their activities. Most S100 genes are organized in a gene cluster on human chromosome 1q21, the basis of the S100 nomenclature approved by the HUGO nomenclature committee (Marenholz et al. 2004). Today, members of the S100 protein family are routinely used in Practical Laboratory Medicine to analyze and monitor disease conditions and are important diagnostic and prognostic biomarkers (Heizmann 2019a, b).

Basic Characteristics

S100 proteins constitute the largest subgroup within the EF-hand protein family. They are involved in many cellular activities (Marenholz et al. 2004; Heizmann 2019a, b; Leclerc and Heizmann 2011; Donato et al. 2002) and are mostly in the form of homo- or hetero-dimers but can also form tetramers and hexamers (Moroz et al. 2002; Ostendorp et al. 2007; Spratt et al. 2019). The crystal structures of S100Z and of other S100 proteins have recently been reviewed (Calderone et al. 2019).

S100A3 is a unique member of this protein family. It binds Ca^{2+} with poor but Zn^{2+} with an exceptionally high affinity. The crystal structure of S100A3 is shown in Fig. 1 and described in detail (Fritz et al. 2002). Molecular genetic investigations, performed on patients with pulmonary fibrosis (one of the leading indications for lung transplantation), revealed a hypomorphic variant in *S100A3* and a novel truncated mutation in *S100A13,* both segregating with the disease in an autosomal manner (Al-Mutairy et al. 2019). These were the first mutations detected in any of the S100 protein family members and suggest a calcium- or zinc-dependent therapeutic approach for the management of this disease of unknown etiology.

Besides their distinct intracellular functions, certain S100 proteins are secreted from cells and exert cytokine-like functions through the binding

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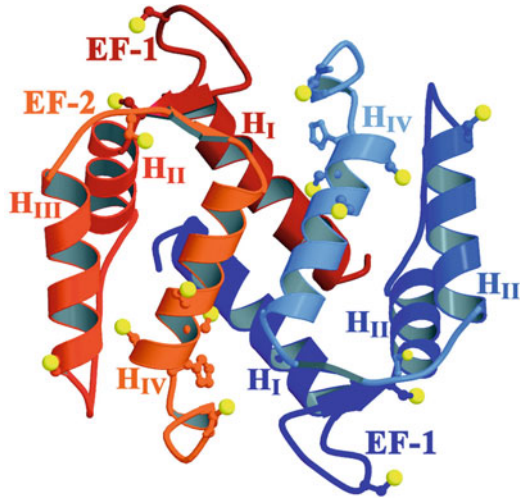
https://doi.org/10.1007/978-3-030-57401-7



S100 Proteins, Fig. 1 Overall structure of the Ca^{2+}-free S100A3 dimer. The subunits are shown in *red* and *blue,* respectively. The N termini are shown in dark colors and the C termini in bright colors. The Cys and His residues are shown as a *ball* and *stick* model. This research was originally published in the *Journal of Biological Chemistry*, G. Fritz et al. (2002) The Crystal Structure of Metal-free Human EF-Hand Protein S100A3 at 1.7-Å Resolution 277, 33092-33098 ©the American Society for Biochemistry and Molecular Biology

and activation of cell surface receptors such as the Receptor for Advanced Glycation End Products (RAGE), Toll-like Receptor 4 (TLR-4), the ErbB4 receptor, the dopamine D2 receptor, amyloid-β, and annexins (Marenholz et al. 2004; Heizmann 2019a, b; Leclerc and Heizmann 2011; Donato et al. 2002; Moroz et al. 2002; Ostendorp et al. 2007; Spratt et al. 2019).

Clinical Importance of S100 Proteins in Human Diseases

Cardiology

S100A1 is predominantly expressed in the heart and a decreased cardiac expression of S00A1 is characteristic of heart failure. S100A1 gene transfer to restore S100A1 protein levels in the failing myocardium is suggested as a future therapeutic strategy to support the injured heart and to reverse or prevent heart failure (Pleger et al. 2007).

S100A2 has the potential to differentially influence Ca^{2+}-cycling and contractility of cardiomyocytes. Therefore, S100A2 and S100A4, in addition to S100A1, may be further candidates for a differentiated heart failure therapy promoting survival of cardiac myocytes (Wang et al. 2014).

Oncology

S100 proteins associated with cancer progression, diagnosis, and treatment are listed in Table 1.

S100B is the best-studied melanoma biomarker incorporated into the American Joint Committee on Cancer (AJCC) melanoma staging system. S100B is routinely analyzed in practical laboratory medicine and elevated S100B levels closely correlate with a poor survival rate.

S100A2-A6. A prognostic significance of S100A2 in laryngeal squamous-cell carcinoma allows discrimination of high-and low-risk patients in the lymph node-negative subgroup for a better adjusted therapy. S100A2 has been found to be a marker for early stages of lung carcinogenesis.

S100A4 (metastasin), a key player in tumor progression and metastasis, is by far the best studied and prominent member of the S100 protein family. The importance of S100A4 in cellular invasion and metastasis is exemplified by its presence in a great variety of different types of cancer, as listed in Table 1. S100A4 can also act via an extracellular route, as an angiogenic factor inducing tumor progression. Inhibiting the process of tumor angiogenesis is a possibility by blocking either S100A4 secretion or its extracellular binding to targets/receptors.

S100A7 (psoriasin) and the homologous protein *S100A15* (koebnerisin) in psoriatric keratinocytes are associated with several malignant tumors.

S100A8/A9 proteins (calprotectin/Mrp8 and Mrp 14) are involved in different types of cancer. Their expression and proposed cytokine-like functions indicate key roles in inflammation-associated cancer and represent promising biomarkers to evaluate the risk potential of various types of cancer in molecular pathology.

S100A11 may become important for the diagnosis and treatment of ovarian cancer.

S100A13 was identified in thyroid tumors and its growth and invasion. A proteomics analysis of cutaneous malignant melanoma (CMM) revealed an association between the expression of

S100 Proteins, Table 1 S100 proteins in oncology

Proteins	Cancer association
S100B	Melanoma metastasis
S100A1	Ovarian cancer
S100A2	Lung carcinogenesis, laryngeal squamous-cell carcinoma
S100A2-A6	Classification of brain tumors
S100A3	Hepatocellular carcinoma, gastric cancer
S100A4/metastasin	Colon, rectal, gastric, ovarian, breast cancers; melanoma; oral squamous cell carcinomas
S100A7/psoriasin	Ovarian, cervical, pancreatic cancers
S100A8/A9/calprotectin	Inflammation-associated cancer, breast, colorectal cancers; ALL[a]; CLL[b]; AML[c]; cutaneous squamous, laryngeal, hepatocellular carcinoma, head, neck, bladder cancer
S100A11	Breast, ovarian, pancreatic cancers
S100A13	Thyroid tumors, cutaneous malignant melanoma
S100A14	Cervical, breast, gastric cancers
S100A16	Colorectal, breast cancers
S100 P	Breast cancer, pancreatic ductal adenocarcinoma, nasopharyngeal carcinoma

[a]Acute lymphoblastic leukemia
[b]Chronic lymphocytic leukemia
[c]Acute myeloid leukemia

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S100A13 and chemotherapy resistance. CMM is commonly unresponsive to standard dacarbazine (DTIC) and temozolomide (TMZ) chemotherapy, and the use of S100A13 as a predictive marker of therapy response would be of great clinical help.

S100A14 is overexpressed in several cancer tissues regulating proliferation, migration, and invasion and suppressing metastasis (e.g., in gastric cancer).

S100P is expressed in many pancreatic tumors, associated with growth and aggressiveness of this cancer type.

Inflammation, Autoimmune Diseases and Allergies

S100B is correlated with active vitiligo depigmentation (Birlea 2017), the most common depigmenting disorder. Serum levels of S100B were increased in patients with active non-segmental vitiligo and associated with disease progression. These findings imply that S100B may be a biomarker for this disease and a new target for treatment.

S100A4/metastasin is implicated in metastatic tumor progression and chronic inflammation. S100A4 induced the expression of acute-phase responsive proteins, serum amyloid A (SAA1), and SAA3 proteins and cytokines in an organ-specific manner. SAA proteins in connection with S100A4 serve as a link between inflammation and tumor progression (Hansen et al. 2014; Ambartsumian et al. 2019). S100A4 was also identified as a candidate gene in allergy. Treatment with an anti-S100A4 antibody resulted in decreased signs of allergy in a mouse model and in allergen-challenged T cells from allergic patients. This strategy may be generally applicable to identify diagnostic and therapeutic candidate genes in allergy.

S100A7/psoriasin, localized in epithelial cells, regulates cell proliferation and differentiation. An increase of S100A7 expression is found in response to inflammatory stimuli, such as in psoriasis (a chronic inflammatory autoimmune-mediated skin disease). This suggests that S100A7 may be a therapeutic target in psoriasis (D'Amico et al. 2016). Serum levels of S100A7 and S100A15 are potential markers of artherosclerosis in patients with psoriasis (Awad et al. 2018).

S100A8 and S100A9 proteins are characterized by a unique expression pattern with strong prevalence in cells of myeloid origin and are associated with a number of chronic inflammatory diseases (Holzinger et al. 2019). Sensitive and automated ELISA calprotectin (S100A8/A9) assays are routinely used in Clinical Chemistry Laboratories to diagnose the two main forms of inflammatory bowel diseases, Crohn's disease and Ulcerative colitis.

S100A11 (calgizzarin) was associated with rheumatoid- and osteoarthritis and is an additional biomarker to S100A/A9 and S100A12 for a more tailored diagnosis of inflammatory diseases.

S100A12/calgranulin C is associated with the Kawasaki disease and Mooren's ulcer as well as atherosclerosis.

Brain Injury and Neuropathologies

S100 proteins were first described as small acidic proteins in a 100% saturated ammonium solution of a brain extract. This extract mainly contained the S100A1 and S100B proteins. S100B represent 0.2% of total brain proteins, mainly synthesized by astrocytes, oligodendrocytes, and Schwann cells. S100 protein members (mainly S100B) are associated with neurodegenerative diseases such as Alzheimer´s disease, Down syndrome and in some psychiatric disorders such as schizophrenia (Table 2) and are routinely used in Practical Laboratory Medicine as neurobiomarkers for head injuries of children and adults.

Drugs

Developing inhibitors against S100B as an antagonist (Khan et al. 2018; Syed et al. 2018) to block the interaction with RAGE is a promising goal for future pharmaceutical interventions and cancer treatment. Targeting the S100A7/A15-receptor axis is suggested as a new targeted strategy for preventing the invasion and metastasis of these types of cancer. Another therapeutic strategy is using neutralizing monoclonal antibodies against S100A7 for cancer treatment. As a consequence

S100 Proteins, Table 2 S100 proteins in brain disorders

Proteins	Brain pathologies
S100B	Neurobiomarker for head injury in high-risk fetuses, newborns, children, and adults biomarker in asphyxiated infants treated with hypothermia intracerebral hemorrhage and ischemic stroke Alzheimer's disease, multiple sclerosis, psychiatric disorders (schizophrenia) acute cerebrovascular insults, seizures, delirium glyphosate and glufosinate poisoning pesticide-induced neurotoxicity
S100A4	Axonal regeneration, neuronal plasticity
S100A6	Amyotrophic lateral sclerosis
S100A7/ A15	Antimicrobial proteins in the CNS
S100A10 (p11)	Biomarker for monitoring the severity of Parkinson's disease

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of the various proinflammatory properties of S100A8/A9 proteins, strategies targeting these molecules are an option for anti-inflammatory therapies, by in vivo administration of S100 antibodies, or by therapeutic drug targeting to inhibit the release of these cytokine-like molecules at sites of inflammation (Vogl et al. 2018).

The antiallergic drug cromolyn binds to S100P preventing RAGE activation inhibiting pancreatic tumor growth and metastasis in animal models. Cytotoxic treatment using cromolyn in combination with gemcitabine or applying a small RAGE antagonistic peptide appears to be a promising to treat pancreatic and possibly other types of cancer. Covalent small molecule inhibitors of S100B have been developed that inhibit S10B-p53 complex and restore active p53 in malignant melanoma (Cavalier et al. 2014). A similar approach was to target the estrogen receptor/S100 interface by a short peptide derived from S100P in order to reduce regression of tumor growth receptor positive breast cancer (Lee et al. 2016; Cho et al. 2016).

Outlook

Today, members of the S100 protein family are routinely used in Practical Laboratory Medicine to analyze and monitor disease conditions and are important diagnostic and prognostic biomarkers. S100 proteins and their targets/receptors are also considered as drug targets for future treatments. So far, only a small number of S100 proteins have been explored for their clinical relevance although more and more human diseases (Tables 1 and 2),

as well as, for example, obesity (Riuzzi et al. 2019) and the sexually-transmitted infection gonorrhea (Maurakis et al. 2019) are linked to S100 proteins. Methods of choice for a simultaneous analysis of Ca^{2+}-binding proteins and their post-translational modifications are the mass spectrometry and the focal molography (a next-generation biosensor that visualizes specific biomolecular interactions in real time). These techniques will have a future impact on the development of point-of-care diagnostic devices applied for analyses of the Ca^{2+}-binding proteins in human diseases.

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SAR-by-NMR

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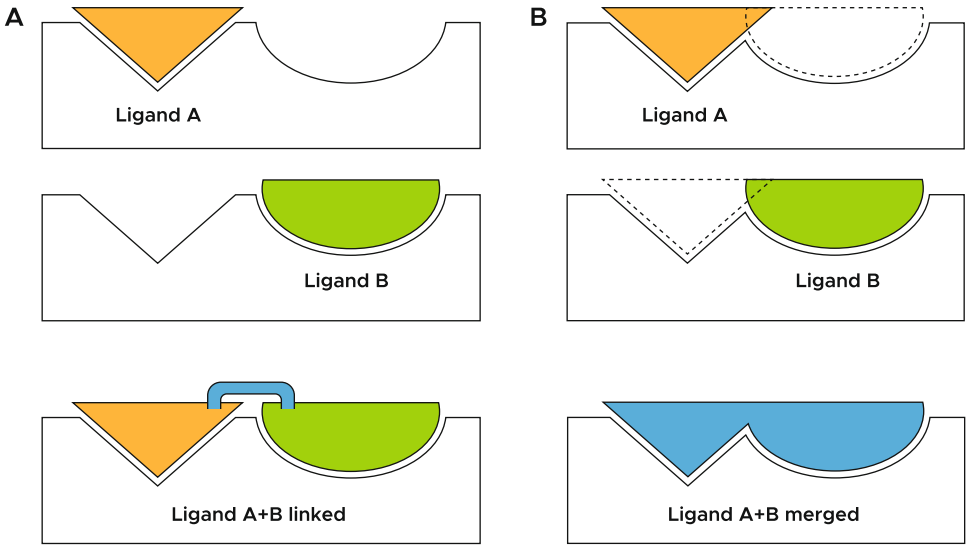
Synonyms

Fragment-based drug discovery; Lead discovery by NMR

Definition

Structure-activity-relationships (SAR)-by-NMR (Shuker et al. 1996) is originally the appellation of a method for generating systematically high-affinity small molecular weight protein ligands from small molecular fragments in the early stages of drug development. NMR is used for detecting weak protein-ligand interactions via chemical shift changes, usually monitored by $^{1}H–^{15}N$ correlation spectroscopy for amide proton and nitrogen signals (Shuker et al. 1996). Such chemical shift perturbations of individual backbone amide signals indicate also the protein binding site. The three-dimensional structure of a protein-ligand complex involving two ligands A and B simultaneously binding at adjacent sites is the basis for the design of a linker between the two fragments (Fig. 1a). This "linking" strategy produces a much tighter binding ligand from weakly binding fragments due to the saving of one entropic factor. In this procedure, protein-ligand interactions are detected via the protein NMR resonances, enabling the measurement or estimation of binding constants and a mapping of the binding site to detect suitably different locations for ligands to be linked.

Over time, SAR-by-NMR became an important element of fragment-based drug design approaches (Erlanson et al. 2016; Ribeiro de Souza Neto et al. 2020), in particular since low-affinity binding in the μM–mM range (as mostly occurring for fragments with a molecular weight between 150 and 300 Da) can be easily detected without creating too many "false positives." It is usually applied in concert with other biophysical techniques including structure determination methods. In this context, different experimental NMR approaches are also often used, detecting effects on the ligand NMR



SAR-by-NMR, Fig. 1 Schematics of SAR-by-NMR linking and merging procedures

spectrum (ligand-observed) rather than site-specific chemical shift changes of protein signals (protein-observed) (Gossert and Jahnke 2016). NMR techniques such as the saturation transfer difference (STD) and waterLOGSY experiments (Gossert and Jahnke 2016) observe ligand resonances to qualitatively detect binding, useful in screening campaigns or for hit validation. The path toward higher affinity is also often modified. Since the linking procedure is not always feasible, fragments A and B with overlapping binding sites may be improved by "merging" as depicted in Fig. 1b. In practice, individual fragments are often developed further by "growing," adding stepwise functional groups.

Basic Characteristics

The original SAR-by-NMR procedure (Fig. 1a) included searches for two fragment-like compounds (MW<250 Da) that bind close to each other near the active center and the structure-guided design of a linker to produce a tighter binding ligand (Shuker et al. 1996), characterized by the following steps:

- Screening of a compound library consisting of fragments in the molecular weight range of 150–250 Da to detect compounds that bind close to the active site of the protein, using correlation spectroscopy for detection
- Screening of derivatives of the initial hits, optionally supported by synthesis of a focused library yielding such derivatives to obtain compounds with improved affinity
- Screening for a second compound that binds in the vicinity of the first one using the same procedure
- Determination of a three-dimensional protein structure with both ligands bound
- Design of a linker for both ligands and synthesis of this compound
- Further improvement by synthesis of derivatives on the basis of the design

Key to the scheme above is the structure-based design of a linker. With its effect accounted for by ΔG_{link}, linking two fragments A and B produces a gain in affinity described by:

$$\Delta G_{AB} = \Delta G_A + \Delta G_B + \Delta G_{link}$$

The binding constant of the compound AB obtained after linking is then:

$$K_d(AB) = K_d(A) \times K_d(B) \times L,$$

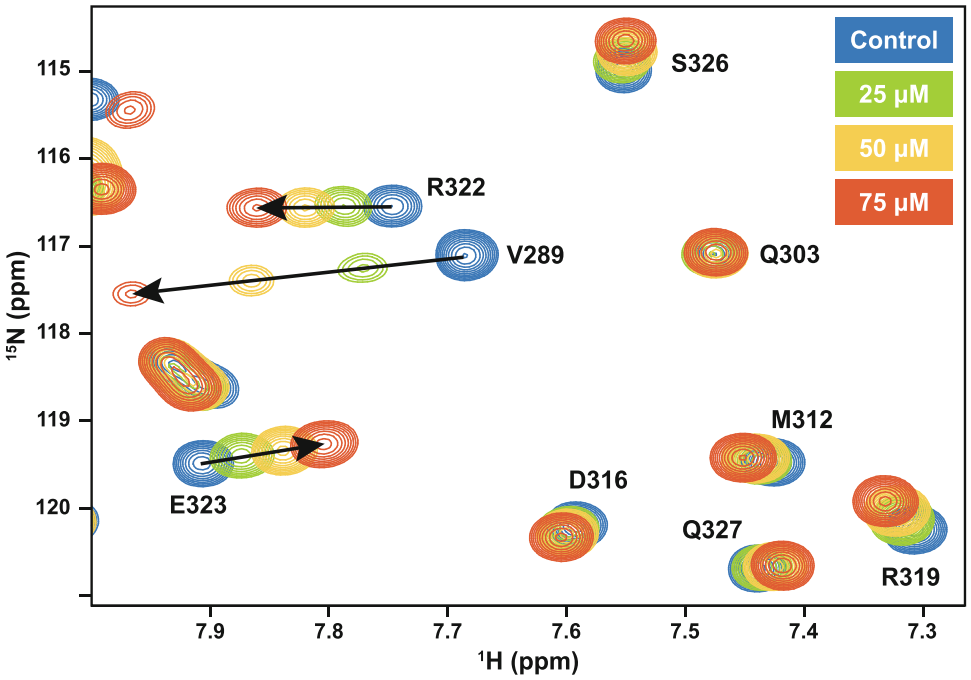
where L is the linking coefficient derived from ΔG_{link}.

In its modified forms (merging, growing), the first two steps of the original procedure are applied to obtain good starting points for choosing larger or merged compounds.

In current applications, between 500 and 10.000 substances are screened to find ligands binding in the mM–nM range. Often, a pre-selection by virtual screening approaches based on existing structural information is applied.

The methodology is often applied in a modified manner. Three-dimensional structures of complexes may also be obtained by X-ray crystallography or by molecular modelling making use of the detected chemical shift changes. The latter is often sufficient when individual hits of the screening phase are stepwise extended toward a tight-binding one. Due to the weak binding of fragments, and possible crystal packing effects, X-ray structures of complexes are sometimes difficult to obtain, requesting alternative methods.

An example for the initial NMR screening step is shown in Fig. 2. A region of a ^{1}H-^{15}N heteronuclear single quantum correlation (HSQC) shows the overlay of four different spectra obtained on ^{15}N-labelled dishevelled PDZ domain samples with four different ligand concentrations (0 mM, 25 mM, 50 mM, and 75 mM). The black NH signals are characteristic for the free protein, and the red signal is close to the chemical shift of the same signal from the complex. Only for a protein signal from an amino acid residue in the binding site, a journey from the signal position of the free to the equivalent of the liganded form may be observed (see R322, V289, and E323). For all other residues, signals in the four different spectra superimpose well within



SAR-by-NMR, Fig. 2 Superposition of regions from ^{1}H–^{15}N correlation spectra of the PDZ domain from the protein dishevelled obtained at different ligand concentrations. Chemical shift changes are indicated by arrows. Each cross peak originates from one particular amide group, as indicated by the amino acid one-letter code and its position in the protein sequence

certain limits, indicating that the chemical environment of the amide protons and nitrogen atoms did not change much through binding of the ligand or induced long-range structural effects.

In the example above, the effects of ligand binding were observed as gradual, concentration-dependent ^{1}H and ^{15}N chemical shift changes which are usually true for weak interactions with small molecular fragments below 300 Da. In general, two different effects may be observed depending on the binding constants and the chemical shift difference between the monitored signal of the complex and the signal of the free protein. At low fragment affinity, one travelling signal is observed that represents the weighted average of the chemical shifts of complex and free form of the protein. In the case of tight binding (nanomolar range and below) and large chemical shift differences, the signal of the free protein may decrease with increasing ligand concentration, and the signal of the complex will appear at its genuine chemical shift. These situations are called "fast exchange" (for the travelling signal) and "slow exchange" regimes. For intermediate cases, the signal may vanish at a certain point, called "coalescence."

The chemical shift changes observed in correlation spectra (the length of the vector in Fig. 2) may be taken directly as a measure of affinity differences when the effects of several ligands are compared. Such chemical shift (δ) changes (Δδ) are then quantified as a linear combination of proton (δ_H) and nitrogen (δ_N) shift contributions (Bertini et al. 2011).

$$\Delta\delta = \sqrt{\frac{(\Delta\delta_H)^2 + (\Delta\delta_N/5)^2}{2}}$$

This provides also a basis for the measurement of binding constants. The dissociation constant may be determined by fitting the observed chemical shift change with increasing ligand concentration, up to very large excess (Gossert and Jahnke 2016).

Early examples of the application of SAR-by-NMR include the design of stromelysin and human papillomavirus E2 protein inhibitors (Hajduk et al. 1997a, b).

A detailed analysis of designed inhibitors for stromelysin by thermodynamical methods (Hajduk et al. 1997a) showed that the combination of two ligands, a biphenyl derivative and acetohydroxamic acid, yield an increase in the enthalpic contributions to the binding energy, whereas the entropic factor remained constant. In the application of the method to the design of ligands for the virus E2 protein (Hajduk et al. 1997b), the potential of NMR to detect low-affinity binding was exploited by starting a ligand refinement from compounds that bound in the millimolar range and combining features of two compounds to one which finally showed an IC_{50} of 10 micromolar in the applied test.

With fragment-based drug design developing into a coherent methodology, making use of many techniques, NMR contributes in several aspects but dominantly with its feature of detecting low-affinity binding and concomitantly the binding site. This is outlined in an analysis of a large number of fragment-based drug design studies by Mortenson et al. and Erlanson et al. for the years 2017 and 2018, respectively (Mortenson et al. 2019; Erlanson et al. 2020).

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Scaffold

► Adaptor Proteins

Second-Generation Antipsychotics

► Atypical Antipsychotics

Sedation

► GABAergic System

sEH

► Soluble Epoxide Hydrolase

Seminiferous Epithelial Barrier

► Blood-Testis Barrier

Senescence

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Synonyms

Cellular senescence

Definition

The term senescence originates from the Latin word *senesce*, meaning "to grow old," and was used for many years to allocate both cellular and organismal senescence (aging). Nowadays it is well established that at the systematic level, senescence is a hallmark of aging, the latter representing the gradual functional decline of proper organismal processes with age. At the cellular level, the terms "senescent" and "aged" are not identical and should not be used interchangeably (López-Otín et al. 2013; Gorgoulis et al. 2019, Table 1). According to the recently proposed consensus definition by the International Cell Senescence Association (ICSA), senescence (from now on used for cellular senescence) is ***a cellular state characterized by prolonged and generally irreversible cell-cycle arrest with secretory features, macromolecular damage, and altered metabolism that is triggered by stressful insults and in certain instances by physiological processes*** (Gorgoulis et al. 2019).

Basic Mechanisms

Inducers

Any stressor that challenges and compromises cellular homeostasis – particularly genome and/or proteome integrity – can potentially induce the senescent phenotype, if the intensity and the duration of the noxious (yet still sublethal) stimuli exceed a specific threshold which overcomes cell's repair capacity (Gorgoulis et al. 2018, 2019). So far myriads of intrinsic and extrinsic senescence inducers have been reported, including, but not limited to, telomere erosion/dysfunction, diverse genotoxic insults, oncogenes, epigenetic modifiers, and oxidative stress/metabolic imbalance stemming from proper protein turnover impairment, mitochondrial dysfunction, and ribosomal and endoplasmic reticulum stress (Gorgoulis et al. 2018, 2019; Myrianthopoulos et al. 2019).

Hallmarks, Molecular Pathways, Markers

Senescence is appreciated as a stress response mechanism characterized by the following **main** interconnected hallmarks: **(1)** ***prolonged and generally irreversible cell-cycle arrest***, mediated predominantly by two signaling pathways that act in a temporal manner: that of p53-p21$^{WAF1/Cip1}$ and Rb-p16^{INK4a} (Gorgoulis et al. 2018, 2019; Myrianthopoulos et al. 2019). The senescence-associated cell cycle withdrawal is not universally an irreversible program as it is currently well established that in certain settings senescent cells can "escape" from the arrested state and re-enter the cell cycle (Gorgoulis et al. 2019; Myrianthopoulos et al. 2019; Galanos et al. 2016; Komseli et al. 2018). **(2)** autocrine or paracrine **secretory properties** termed ***senescence associated secretory phenotype (SASP)***. The senescence secretome consists of cytokines, chemokines, growth factors, proteases, and other factors, depending on the nature of the stressor, cell type, and senescence endurance (Gorgoulis et al. 2019; Myrianthopoulos et al. 2019). This feature exerts immunomodulatory functions that affect the tissue microenvironment and is controlled by various signalling cascades [e.g., mammalian target of rapamycin (mTOR), mitogen-

Senescence, Table 1 Differences between senescence and aging

Senescence	Aging
Well-orchestrated response	Unprogrammed, random process
Although the initial factors which trigger senescence (e.g., DNA damage) may itself be random, the accompanying cellular changes associated with cell senescence are not random, but a rather orchestrated response	Results from the accumulation of random(stochastic) damage leading to impairment in cell function with time
Senescence occurs throughout the lifespan, including during embryogenesis. Cells of any age can undergo senescence	Time (age)-dependent functional decline
Can be triggered not only by telomere attrition (which is definitely related to age), but also by nontelomeric, not aging-associated, acute genotoxic insults (stress-induced senescence)	Telomere attrition is a hallmark of aging, but other acute stimuli cannot elicit a specific aged cell phenotype
Cell-cycle arrested in G1, G1/S, or G2, depending on the activated checkpoint	May not be cell-cycle arrested??
It can be considered as a "sudden" phenomenon meaning that whatever the trigger (telomeric or nontelomeric) or the duration of the phenomenon, the establishment of the cell-cycle arrest coupled to phenotypic modifications is a rather immediate response, induced within relatively short periods of times	The loss of physiological cellular integrity that characterizes aged cell is gradual and progressive, not an "on-off" phenomenon
Both detrimental and beneficial depending on the biological context	Detrimental to the function of normal biological processes
Reversible ("escape" from senescence)	Terminal, permanent, nonreversible phenomenon (regeneration?)
Hyper-secretory phenotype, hyperfunction	–
SA-β-gal positive	?

activated protein kinase (MAPK)-p38, phosphoinositide 3 kinase (PI3K)/AKT, Notch1, and NOTCH/JAG1], eventually leading to NF-κB and CCAAT/enhancer binding protein beta (C/EBPβ) transcriptional upregulation. Cytoplasmic bridges and extracellular vesicles are also involved (Gorgoulis et al. 2019; Myrianthopoulos et al. 2019). **(3)** ***macromolecular damage***, in the form of DNA, protein, and lipid damage, as a result of decline in quality control and damaged turnover mechanisms, commonly evident in the stressful environment of senescence (Gorgoulis et al. 2019; Myrianthopoulos et al. 2019). Of note, macromolecular damage is a unique feature of senescent cells allowing their distinction from other non-dividing cells, such as quiescent or terminally differentiated cells (Gorgoulis et al. 2019). DNA damage, mainly in the form of persistent DNA damage foci, is the most well-established type of macromolecular damage occurring in most types of senescence (for instance, in developmental senescence DNA damage and DDR are absent). Persistent DNA damage foci include irreparable lesions principally related to telomeric DNA (TIFs, telomere dysfunction-induced foci; TAFs, telomere-associated foci) and DNA-SCARSs (DNA segments with chromatin alterations reinforcing senescence). DNA damage elicits the activation of the DNA damage response pathway (DDR), which in the case of oncogene-induced senescence (OIS) cooperates with members of the *CDKN2A* locus (*ARF*) to exert anti-tumor functions (Gorgoulis et al. 2018, 2019; Myrianthopoulos et al. 2019; Evangelou et al. 2013; Velimezi et al. 2013). Cytoplasmic chromatin fragments (CCFs), another form of DNA damage in the senescence context, are related to the activation of a proinflammatory reaction by the cGAS-cGAMP-STING network. Regarding protein and lipid damage, they are the result of damaged biomolecule accumulation due to excessive reactive oxygen species (ROS)

production and improper degradation (Gorgoulis et al. 2018, 2019; Myrianthopoulos et al. 2019; Vasileiou et al. 2019). **(4)** ***Altered metabolism***, related to (i) changes in mitochondrial function, dynamics, and morphology. Mitochondria appear abundant but dysfunctional, producing increased ROS levels and (ii) lysosomal aggregation and malfunction, associated with elevated senescence-associated b-galactosidase (SA-b-Gal) activity and intralysosomal accumulation of lipofuscin (Georgakopoulou et al. 2013; Evangelou et al. 2017). Altogether, aggregation within senescent cells of large dysfunctional mitochondria and lysosomes, the latter enriched in lipofuscin granules, is reflected by increased cytoplasmic granularity (Gorgoulis et al. 2019; Vasileiou et al. 2019).

Another potent property of senescent cells is their ***resistance to apoptosis***, and inhibition of the BCL-2 cell death regulator family of proteins has been exploited in senolytic strategies. The BCL-2 family consists of both pro-apoptotic and anti-apoptotic factors. Among anti-apoptotic ones are BCL-2, BCL-XL, BCL-w, BCL-2-related protein A1 BFL-1 (A1), myeloid cell leukemia 1 (MCL-1), and BCL-B/Boo, featuring four BCL-2 homology (BH) domains (BH1, BH2, BH3, and BH4). The pro-apoptotic fraction includes the multi-domain pro-apoptotic effectors, such as BAK and BAX and the so-called "BH3-only proteins" such as BAD, BID, BIK, BIM, BMF, HRK, PUMA, and NOXA, which harbor a short BH3 domain (Gorgoulis et al. 2019; Myrianthopoulos et al. 2019). Depending on the initial stressor/signal and cell type, senescent cells can acquire numerous additional salient cellular and subcellular features (Gorgoulis et al. 2019; Myrianthopoulos et al. 2019) related to: ***(i) morphological aspects***, such as cellular flattening/elongation, irregular shape, and enlargement due to cytoskeleton rearrangements and alterations in plasma membrane composition and increase in nuclear and nucleolar size as well as fragmentation of the nuclei (polyploidy), mitochondria, and endoplasmic reticulum; ***(ii) epigenetic traits*** that include chromatin modifications (e.g., elevated H4K20me3 and H3K9me, H4K16ac, H3K27me3 "canyons," H3K4me3 "mesas," senescence-associated distension of satellites (SADSs), senescence-associated heterochromatin foci (SAFHs), and others), accompanied by characteristic transcriptional signatures, miRNAs, and non-coding RNA expression; and ***(iii) protein markers*** that are either present (DCR2, NKG2D ligands) or absent (for instance, proliferation markers, Lamin B1). For the time being, none of the above-described markers (stemming from features and signaling pathways) is sufficient alone for the identification of senescent cells with absolute specificity. Based on the fact that some markers have a broader spectrum while others depict specific senescence types, the ICSA consortium has proposed a specific multi-marker approach for robust detection (Gorgoulis et al. 2019) that allows accurate estimation of the effectiveness of the anti-senescence strategies.

Physiological and Pathological Implications

Senescence could be metaphorically described as the Greek god Hermathena or the Roman god Janus both facing to two opposite directions depending on the situation. Of clinical importance, short-term induction of senescence has a favorable outcome during development (*developmentally programmed or embryonic senescence*) and in normal processes, like tissue repair *(acute senescence*) (Gorgoulis et al. 2019; Myrianthopoulos et al. 2019). Additionally, cellular senescence plays a crucial role as an anti-tumor barrier at the early stages of tumorigenesis, preventing neoplastic cells from further expansion and propagation (Gorgoulis et al. 2019; Bartkova et al. 2006; Halazonetis et al. 2008; Gorgoulis and Halazonetis 2010). On the other hand, persistence of senescence is associated with harmful properties mediated mainly via paracrine and/or systematic SASP, while escape from OIS is implicated in tumor progression and remission (Galanos et al. 2016; Komseli et al. 2018). Accumulation of senescent cells results from chronic stress damage and/or insufficient clearance by the immune system (*chronic senescence*). As a consequence, not only the regenerative potential and function of tissues/organs are impaired, but also a "slow burning" inflammatory milieu is maintained, triggering the

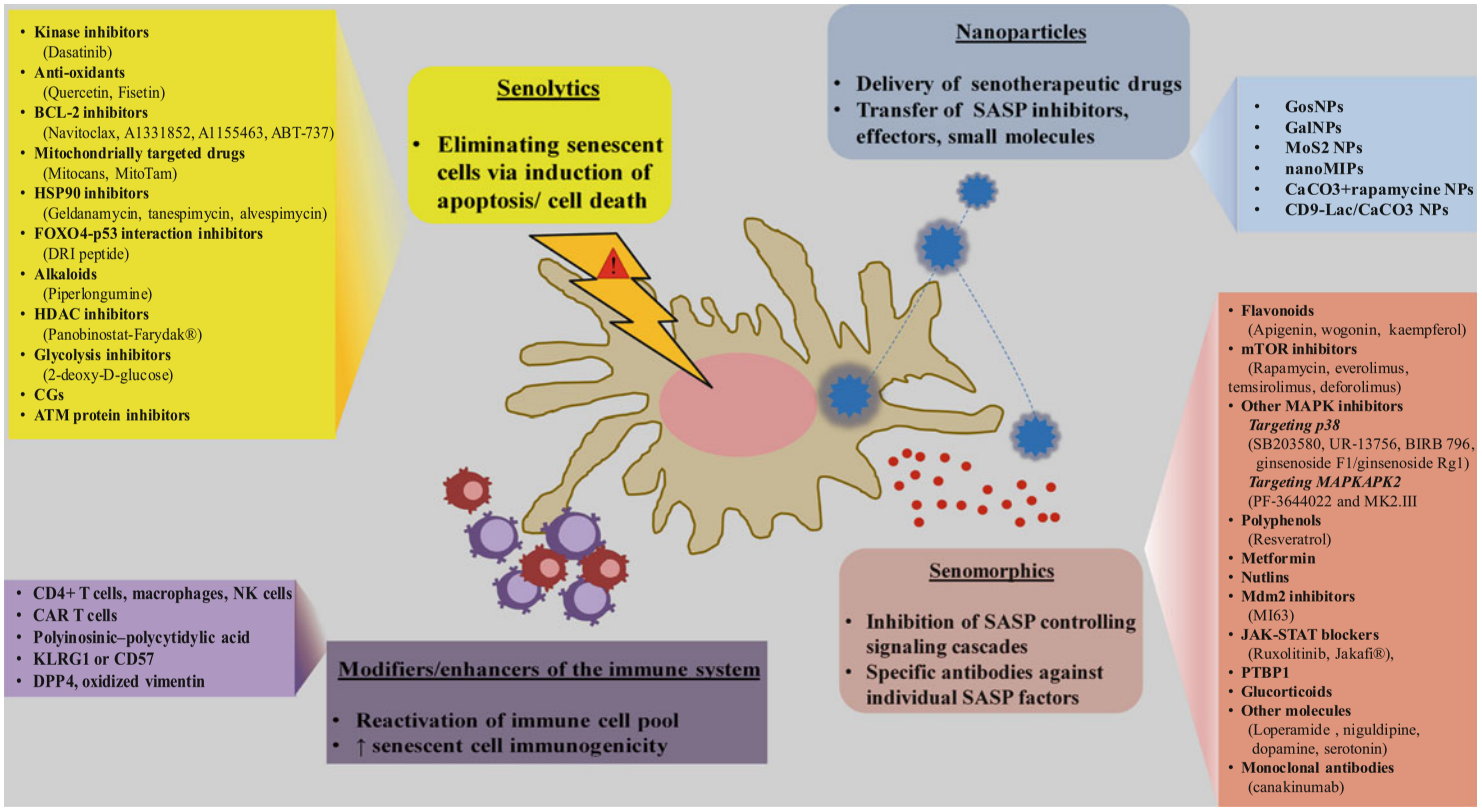
aging process and various pathological conditions such as cardiovascular, kidney, liver, and neurodegenerative diseases, cancer, type 2 diabetes, osteoarthritis, cataract, idiopathic pulmonary fibrosis, metabolic syndrome, and cahexia (Gorgoulis et al. 2019; Myrianthopoulos et al. 2019; Munoz-Espin and Serrano 2014).

Pharmacological (Therapeutic) Intervention

Given the continuously growing knowledge on the wide spectrum of adverse effects of chronic senescence, as already mentioned, the development of new therapies that selectively target senescent cells or neutralize their undesirable properties emerged as an attractive challenge. Research on senotherapeutic agents and their precise mode of action (molecular key players, pathways, and mechanisms, Fig. 1), although quite restricted, is rapidly expanding, and this information is currently exploited for clinical testing (Table 2). This exciting and relatively new field encompassing molecular and chemical biology and pharmaceutical chemistry along with sophisticated methodologies and advanced technologies (e.g., efficient process of "big data" via machine learning) for drug discovery (Sakellaropoulos et al. 2019; Vougas et al. 2019) has already provide aspects of its potential and future therapeutic opportunities. For the time being, the vast majority of available senotherapeutic compounds are either drugs already used for other research and clinical purposes or extensively investigated natural derivatives. Traditional and next-generation, direct or indirect, senotherapies are based on the following classes of molecules: ***(1) senolytics***, ***(2) senomorphics or senostatics*** (SASP inhibitors), ***(3) modifiers/enhancers of the immune system,*** and ***(4) nanoparticles***.

Senolytics

Senolytics are molecules that impede cardinal senescence pro-survival pathways rendering senescent cells vulnerable to cell death. The first described compounds with senolytic properties were dasatinib and quercetin (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019). Dasatinib is a kinase inhibitor that was traditionally used as an anticancer drug. Apart from the activity of various protein tyrosine kinases such as BCR/Abl, c-Kit, and Scr family members, dasatinib blocks Ephrin receptors, as well as promotes apoptosis. These receptors interact with two ligands known as Ephrin 1 (EFNB1) and Ephrin 3 (EFNB3) that have been shown to be upregulated in senescent cells, generating thus critical pro-survival (anti-apoptotic) signals. Quercetin, in turn, is a flavonoid with well-known antioxidant properties that can modulate various molecular cascades such as the NF-kB, PI3K/AKT, mTOR, p53/p21$^{WAF/CIP1}$, serpin, and estrogen receptor signaling pathways (Gorgoulis et al. 2019; Paez-Ribes et al. 2019; von Kobbe 2019). Regarding senolysis by quercetin, inhibition of the PI3Kδ isoform has been favored as the most prevalent mechanism for apoptosis induction. Administration of dasatinib or quercetin has been reported to effectively but variably, depending on the cell type, alleviate aging and senescence *in vitro* and *in vivo* (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019). Interestingly, their combination exerts a broader senolytic outcome and has been demonstrated in clinical trials to effectively reduce the senescent cell "load" in humans (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019; von Kobbe 2019, Table 2). Among other recently demonstrated flavonoids exerting senolytic efficiency is fisetin that behaves similarly to quercetin, as expected by their slight structural differences (hydroxyl group at position 5 of the chromone scaffold). Fisetin affects a variety of cellular functions as well as molecular key players/pathways including topoisomerases; cyclin-dependent kinases; the NF-kB, PI3K/AKT/mTOR, PPAR, and PARP1 signaling pathways; and epigenetic modifiers (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019; von Kobbe 2019). Based on such a multilevel activity, its several beneficial effects, for instance, on cancer prevention and neurodegenerative processes, seem to rely not only on the antioxidant potential but also on their impact on senescence and SASP, a feature that seems valid for the majority of flavonoids in promoting well-being and health.



Senescence, Fig. 1 Schematic overview of direct and indirect senotherapeutic approaches and corresponding drugs/agents currently applied or proposed (for details see text)

Senescence, Table 2 Senotherapeutics in clinical trials

Drug-drug combination	Mechanism of action	Clinical trial Id	Clinical entity under investigation	Clinical trial phase
Senolytics				
Dasatinib + quercetin	PI3K-AKT pathway inhibitor	NCT02848131	Chronic kidney disease	
		NCT02874989	Idiopathic pulmonary fibrosis	
		NCT02652052	Hematopoietic stem cell transplant survivors	
		NCT04063124	Alzheimer's	Phase 1 and 2
Navitoclax + cisplatin and etoposide	Bcl-2 inhibitor	NCT00878449	Small cell lung cancer (SCLC)	Phase 1 and 1/2
Navitoclax + dabrafenib and trametinib	B-RAF inhibitor and MEK inhibitor	NCT01989585	BRAF mutant metastatic melanoma Stage III–IV cutaneous melanoma AJCC v7 Unresectable melanoma	Phase 1 and 1/2
Navitoclax + osimertinib	Tyrosine kinase inhibitor acting on EGFR (T790M + mutation)	NCT02520778	EGFR mutant-positive advanced NSCLC (previously treated with another EGFR inhibitor, including metastatic cancer) Stage III–IV lung non-small cell cancer AJCC v7	Phase 1 and 1/2
Navitoclax + gemcitabine	Nucleoside analogue	NCT00887757	Advanced solid tumors	N/A
Navitoclax + paclitaxel	Microtubule inhibitor (block depolymerization of microtubules)	NCT00891605	Advanced solid tumors	Phase 1
Navitoclax + docetaxel	Microtubule inhibitor (block depolymerization of microtubules)	NCT00888108	Advanced solid tumors	Phase 1
Navitoclax + irinotecan	Topoisomerase 1 inhibitor	NCT01009073	Advanced solid tumors	Phase 1
Navitoclax + erlotinib	Tyrosine kinase inhibitor acting on EGFR	NCT01009073	Advanced solid tumors	Phase 1
Navitoclax + sorafenib	Protein kinase inhibitor of VEGFR, PDGFR, RAF kinases (c-RAF > B-RAF)	NCT02143401	Advanced solid tumors (relapsed or refractory) Metastatic malignant solid neoplasm Recurrent hepatocellular carcinoma Recurrent malignant solid neoplasm Refractory malignant neoplasm Stage IV hepatocellular carcinoma AJCC v7 Unresectable solid neoplasm	Phase 1
Navitoclax + trametinib	MEK inhibitor (MAPK/ERK pathway inhibitors, both MEK1 and MEK2)	NCT02079740	KRAS or NRAS mutation-positive advanced solid tumors (including metastasis) Advanced lung carcinoma Advanced malignant solid neoplasm Advanced pancreatic carcinoma Malignant female reproductive system neoplasm Metastatic malignant solid neoplasm	Phase 1b and 2

(continued)

Senescence, Table 2 (continued)

Drug-drug combination	Mechanism of action	Clinical trial Id	Clinical entity under investigation	Clinical trial phase
			Recurrent lung carcinoma Recurrent malignant solid neoplasm Recurrent pancreatic carcinoma Stage III–IV lung cancer AJCC v7 Stage III–IV pancreatic cancer AJCC v6 and v7 Unresectable malignant solid neoplasm	
Leniolisib (CDZ173)	Selective PI3Kδ inhibitor	NCT02435173	CVID: common variable immunodeficiency KRAS APDS: Activated PI3K-delta syndrome Advanced Lung PASLI: p110 delta activating mutation causing senescent T cells, lymphadenopathy, and immunodeficiency	Phase 2 and 3
		NCT02859727	Extension to the study above (patients with genetically activated PI3Kδ carcinoma-APDS/PASLI)	Phase 2 and 3
Metformin	mTOR inhibitor	NCT03309007	Pre-diabetes in the elderly	Phase 3
Fisetin	PI3K/AKT and ROS inhibitor	NCT04210986	Osteoarthritis, knee	Phase 1 and 2
Senomorphics				
Metformin	mTOR inhibitor, IKK/NF-κB inhibitor	NCT03309007	Pre-diabetes in the elderly	Phase 3
		NCT03451006	Aging, inflammation, frailty	Phase 2
Topical rapamycin (sirolimus)	mTOR inhibitor	NCT03103893	Human skin senescence	Completed
Rapamycin (sirolimus)		NCT01649960	Coronary artery disease	Phase2

Another potent group of senolytic drugs includes first- and second-generation inhibitors of the BCL-2 (B CELL LYMPHOMA-2) cell death regulator family of proteins, implicated in triggering the intrinsic mitochondrial apoptosis pathway. Navitoclax (ABT263), an anticancer drug that specifically targets BCL-2, BCL-W, and BCL-XL, was the first described inhibitor of the BCL-2 family with senolytic properties (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019; von Kobbe 2019). The molecule behaves as a protein-protein interaction inhibitor (PPI), disrupting the interaction between BCL-2 proteins and their endogenous inhibitory regulators (BH3 domain-containing proteins). Despite the severe side effects (platelet and neutrophil toxicity) that rendered clinical testing unfeasible, establishment of Navitoclax as a senolytic opened new horizons for the identification of other novel selective BCL-XL inhibitors that simultaneously eliminate senescent cells. Indeed, additional such compounds entered the scenery. A1331852 and A1155463 are members of the flavonoid family that were initially applied for cancer treatment (Paez-Ribes et al. 2019; von Kobbe 2019). They are high-affinity BH3 mimetic ligands of BCL-XL and selectively disrupt the BCL-XL-BIM complex, exerting senolytic properties in a cell type-specific manner but with lower toxicity that renders them attractive candidates for clinical applications (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019; von Kobbe 2019). An additional BH3 mimetic compound and PPI inhibitor, the anticancer drug ABT-737, binds to the hydrophobic groove of BCL-2, BCL-XL, and BCL-W in contrast to pro-survival proteins MCL-1, BCL-B,

and BFL-1 (A1) that are resistant. Additionally, mitocans (mitochondrially targeted anti-cancer drugs) are also BH3 mimetics known to induce apoptosis in malignant cells. Given that senescent cells exhibit an increased mitochondrial load, these compounds emerged as intriguing candidates for putative senolytic interventions. Interestingly, the mitochondria-targeted tamoxifen (MitoTam) has been reported to selectively eliminate senescent cells *in vitro* and *in vivo* (Paez-Ribes et al. 2019; von Kobbe 2019). The molecule impedes mitochondrial oxidative phosphorylation (OXPHOS) in senescent cells resulting in the loss of mitochondrial integrity and eventually apoptosis.

The wide range of senolytics also includes molecules that belong to the following subclasses (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019; von Kobbe 2019): **(i)** FOXO4-p53 interaction inhibitors (DRI peptide), **(ii)** alkaloids (piperlongumine and analogues), **(iii)** heat shock protein (HSP90) inhibitors, **(iv)** histone deacetylase (HDAC) inhibitors (Panobinostat-Farydak®), **(v)** glycolysis inhibitors (2-deoxy-D-glucose), **(vi)** cardiac glycosides (CGs), and **(vii)** ataxia-telangiectasia mutated (ATM) protein inhibitors. FOXO4 (Forkhead box protein O4), a member of the forkhead family transcription factors, interacts with p53 at sites of DNA damage, up regulating the senescence master regulator $p21^{WAF1/CIP1}$. This interaction can be disrupted by a FOXO4 D-retro-inverso (DRI) peptide that selectively competes with FOXO4 for p53 binding, causing p53 nuclear exclusion in senescent cells and thus promoting their apoptosis, as shown in both *in vitro* and *in vivo* settings (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019). Piperlongumine is a natural compound shown to induce apoptosis in cancer cells by generating reactive oxygen species (ROS) or through modulation of the NF-kB cascade. The latter along with the fact that the exact mechanism of senolytic activity remains still unraveled but seems independent from ROS production raises the possibility of a common behavior between alkaloids and flavonoids in eliminating senescent cells (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019). HSP90 inhibitors include the natural antitumor antibiotic geldanamycin and its analogues tanespimycin (17-AAG) and alvespimycin (17-DMAG). They belong to the macrocyclic lactam class of agents and inhibit HSP90 through binding to the N-terminal ATP pocket. This inhibition seems to be associated with AKT (protein kinase b) destabilization and apoptosis induction, reminding the mode of action of other senolytics such as quercetin (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019). Interestingly, in contrast to other senolytics, their spectrum of efficacy is likely broader in terms of cell type affected. Among the HDAC inhibitors recently described as senolytics, panobinostat is the most prevalent compound. Panobinostat has been shown to exert a senolytic potential and a synergistic effect on persistent senescent cells after their treatment with taxol or cisplatin (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019; von Kobbe 2019). At a different level, altered (mainly hypermetabolic) activity and elevated glycolysis commonly evident in senescent cells are essential for their survival. In the context of therapy-induced senescence or OIS, treatment of senescent cells with 2-deoxy-D-glucose (2-DG), a pseudo-substrate for glycolysis, renders them selectively susceptible to apoptosis (Paez-Ribes et al. 2019). Cardiac glycosides (CGs) impose a similar vulnerability to senescent cells by affecting cell membrane Na/K-ATPas and altering thus the electrochemical gradient balance. Lastly, KU-60019 an ATM and DDR inhibitor seems also to exert an effective anti-senescence potential in *in vivo* settings recapitulating age-related processes, via regulation of lysosomal acidification, removal of dysfunctional mitochondria, and metabolic reprogramming (Paez-Ribes et al. 2019).

Senomorphics or Senostatics (SASP Inhibitors)

Senomorphics or senostatics are agents that exert inhibitory effects on SASP functions and not on senescent cell viability itself. They include compounds that target various SASP controlling signalling cascades or specific antibodies against individual SASP factors such as IL-1b, IL-6,

and IL-8. Given the huge diversity of putative targets, the number and the spectrum of described senomorphic compounds are extended (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019; von Kobbe 2019). Flavonoids such as apigenin, wogonin, or kaempferol mediate suppression of SASP components and particularly IL-1a, IL-1b, IL-6, IL-8, GM-CSF, CXCL1, monocyte chemoattractant protein-2 (MCP-2), and MMP-3, partially via the NF-kB p65 subunit and the NF-kB inhibitor IkBz (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019). Rapamycin (Rapamune®) has also been shown to exert senomorphic functions. This natural macrolide is a well-known selective inhibitor of the mTOR pathway. mTOR activates MAPKAPK2 (MAPK activated protein kinase 2) translation, resulting in phosphorylation of the member of the TIS11 family of early response proteins ZFP36L1 (ZFP36 ring finger protein like 1) and direct induction of various SASP components (Paez-Ribes et al. 2019; von Kobbe 2019). In addition, IL-1α translation inhibition and IL-6 downregulation by rapamycin can be exerted via an Nrf2-independent mechanism. Rapamycin is also known to regulate prelamin A, a factor implicated in longevity. Novel mTOR inhibitors such as everolimus, temsirolimus, and deforolimus exerting superior pharmacological properties have also entered the scenery of senomorphics (Paez-Ribes et al. 2019). Among other inhibitors of the MAPK pathway, those targeting p38 (SB203580, UR-13756, BIRB 796, and ginsenoside F1/ginsenoside Rg1) and MAPKAPK2 (PF-3644022 and MK2.III) have been demonstrated to suppress several SASP components (for instance, IL-6 and IL-8) by inhibiting NF-κB transcriptional activation and paracrine effects in human senescent cells (Paez-Ribes et al. 2019; von Kobbe 2019).

A different class of agents presenting senomorphic activity includes polyphenols and especially resveratrol, a natural trans-stilbene polyphenol with a wide spectrum of bioactivities. Regarding its senomorpic properties, resveratrol modulates potent cellular signaling pathways such as NF-kB (IkB inhibition) and PI3K/AKT, controlling thus the release of multiple pro-inflammatory cytokines (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019; von Kobbe 2019). Resveratrol also activates SIRT1, a class III histone deacetylase, the former with notorious properties in lifespan and AMPK (AMP-activated protein kinase) and the latter implicated in various processes including metabolism and aging. In addition, the molecule and its synthetic analogues (polar substitution on the 4' position) can restore the expression of mRNA splicing factors, and therefore these agents have been proposed as potential drugs for reversing the senescent phenotype (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019; von Kobbe 2019). This function was independent of SIRT1 activation and most probably related to enhanced telomere maintenance.

The intriguing beneficial impact of the antidiabetic drug metformin (Glucophage®) on aging and life span expansion led to initiation of drug repurposing research seeking for its putative role on senescence. These efforts revealed some targets/levels of senescence regulation by metformin (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019; von Kobbe 2019): ***(i)*** decrease inNF-κB nuclear translocation and inhibition of phosphorylation of the two catalytic subunits of IkB kinase, namely, IKK α and β, leading to downregulation of several SASP factors, namely, CXCL-5, IL-6, IL-8, and IL-1β; ***(ii)*** AMPKa stimulation causing mTOR pathway inhibition; ***(iii)*** tuning of mitochondrial electron transport, known to eventually affect regulation of pro-inflammatory cytokines such as IL-6; and ***(iv)*** mitochondrial glycerol-3-phosphate dehydrogenase (GPDH) and lysine-specific demethylase 6A (KDM6A/UTX) control. Based on the aforementioned beneficial effects on health and life span, testing for additional therapeutic opportunities in the frame of clinical trials is currently ongoing (Targeting Aging with MEtformin – TAME study, Table 2).

Nutlins utilized for cancer treatment can impose apoptosis and/or senescence by inhibiting Mdm2 and stabilizing p53. However, these molecules apart from inducing senescence exert also some anti-senescence properties. Nutlin-3a is known to suppress NF-κB in a p53-dependent manner, driving downregulation of certain SASP interleukins in human cells (Paez-Ribes et al.

2019; von Kobbe 2019). A similar potential for SASP inhibition has been reported for the next-generation Mdm2 inhibitor MI-63 (Paez-Ribes et al. 2019). Blocking of the JAK/STAT pathway by targeting JAK kinases (ruxolitinib, Jakafi®), eventually leading to inactivation of C/EBPβ, has also been linked with SASP alleviation. Among the JAK kinases, blocking of JAK1/2 drives the decrease of the SASP modulator activin A that is frequently found upregulated during aging (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019). Likewise, NOTCH1 upregulation has been associated with SASP suppression, via C/EBPβ downregulation, in the context of OIS (Paez-Ribes et al. 2019). In addition, the alternative splicing modulator polypyrimidine tract binding protein 1 (PTBP1) has emerged as promising therapeutic target for SASP inhibition and is currently under investigation (Paez-Ribes et al. 2019). Another class of drugs harboring SASP inhibitory potential includes the glucocorticoids, steroid hormones with well-characterized anti-inflammatory properties (Myrianthopoulos et al. 2019; Paez-Ribes et al. 2019). The most effective compounds for SASP (IL-1a and IL-6) suppression, via control of NF-kB transcription, are corticosterone and cortisol. In contrast, dexamethasone has been demonstrated to induce senescence via SIRT1 inhibition and activation of the $p53/p21^{WAF/CIP1}$ axis, proving a putative explanation for its adverse side effects. Notably, some other clinically applied drugs such as loperamide and niguldipine as well as dopamine and serotonin antagonists have been reported to exhibit senomorphic properties (Myrianthopoulos et al. 2019). Lastly, an additional way to directly target SASP components (ligands or receptors) encompasses the use of monoclonal antibodies. Canakinumab that reacts against IL-1b and has been extensively used to treat autoimmune diseases comprises such an example (Paez-Ribes et al. 2019).

Modifiers/Enhancers of the Immune System

The immune system undergoes an age-related decline that has been associated with accumulation of senescent cells in tissues. Given that senescent cells are immunogenic, stimulation of the immune system by immunotherapy, currently used for treatment of cancer and other diseases, has also emerged as an attractive strategy. Senescent cell clearance is achieved by interventions that aim at two levels: reactivation of the immune cell pool or increase in senescent cell immunogenicity. Senescent cells are eliminated by $CD4^+$ T cells, macrophages, and NK cells (senescence surveillance). Chimeric antigen receptor (CAR) T cells against senescence occurring agents, similar to those developed for cancer therapies, seem a promising choice (Paez-Ribes et al. 2019; von Kobbe 2019). Moreover, restoration of T cell and NK functions that decline during the development of age-related pathologies and aging can be achieved via inhibition of AMPK by an p38 MAPK inhibitor or via downregulation of the killer cell lectin-like receptor G1 (KLRG1 or CD57) (Paez-Ribes et al. 2019; von Kobbe 2019). Treatment with polyinosinic-polycytidylic acid, a NK toll-like receptor 3 (TLR3) ligand and interferon-γ, increases in certain settings the cytotoxic activity of NK cells via the NK cell receptor NKG2D (Paez-Ribes et al. 2019; von Kobbe 2019). The latter interacts with corresponding ligands frequently upregulated in senescent cells. As for enhancing senescent cell immunogenicity, the surface marker DPP4 can be exploited in the context of an antibody-dependent NK cell-mediated cytotoxicity, for senescent cell elimination (Paez-Ribes et al. 2019; von Kobbe 2019). Similarly, an oxidized form of membrane-bound vimentin discovered in senescent fibroblasts could be recognized and targeted by humoral innate immunity processes (Paez-Ribes et al. 2019).

Nanoparticles

Nanoparticles (NPs) are not considered in a strict manner, as agents that can alone influence the viability or the function of senescent cells. In fact, NPs behave as carriers, able to efficiently deliver among other substrates, a senotherapeutic drug for targeted clearance or manipulation of the deleterious potential of the senescent cell. In this context, mesoporous silica nanoparticles capped with a mixture of galacto-oligosaccharides (GosNPs) of different lengths were the pioneer cargo delivery system

generated. GosNPs enter cells via endocytosis and fuse with lysosomes that in senescent cells are associated with elevated endogenous SA-βgal activity (Paez-Ribes et al. 2019). This interaction results in enzymatic hydrolysis of the cap and release of the cargo selectively in senescent cells. Subsequently, silica beads capped with 6-mer galacto-oligosaccharides (GalNPs) have been reported as superior mediators for effective and specific (with lower toxicity) transport of drugs in *in vivo* models of damage- and chemotherapy-induced senescence (Paez-Ribes et al. 2019). Such evidence opens new perspectives in enhancing the beneficial effect of chemotherapy by elimination of cancer cells undergoing chemotherapy-induced senescence. Moreover, molybdenum disulfide nanoparticles (MoS2 NPs) as well as molecularly imprinted nanoparticles (nanoMIPs) have been generated and tested *in vitro* (Paez-Ribes et al. 2019). MoS2 NPs seem to exhibit low preference for senescent cells. In contrast, nanoMIPs loaded with dasatinib can eliminate senescent cells by internalizing the drug, after the recognition of surface molecules such as b2 microglobulin (B2M) (Paez-Ribes et al. 2019).

Alternatively, other emerging nanovehicles aim to restrain SASP by transferring encapsulated inhibitors, effectors, and small molecules. Examples applied and tested in *in vitro* settings but still lacking in vivo evidence include (Paez-Ribes et al. 2019) (i) rapamycin containing porous calcium carbonate nanoparticles (CaCO3). These NPs are coated with a conjugate of lactose (Lac, galactose/glucose dimers linked with β-1,4- glycosidic units) that allows cargo release in the presence of increased SAβ-gal activity and polyethylene glycol imposing stability in the blood and opsonisation avoidance. (ii) The latter nanodevices, conjugated with a monoclonal antibody against CD9. CD9-Lac/CaCO3 exerts their delivery properties by interacting with CD9, commonly located at the surface senescent cells that simultaneously exhibit elevated SAβ-gal activity, thus strengthening cell specific uptake and cargo release.

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Sepsis

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Synonyms

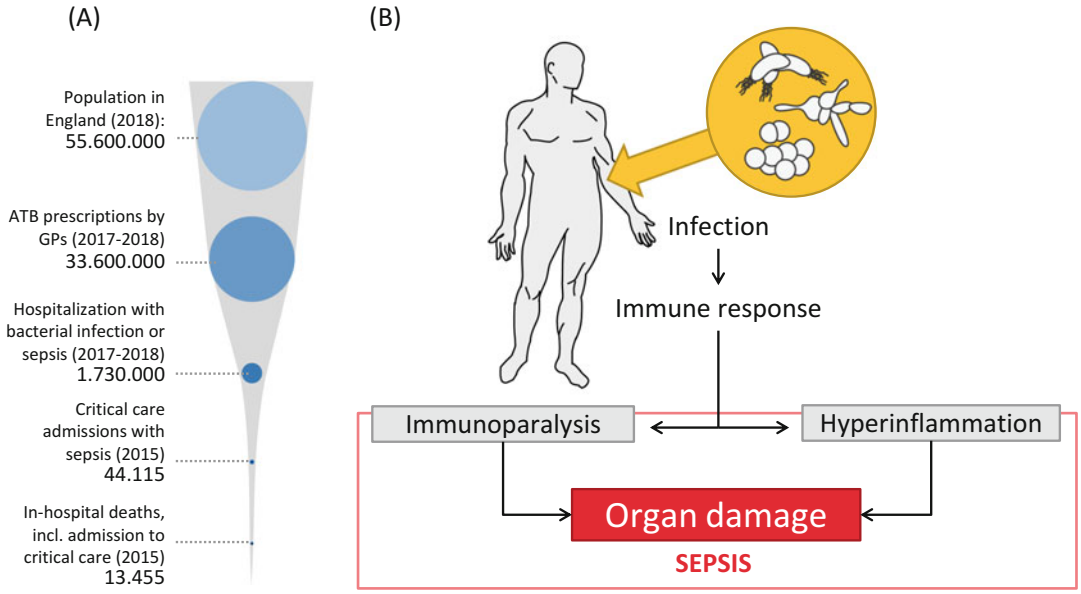
Blood poisoning; Infection; Organ dysfunction; Systemic inflammation; Septic shock

Definition

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection. Organ dysfunction is the criterion that distinguishes sepsis from minor, self-limiting infections (Singer et al. 2016). Main clinical manifestations are altered mentation, high respiratory rates, and low blood pressure that occur in patients with suspected infection (Seymour et al. 2016).

Basic Mechanisms

Sepsis has long been interpreted as an excessive systemic inflammatory response due to infection ("systemic inflammatory response syndrome, SIRS"; Bone et al. 1992). Failing containment of invading pathogens and pathologic hyperinflammatory immune responses were thought causative of impaired organ function. The exclusive reliance on SIRS as the hallmark of sepsis has been challenged by the finding that SIRS criteria are absent in a significant proportion of patients (Kaukonen et al. 2015). Moreover, reduced immune responsiveness ("immunoparalysis") has been shown to dominate sepsis pathology, at least in later stages of the disease process (Hotchkiss et al. 2013). These opposing response patterns are both able to provoke remote organ



Sepsis, Fig. 1 Dysfunctional immune responses in sepsis (**a**) Infection is a frequent and typically self-limiting event. Approximately 2.5% of patients presenting in hospitals with infection will be admitted to an intensive care unit due to infection-triggered organ failure, i.e., sepsis (data adapted from (Singer et al. 2019), circle areas are proportional to population and case numbers). (**b**) Organ damage in sepsis can be provoked by either reduced immune responsiveness (immunoparalysis) or hyper-inflammatory responses to infections (immunopathology) in a minority of patients. Abbreviations: *ATB* antibiotic, *GP* general practitioner

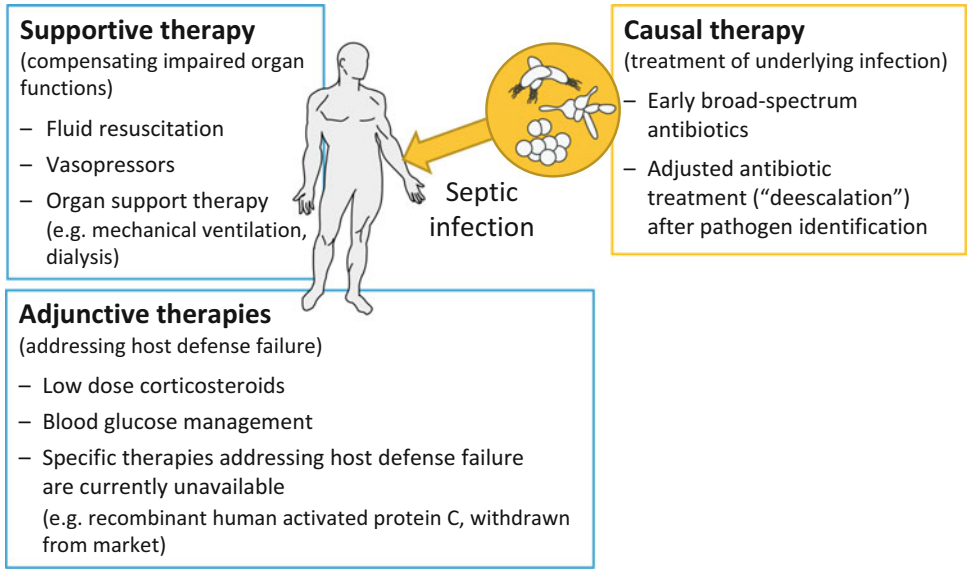
failure. The molecular mechanisms underlying sepsis are, though, not fully understood. However, epidemiological data point toward a rare event in the light of frequent uncomplicated infections receiving antibiotic prescriptions across the population (Singer et al. 2019) (Fig. 1).

Currently, sepsis is best conceptualized as an imbalanced and dysfunctional host response induced by failed control of pathogens at the site of infection. Numerous factors including age, gender, genetic predisposition, and comorbidities influence susceptibility to the disease (Paoli et al. 2018; Taudien et al. 2016). Importantly, damaging processes in the course of sepsis seem to differ depending on tissues, their regenerative capacity, and cell type. For example, sepsis-associated organ dysfunction is often reversible, and necrotic or apoptotic cell death is a rare event in parenchymal organs of septic patients (Hotchkiss et al. 1999). In contrast, immunoparalysis can be attributed to cell death of immune cells, in particular lymphocytes (Hotchkiss et al. 2013).

Despite tremendous research efforts during recent decades, no host-directed *adjunctive therapy* for sepsis exists (Fig. 2). Numerous clinical studies on candidate mediators failed, and only recently, meta-analyses of the clinical trials indicated that certain beneficial effects might have escaped notice due to the highly individual disease courses and etiologies. For example, analysis of data on antibodies neutralizing the pro-inflammatory cytokine anti-TNFα suggests a benefit for a subset of patients (Marshall 2014). Current efforts aim at identification of subgroups of patients that will respond to specific interventions, e.g., immune modulatory therapy or adjunctive therapies (Marshall 2014; Seymour et al. 2019). Besides individualized treatment approaches, targeted drug delivery might hold potential for improved sepsis therapies.

Immune Responses

The occurrence of hyperinflammation and immunoparalysis in sepsis patients raises



Sepsis, Fig. 2 Current options to treat sepsis
Sepsis therapy includes "causal" (anti-infective), "supportive" (replacing impaired organ function), and "adjunctive" (interfering with the failing host response) treatment options

questions concerning the causes of these fatal dysfunctions of the immune system.

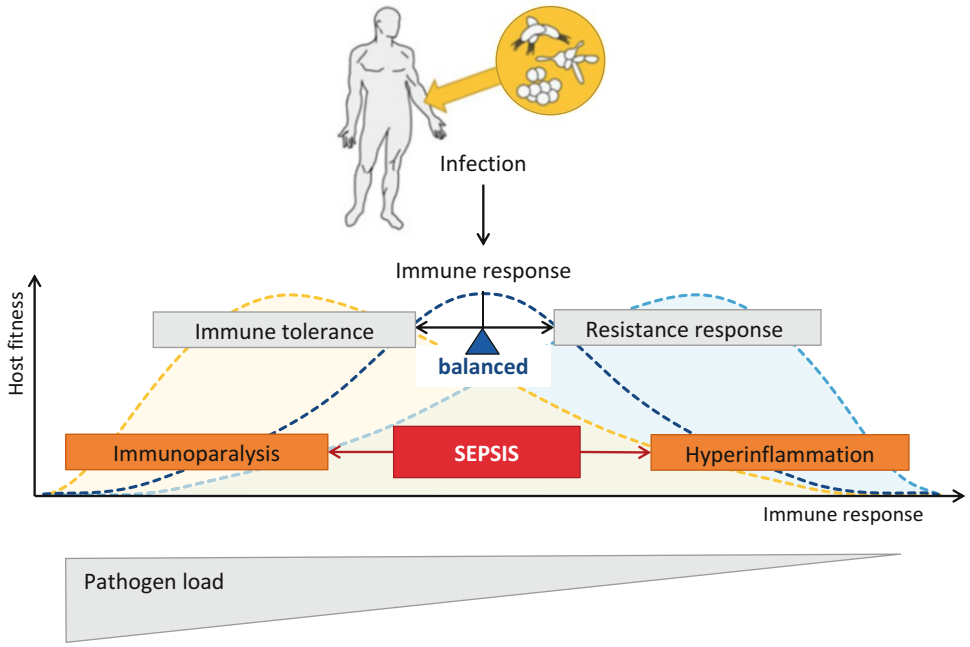
Regularly, higher organisms react to invading microbes with two alternative response patterns. In addition to the well-known resistance responses of the immune system, which result in pathogen clearance, "disease tolerance" has been acknowledged as an alternative adaptive strategy of the host (Medzhitov et al. 2012). Disease tolerance enables the organism to sustain a certain pathogen load without marked damage or disease symptoms (i.e., organ failure). Disease tolerance requires maintenance of cell and organ functions of the infected host by continuous repair reactions despite persistent pathogen burden. Immune resistance and disease tolerance responses enable the resolution of infection and the restoration of health.

In sepsis, the concerted resistance and tolerance responses of the host fail. Exuberant resistance responses cause a hyperinflammatory state, i.e., SIRS, and collateral tissue damage. Dysregulated disease tolerance might result in immunoparalysis, recurrent or chronic infections, and corresponding damage caused by disseminating pathogens (Fig. 3).

Significant progress has been made regarding the understanding of the causes of dysregulated resistance and tolerance and consequently of hyperinflammation and immunoparalysis in sepsis patients. Both response patterns have been attributed to specific traits of the infecting pathogen or the host. However, increasing evidence indicates pathogen dose as key for causing these opposing reactions (Bauer et al. 2018). Innate immune cells challenged with low amounts of pathogens were shown to generate energy-demanding resistance responses, whereas high pathogen load will increasingly perturb cell metabolism. Under these conditions, a shift from resistance reactions toward maintenance and repair activities seems to occur, which ultimately enables tolerance toward pathogens. Induction of resistance and tolerance depending on the pathogen burden might help the cells and tissues to integrate challenges by various microbes and other stressors, in particular in the critically ill, and thus fulfill the basic fitness and survival requirements of cells and organs (Fig. 3).

The Role of Parenchymal Tissues

While the activated immune system during phases of acute infections is highly energy demanding, the overall limited energy supply has to be



Sepsis, Fig. 3 Immune response patterns to infection and development of sepsis
In sepsis, the balanced interaction of immune cells, pathogens, and parenchyma upon local, uncomplicated infections gets out of control. Resistance responses progress to systemic hyperinflammation, whereas excessive tolerance might result in immunoparalysis. Both immune response patterns impact host fitness by causing organ damage. Pathogen load and energy metabolism have been suggested as key mediators of the dichotomous immune responses

redistributed, which might result in parenchymal metabolic shut-down. Indeed, critical illness is frequently associated with reduced oxygen consumption, metabolic adjustments, and decreased organ function. Importantly, this organ dysfunction is not associated with markedly increased cell death and is usually quickly restored after episodes of sepsis (Hotchkiss et al. 1999). This, together with drastically altered metabolism, has led to the concept of "hibernation" of the failing organs (Stanzani et al. 2020). These topics are active research areas aiming at alternative treatment approaches for the attainment of metabolic homeostasis and restoration of organ function during and after sepsis.

Molecular Mechanisms Mediating (Mal-) adaptive Responses to Infection

Molecular signaling events mediating resistance and tolerance responses to infections have been investigated in cellular and animal models. Given the crucial role of a balanced energy budget, the emerging involvement of energy sensing signaling pathways does not come as a surprise. Both in vitro and in vivo data indicate involvement of the mechanistic target of rapamycin (mTOR) signaling pathway in the induction of **resistance** responses to infectious microbes. mTOR is known as a central mediator of anabolic processes. Together with the signaling proteins of the phosphoinositide 3-kinase (PI3K) family, mTOR is controlling energy demanding cell functions, such as proliferation or differentiation, in addition to resistance responses of immune cells (Cheng et al. 2014).

Experimentally, sepsis-induced hyperinflammation could already be effectively treated by drugs targeting mTOR as the key mediator of enduring resistance responses (Yen et al. 2013). In septic mice, the mTOR inhibitor rapamycin significantly suppressed the hyperinflammatory response and decreased mortality.

Signaling reactions that trigger the development of disease **tolerance** are less well understood. Recent investigations revealed AMP-activated protein kinase (AMPK) as a critical mediator of tolerance responses to infections (Bauer et al. 2018). In contrast to mTOR, AMPK activity is induced at low cellular energy levels. In pathogen-challenged cells, the signaling protein mediates maintenance and repair reactions, including autophagy of cellular organelles. A key role of AMPK has been proposed for tissue damage control in parenchymal cells in states of disease tolerance (Soares et al. 2014).

Taken together, resistance and tolerance responses triggered by invading pathogens appear to be part of evolutionary conserved and interdependent reaction patterns of the organism to environmental stress in general (Bauer et al. 2018; Stanzani et al. 2020).

Pharmacological Intervention

Current Standard of Care

Current therapeutic concepts mainly focus on "*causal" therapy*, i.e., early anti-infective therapy, combined with circulatory support by volume resuscitation and additional vasopressors (*"supportive" therapy*), if a mean arterial pressure of 65 mm Hg cannot be achieved by volume resuscitation alone (Rhodes et al. 2017).

Antimicrobial therapy should be initiated as early as possible after diagnosis. Importantly, standard samples for microbiological assessment (including blood cultures) should be taken before starting antibiotics. Initial therapy will, in general, rely on empirical broad-spectrum therapy with one or more antimicrobials (including antifungal or antiviral therapy, if applicable). After identification of the causative pathogen, the antibiotic regimen should be narrowed as appropriate (Rhodes et al. 2017).

According to the Surviving Sepsis Campaign (SSC) guidelines, initial resuscitation from sepsis-induced hypoperfusion should be initiated immediately after sepsis diagnosis using IV crystalloids. If a target mean arterial pressure of 65 mm Hg cannot be achieved by fluid resuscitation alone, norepinephrine is recommended as first-choice vasopressor. Vasopressin or epinephrine can be added to raise the arterial pressure to the target values or to reduce norepinephrine dosage (Rhodes et al. 2017). The SSC as a "one world" initiative has been criticized as primarily suitable for limited income countries, and a more individualized approach regarding hemodynamic monitoring and catecholamine support will be applicable in high-income countries.

The present therapeutic approaches are summarized in Fig. 2.

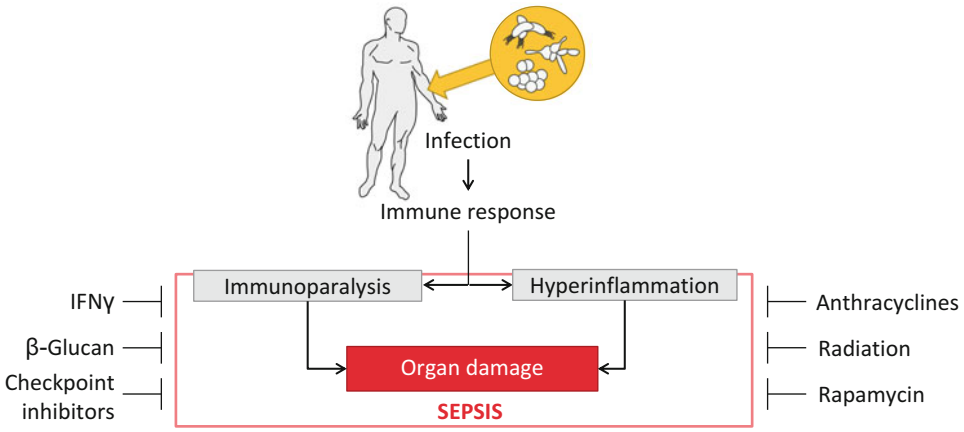
The only currently used immunomodulatory intervention in septic shock is the administration of "stress doses" of hydrocortisone. This approach yielded controversial results (Suffredini 2018) and reflects a prominent example for the need to define subgroups of patients prior to initiation of therapy (personalized medicine, "theranostics").

There is currently no approved therapy that targets the dysbalanced immune response. Moreover, no specific therapies exist to pharmacologically treat organ dysfunction, since recombinant human activated protein C was withdrawn from the market in 2011.

Novel Treatment Approaches – Restoring Immune and Organ Function

From the immunological point of view, the sepsis paradigm currently shifts from a focus on simple "hyperinflammation" to a multifaceted disruption of immune balance, as many patients present with signs of "immunoparalysis." This is best reflected in current trials studying the potential of immunostimulatory therapies, such as growth factors or check point inhibitors as adjunct options in sepsis therapy (Hotchkiss et al. 2013). Leentjens et al. demonstrated partial reversion of immunoparalysis of humans by treatment with IFN-γ in vivo (Leentjens et al. 2012). Immunostimulatory agents used in preclinical models cover a broad spectrum of agents, including pathogenic yeast *Candida albicans* and its cell wall component β-glucan (Novakovic et al. 2016). However, these approaches will have to rely on improved patient stratification.

In line with the critical balance of mTOR and AMPK, hyperinflammation during sepsis can be



Sepsis, Fig. 4 Evolving therapeutic concepts to modulate resistance and tolerance responses in sepsis models Currently studied options to modulate failing immune responses in sepsis. These specific interventions aim to suppress organ damage provoked by either immunopathology or immunoparalysis in the course of sepsis

suppressed by the immunosuppressive and anti-proliferative agent rapamycin (Yen et al. 2013). Additional options to treat hyperinflammatory sepsis have been revealed by Figueiredo et al. (2013). In a groundbreaking study, these authors screened a drug library for candidates that affect inflammatory reactions in a macrophage cell line. Anthracyclines – commonly applied for the therapy of solid cancers – were identified to prevent organ dysfunction in mouse models of polymicrobial sepsis independent of changes in pathogen burden. Apparently, low doses of anthracyclines induce a DNA damage response and autophagy, which are assumed to be the mechanisms underlying tissue protection and anti-inflammatory effects. Interestingly, irradiation produced similar effects. Together, these data indicate that low doses of stressors, such as toxins or irradiation, stimulate cellular repair and thereby induce cross-protection, i.e., generally increased resilience. The anti-inflammatory effects of anthracylines or radiation might be connected to energy demands. Reactions to environmental stressors and inflammatory responses compete for energy sources. The additional energy requirement also affects protein synthesis and cell proliferation. Accordingly, treatment of cells with anthracylines or radiation seems to lower the immune response in favor of tolerance reactions. Morbidity induced by systemic hyper-inflammation decreases, and the organism exhibits a state of "disease tolerance."

Together, these interventions (Fig. 4) targeting immunopathology and immunoparalysis during the course of sepsis might add to the therapeutic armamentarium of personalized care in the field of infection-driven organ dysfunction, which discriminates sepsis from uncomplicated infection.

Cross-References

- AMP-activated Protein Kinase
- Cytokines
- Inflammation
- mTOR Signaling Pathways
- β-Lactam Antibiotics

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Septic Shock

► Sepsis

Serine/Threonine Protein Kinase Phosphorelay Modules

▶ MAP Kinase Cascades

Serotonergic Hallucinogen

▶ Psychedelic Drugs

Serotonin

▶ Serotoninergic System

Serotoninergic System

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Synonyms

5-hydroxytryptamine = 5-HT (= enteramine); Serotonin

Definition

The serotoninergic system is one of the oldest neurotransmitter/hormone systems in evolution, which may explain why 5-HT interacts with such a diversity of receptors of the G protein-coupled family (type A) and the ligand-gated family Cys-loop family, similarly to acetylcholine, GABA, or glutamate. 5-HT was discovered in the gut in the 1930s and called enteramine and then rediscovered in the 1940s in the blood and called serotonin, since it also constricted smooth muscle in the vessels. 5-HT is synthesized from l-tryptophan; there are two tryptophan hydroxylases forming 5-hydroxytrytophan (5-HTP), which by the l-amino acid decarboxylase leads to 5-HT. 5-HT can be conjugated with glucuronide or sulfate. In the nervous system, 5-HT is metabolized via monoamine oxidase to 5-hydroxyindolacetaldehyde and finally to 5-hydroxyindolacetic acid (via aldehyde dehydrogenase). It can also lead to 5-hydroxytryptophol by an aldehyde reductase in some peripheral nerves. Thus, 5-HT acts as a neurotransmitter with all the typical features, such as intracellular storage, activity-dependent release, the existence of both pre- and post-synaptic receptors, and an active uptake system, via the serotonin transporter and metabolizing/inactivating enzymes. 5-HT also acts as a hormone, released into the blood or gut to work more distantly.

Basic Characteristics

Physiology

The main source of 5-HT (95%) is from enterochromaffin cells of the gut, where 5-HT is synthesized from tryptophan. It can be released into the gut lumen, e.g., as a reaction to pressure and act on receptors located on the smooth muscle, or released into the portal blood circulation, by a variety of nervous or alimentary stimuli. 5-HT is also found in enteric neurons. In the blood, the vast majority of 5-HT is in storage granules from platelets, which are endowed with a very active uptake system (they do not synthesize 5-HT). Large amounts of 5-HT are released during platelet aggregation, and it can act locally on endothelial cells and vascular smooth muscle. 5-HT is also present in mast cells. In the central nervous system (CNS) and peripheral nervous system (PNS), 5-HT acts as a neurotransmitter on a variety of

receptors, which may be located pre- or postsynaptically. 5-HT is also found in the pineal gland, where it serves as a precursor for the synthesis of melatonin by 5-HT-*N*-acetyltransferase and hydroxyindole-*O*-methyltransferase, under the control of the clock genes of the suprachiasmatic nucleus, which modulates enzyme activity levels up to 50-fold during the circadian cycle.

Multiple 5-HT Receptor Subtypes

There are at least 15 different 5-HT receptors, and the system may be more complex (see Tables 1, 2, 3 and 4). With the exception of 5-HT_3 receptors (ligand-gated ion channels), 5-HT receptors belong to the G-Protein-Coupled Receptors (GPCR) superfamily, which is one of the most complex families of neurotransmitter receptors. Some 5-HT receptors have multiple splice variants (5-HT_{2C}, 5-HT_4, 5-HT_7) or RNA-edited isoforms (5-HT_{2C}). There is evidence that homo- and hetero-dimerization (e.g., 5-HT_{2C}, 5-$HT_{1B/1D}$, 5-HT_{2C}/GHS-R1a) can occur. We have proposed a classification system based on operational, structural, and transductional criteria (Humphrey et al. 1993; Hoyer et al. 1994; Hartig et al. 1996) which has been progressively refined (Hoyer and Martin 1997; Hannon and Hoyer 2008). The current classification (Barnes et al. 2021) favors an alignment of nomenclature with the human genome to account for species differences. Seven families of 5-HT receptors have been recognized, 5-HT_1 to 5-HT_7. Yearly updates about 5-HT receptors and ligands can be found in the BJP Concise Guide to PHARMACOLOGY (Alexander et al. 2019).

Most 5-HT receptors, except 5-HT_3 which are Cys-loop ligand-gated channels with structural analogy to nicotinic or $GABA_A$ receptors, belong to the GPCR family A. Lowercase denomination means that the receptor is not functionally defined in native in vitro or in vivo systems. The 5-HT_{1C} slot is empty, as it was renamed 5-HT_{2C}. There are no lowercase for 5-HT_3 receptor subunits, as they may form heteromers for which in vivo proof is not yet established, although probable.

The 5-HT_1 receptor class comprises five receptors (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1E}, and 5-HT_{1F}) which, in humans, share 40–63% overall sequence identity and couple somewhat preferentially to $G_{i/o}$ to inhibit cAMP formation (see Table 2). 5-ht_{1E} receptors have a lowercase appellation to denote that endogenous receptors with a physiological role have not yet been reported or may not even exist in rodents (rat, mouse). In contrast, 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, and 5-HT_{1F} receptors have been documented functionally. The 5-HT_{1C} designation is vacant, as the receptor was renamed 5-HT_{2C}, due to structural, operational, and transductional similarities with the other two members of the 5-HT_2 receptor family.

5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors exhibit 46–50% overall sequence identity and couple preferentially to $G_{q/11}$ to increase inositol phosphates and cytosolic [Ca^{2+}] (see Table 3).

5-HT_3 receptors belong to the ligand-gated Cys-loop ion channel receptor superfamily, similar to the nicotinic acetylcholine or $GABA_A$ receptors, and share electrophysiological and structural patterns. The receptors are found on central and peripheral neurons where they trigger rapid depolarization due to the opening of non-selective cation channels (Na^+, Ca^{2+} influx, K^+ efflux). The response desensitizes and resensitizes rapidly. The native 5-HT_3 receptor, as revealed by electron microscopy, is a pentamer. There are five potential different subunits, 5-HT_{3A}, 5-HT_{3B},

Serotoninergic System, Table 1 5-HT receptor families and receptor subtypes

5-HT_1	5-HT_2	5-HT_3	5-HT_4	5-ht_5	5-HT_6	5-HT_7
5-HT_{1A}	5-HT_{2A}	5-HT_{3A}	5-HT_4	5-ht_{5A}	5-HT_6	5-HT_7
5-HT_{1B}	5-HT_{2B}	5-HT_{3B}		5-ht_{5B}		
5-HT_{1D}	5-HT_{2C}	5-HT_{3C}				
5-ht_{1E}		5-HT_{3D}				
5-HT_{1F}		5-HT_{3E}				

Serotoninergic System, Table 2 5-HT_1 receptor nomenclature as proposed by the NC-IUPHAR Subcommittee on 5-HT receptors

Nomenclature	5-HT_{1A}	\$5-HT_{1B}	5-HT_{1D}	5-ht_{1E}	5-HT_{1F}
Previous names	–	5-$HT1_{D\beta}$	5-$HT1_{D\alpha}$	–	5-$ht_{1E\beta}$, 5-HT_6
Selective agonists	8-OH-DPAT	Sumatriptan	Sumatriptan	–	LY 334370
	(R)-UH301	Eletriptan	Eletriptan		LY 334864
	F13714	L 694247	PNU 109291		
	Befiradol	CP93129(rodent)	L775606		
Selective antagonists (pKB)	WAY100635 (8.7)	GR 55562 (7.4)	BRL 15572 (7.9)	–	–
	Robalzotan (9.2)	SB 224289 (8.5)			
	NAN190 (9.4)	SB 236057 (8.9)			
Radioligands	[^{3}H] WAY100635	[^{125}I]GTI	[^{125}I]GTI	[^{3}H]5-HT	[^{125}I]LSD
	[^{3}H]8-OH-DPAT	[^{125}I]CYP (rodent)	[^{3}H] Sumatriptan		[^{3}H]LY 334370
	[^{3}H] Robalzotan	[^{3}H]Sumatriptan	[^{3}H]GR 125743		
	[^{3}H]pMPPF	[^{3}H]GR 125743			
G protein effector	Gi/o	Gi/o	Gi/o	Gi/o	Gi/o
Gene/chromosomal localization	HTR1A/ 5q11.2-q13	HTR1B/6q13	HTR1D/ 1p34.3-36.3	HTR1E/ 6q14-15	HTR1F/3p11-p14.1
Structural information	h421 P8908	h390 P28222	h377 P28221	h365 P28566	h366 P30939
	m421 Q64264	m386 P28334	m374 Q61224		m366 Q02284
	r422 P19327	r386 P28564	r374 P28565		r366 P30940

5-HT_{3C}, 5-HT_{3D}, and 5-HT_{3E}. Currently, the consensus is that native receptors are best reproduced by the heteromeric combination of 5-HT_{3A} and 5-HT_{3B} subunits. 5-HT_{3B} homomers lack functions (as is probably the case for either of 5-$HT_{3C,3D,3E}$). 5-HT_{3A} subunits can form homopentamers, but to become really functional, they have to form heteropentamers (with, e.g., 5-HT_{3B}) with two 5-HT_{3A} subunits. It has been suggested that 5-HT_3 receptors can heterodimerize with other members of the superfamily (e.g., nicotinic alpha$_7$), but this has not been demonstrated in situ.

5-HT_4, 5-HT_6, and 5-HT_7 receptors all couple preferentially to G_s and promote cAMP formation, yet they are regarded as distinct receptor classes due to their limited (<35%) overall sequence identities. This subdivision is arbitrary and may be subject to future modification.

Two subtypes of the 5-ht_5 receptor (5-ht_{5A} and 5-ht_{5B}), sharing 70% overall sequence identity, have been found in rodents. The human 5-ht_{5B} receptor gene does not encode a functional protein, due to the presence of stop codons in its coding sequence. Human recombinant 5-ht_{5A} receptors inhibit forskolin-stimulated cAMP production, although the receptor may also couple positively to cAMP production. Currently, a physiological readout for this receptor is still missing, although A-843277, a selective 5-ht_{5A} antagonist, has antidepressant/antipsychotic properties in rodent models.

Additional 5-HT receptors have been defined phenotypically and pharmacologically, although a corresponding gene product encoding the receptor has not yet been identified. As long as their structure is unknown, these receptors are regarded as orphans. However the so-called "5-HT_1-like" receptor mediating direct vasorelaxation actually corresponds to the 5-HT_7 receptor. The situation with the remaining orphan receptors (see Hoyer

Serotoninergic System, Table 3 5-$HT_{2, 3, 4}$ receptor nomenclature as proposed by the NC-IUPHAR Subcommittee on 5-HT receptors

Nomenclature	5-HT_{2A}	5-HT_{2B}	5-HT_{2C}[b]	5-HT_3	5-HT_4
Previous names	D/5-HT_2	5-HT_{2F}	5-HT_{1C}	M	–
Selective agonists	α-Methyl-5-HT	BW 723C86	Ro 600175	SR 57227	BIMU 8
	DOI[a]	Ro 600175	DOI	m-chlorophenyl-biguanide	RS 67506
	DOB	DOI			ML 10302
Selective antagonists (pKB)	Ketanserin (8.5–9.5)	LY266097 (9.7)	Mesulergine (9.1)	Granisetron (10)	GR 113808 (9–9.5)
	Volinanserin (9.4)	RS127445 (9.5)	SB 242084 (9.0)	Ondansetron (8–10)	SB 204070 (10.8)
	Pimavanserin (9.4)	SB 204741 (7.8)	RS 102221 (8.4)	Tropisetron (10–11)	RS 100235 (11.2)
Radioligands	[^{125}I]DOI	[^{3}H]5-HT	[^{125}I]LSD	[^{3}H](S)-zacopride	[125I]SB 207710
	[^{3}H]DOB		[^{3}H] Mesulergine	[^{3}H]tropisetron	[3H]GR 113808
	[^{3}H] Ketanserin			[^{3}H]granisetron	[3H]RS 57639
	[^{3}H]MDL 100907			[^{3}H]GR 65630	
				[^{3}H]LY 278584	
G protein effector	Gq/11	Gq/11	Gq/11	[c]	Gs
Gene/chromosomal localization	HTR2A/ 13q14-q21	HTR2B/ 2q36.3-q37.1	HTR2C/ Xq24	HTR3/11q23.1-q23.2	HTR4/5q31-33
Structural information	h471 P28223	h481 P41595	h458 P28335	Multi-subunit[d]	h387 Y09756AS
	m471 P35362	m504 Q02152	m459 P34968	5-HT_{3A}, 5-HT_{3B},	m387 Y09587AS
	r471 P14842	r479 P30994	r460 P08909	5-$HT_{3C,}$ 5-HT_{3D},	r387 U20906AS
				5-$HT_{3E.}$	

[a]Also activates the 5-HT_{2C} receptor. [b]Multiple isoforms of the 5-HT_{2C} receptor are produced by RNA editing. [c]The 5-HT_3 receptor is a ligand-gated cation channel that exists as a pentamer of 4TM subunits. [d]Human, rat, mouse, guinea pig, and ferret homologues of the 5-HT_{3A} receptor have been cloned that exhibit species variation in pharmacological profile. A second 5-HT_3 receptor subunit, 5-HT_{3B}, imparts distinctive biophysical properties upon hetero-oligomeric (5-HT_{3A}/5-HT_{3B}) versus homo-oligomeric (5-HT_{3A}) recombinant receptors. The function of the 5-$HT_{3C,3D,3E}$ subunits and other putative members of the family requires further investigations

et al. 1994; Barnes et al. 2021) has not evolved further, and thus the status quo ante remains. In particular, no progress has been made with the "5-HT_{1P} receptor," which is described in the gut and whose pharmacology is reminiscent of the 5-HT_4 receptor, with the restriction that some of the ligands described as 5-HT_{1P} selective, like the 5-HT dipeptides, do not affect 5-HT_4 receptors. The 5-HT_{1P} receptor could be a heterodimer, hence the separate pharmacological profile; however the putative partners remain to be identified.

Pathophysiology/Clinical Applications

5-HT has been implicated in the etiology of numerous disease states, including depression, anxiety, social phobia, schizophrenia, Parkinson's disease and other movement disorders, obsessive compulsive disorders, aggression, panic and sleep disorders, migraine, vascular and pulmonary hypertension, eating disorders, addiction, nausea/vomiting, irritable bowel syndrome (IBS), and other functional GI disorders, by interacting at different receptors or transporters (see 6).

Serotoninergic System, Table 4 5-$HT_{5, 6, 7}$ receptor nomenclature as proposed by the NC-IUPHAR Subcommittee on 5-HT receptors

Nomenclature	5-ht_{5A}	5-ht_{5B}	5-HT_6	5-HT_7
Previous names	5-$HT_{5\alpha}$	–	–	5-HT_X, 5-HT_1-like
Selective agonists	–	–	–	–
Selective antagonists (pK_B)	SB 699551 (8.0)	–	Ro 630563 (7.9)	SB 258719 (7.9)
			SB 271046 (7.8)	SB 269970 (9.0)
			SB 357134 (8.5)	
Radioligands	[^{125}I]LSD	[^{125}I]LSD	[^{125}I]SB 258585	[^{125}I]LSD
	[^{3}H]5-CT	[^{3}H]5-CT	[^{125}I]LSD	[^{3}H]SB 269970
			[^{3}H]5-HT	[^{3}H]5-CT
				[^{3}H]5-HT
G protein effector	$G_{i/o}$	None identified	G_s	G_s
Gene/chromosomal localization	HTR5A/7q36.1	htr5b/2q11-q13	HTR6/1p35-36	HTR7/10q23.3-24.3
Structural information	h357 P47898	m370 P31387	h440 P50406	h445 P34969^{AS}
	m357 P30966	r370 P35365	m440 NP_067333	m448 P32304
	r357 P35364		r438 P31388	r448 P32305^{AS}

Indeed, 5-HT is also a substrate for the 5-HT transporter, itself an important target in the treatment of depression and the extended range of anxiety disorder spectrum (GAD, OCD, social and other phobias, panic and post-traumatic stress disorders). The 5-HT transporter is the target for SSRIs (selective serotonin reuptake inhibitors) such as fluoxetine, paroxetine, fluvoxamine, and citalopram or the more recent dual reuptake inhibitors (for 5-HT and noradrenaline, also known as SNRIs) such as venlafaxine. There are still efforts to develop triple uptake inhibitors (5-HT, NE, and DA) and/or drugs combining receptor modulation and uptake blockade.

5-HT_{1A} receptor agonists, such as buspirone, tandospirone, or gepirone, were developed for the treatment of anxiety and depression. Furthermore, the 5-HT_{1A} receptor and β-adrenoceptor antagonist, pindolol, was reported to enhance the therapeutic efficacy and shorten the onset of action of SSRIs upon co-administration in severely depressed patients. It is suggested that the combination therapy functions provided that sufficient 5-HT_{1A} receptor occupancy is reached. The antidepressants vilazodone and vortioxetine combine 5-HT uptake inhibition and 5-HT_{1A} agonism. A number of antipsychotics such as clozapine, ziprasidone, quetiapine, aripiprazole, lurasidone, and cariprazine also share 5-HT_{1A} agonism, which may help with hypofrontality and memory impairment.

5-HT_{1B} receptors have both a CNS and a vascular distribution and are the targets of the antimigraine drug sumatriptan, a non-selective 5-$HT_{1D/1B}$ receptor agonist, ergolines, and many other triptans (ergotamine, dihydroergotamine (DHE), zolmitriptan, naratriptan, rizatriptan, eletriptan, almotriptan, donitriptan, etc.).

However, most of the triptans have at least equal affinity to 5-HT_{1D} receptors, which are not expressed in vessels. The 5-HT_{1D} approach to target migraine with 5-HT_{1D} agonists, as a non-vascular target, did not succeed. Indeed, the selective 5-HT_{1D} receptor agonist PNU-142633 displayed no efficacy in migraine patients.

There is no evidence for any clinical relevance of the 5-ht_{1E} receptor. By contrast, since sumatriptan and naratriptan also bind to the 5-HT_{1F} receptor, it has been hypothesized that 5-HT_{1F} might be a target for antimigraine drugs. 5-HT_{1F} receptor mRNA is present in the trigeminal ganglia, stimulation of which leads to plasma extravasation in the dura, a component of neurogenic inflammation linked to migraine. Lasmiditan

(COL-144, LY573144), a selective 5-HT_{1F} receptor agonist, has been developed and registered for the treatment of migraine, with no evidence of cardiovascular side effects.

Ketanserin, a selective 5-HT_{2A} antagonist was initially developed to treat hypertension, but 5-HT_{2A} receptor antagonism as antihypertensive principle has failed. LSD and other psychedelics (e.g., psilocybin, MDMA, and N,N DMT) most probably produce their effects by activating central 5-HT_{2A} receptors, although their selectivity vis- à -vis 5-HT_{2B} and 5-HT_{2C} receptors is limited. Psilocybin and MDMA received FDA breakthrough therapy status for the treatment of treatment-resistant depression and PTSD, respectively. There is a renewed interest in LSD and other psychedelics for the treatment of a number of neuropsychiatric conditions, including substance use disorders. 5-HT_{2A} receptor antagonists such as risperidone, ritanserin, seroquel, olanzapine, volinanserin (MDL 100907), or cariprazine have been developed for the treatment of schizophrenia. The combination of dopamine D_2 and 5-HT_{2A} receptor antagonism may still explain the beneficial antipsychotic activity of drugs such as clozapine, olanzapine, seroquel, and many others. More recently, pimavanserin was approved by the US FDA to treat hallucinations and delusions associated with Parkinson's disease, although it did not meet clinical endpoints in schizophrenia.

BW 723C86 is a selective agonist for the rat 5-HT_{2B} receptor, less selective at human receptors, and was investigated in various neuropsychiatric indications. However, 5-HT_{2B} receptor activation is responsible for the valvulopathies reported for appetite suppressant preparations containing fenfluramine, dex-fenfluramine, or benfluorex; therefore 5-HT_{2B} agonism is strictly banned from all drug development programs. 5-HT_{2B} antagonists have been in clinical trials with indications such as migraine, pulmonary hypertension, irritable bowel syndrome, and various forms of fibrosis, but no compound has been registered so far.

The anxiogenic component of mCPP may be mediated by 5-HT_{2C} receptor activation, and selective 5-HT_{2C} receptor antagonists such as agomelatine display anxiolytic/antidepressant properties in animal models. Central 5-HT_{2C} receptor activation produces a tonic, inhibitory influence upon frontocortical dopaminergic and adrenergic, but not serotonergic transmission. Agomelatine a 5-HT_{2C} antagonist, combined with melatonin 1/2 receptor agonist, is a clinically effective antidepressant. Lorcaserin, a 5-HT_{2C} receptor agonist, was registered to treat obesity and eating disorders but was withdrawn (Tox issues). 5-HT_{2C} agonists are being investigated in obesity, substance use disorders, depression, and schizophrenia. The 5-HT_{2C} agonist vabicaserin entered phase II trials in schizophrenia, but the clinical outcomes were moderate, and development stopped.

5-HT_3 receptor antagonists, e.g., ondansetron, granisetron, and tropisetron, are used clinically to treat chemotherapy- and radiotherapy-induced nausea and vomiting. Since 5-HT_3 receptor activation in the brain leads to dopamine release, and 5-HT_3 receptor antagonists produce central effects similar to those of antipsychotics and anxiolytics, schizophrenia and anxiety were also considered as potential indications. 5-HT_3 receptor antagonists have been reported to have cognition enhancing effects and active in pain and migraine models, but neither of these did materialize in clinical studies. On the other hand, alosetron is now indicated for women suffering from IBS with diarrhea (IBS-D), although under very strict conditions (black box).

Selective 5-HT_4 receptor ligands have therapeutic utility in a number of disorders, including cardiac arrhythmia, neuro-degenerative diseases, depression, urinary incontinence, and GI motility disorders. Cisapride, a gastroprokinetic agent, acts as an agonist at the 5-HT_4 receptor but was withdrawn due to side effects (QT prolongation). Metoclopramide is a 5-HT_4 agonist, with dopaminergic activity. Tegaserod, a 5-HT_4 receptor partial agonist, was used to treat constipation-predominant IBS (IBS-C) and constipation and, after a withdrawal, has been reintroduced under a limited schedule. The 5-HT_4 agonist prucalopride increases bowel movement in volunteers and is registered to treat chronic constipation. Finally, the 5-HT_4 antagonist piboserod is used to treat atrial fibrillation.

Multiple antipsychotics (clozapine, olanzapine, fluperlapine, and seroquel) and

antidepressants (clomipramine, amitriptyline, doxepin, and nortriptyline) are 5-HT_6 receptor antagonists. This attribute suggested an involvement of the 5-HT_6 receptor in psychiatric disorders. Selective 5-HT_6 receptor antagonists have positive effects in preclinical models of memory impairment and cognition. The 5-HT_6 receptor antagonist idalopirdine shows positive clinical effects in Alzheimer's disease when administered in combination with the cholinesterase inhibitor donepezil.

The 5-HT_7 receptor has affinity for multiple atypical antipsychotics, e.g., clozapine, risperidone, amisulpride, aripiprazole, or lurasidone, and various antidepressants. The presence of 5-HT_7 sites in the limbic system and thalamocortical regions suggests a role in affective disorders, whereas the expression in the supra chiasmatic nucleus supports a role in circadian rhythms. Various 5-HT_7 antagonists were/are investigated in depression, psychosis, schizophrenia, and circadian disorders.

Cross-References

► Antidepressant Drugs

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Sertoli Cell Barrier

► Blood-Testis Barrier

Severe Eosinophilic Asthma

► Interleukin-5

Sex Steroid Receptors: Androgen Receptor, Estrogen Receptors, Progesterone Receptor

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Definition

Sex steroid receptors are members of the steroid hormone receptor (SHR) family that ligand-

dependently regulate functions of the sexual organs. Sex steroid receptors are the androgen receptor (AR), the estrogen receptor α and β (ERα, ERβ), and the progesterone receptor (PR) (OMIM 313700, 2019; OMIM 133430, 2020; OMIM 601663, 2020; OMIM 607311, 2019; respectively).

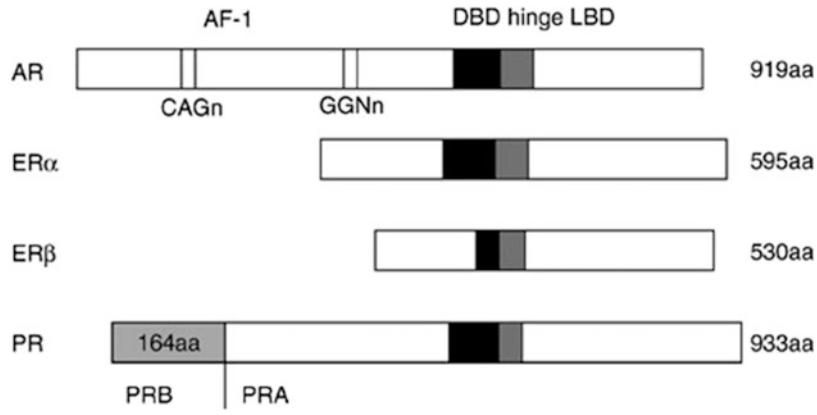
Basic Characteristics

The subgroup of SHRs belongs to the superfamily of nuclear receptors, which transactivate target genes ligand-dependently. Unliganded SHRs are associated with large multiprotein complexes of chaperones in the cytoplasm, in contrast to other nuclear receptors. SHRs comprise the glucocorticoid receptor and the mineralocorticoid receptor and the sex steroid receptors.

SHRs are built in a modular structure with similar structure elements. They contain a DNA-binding domain (DBD), a hinge region with a nuclear location signal (NLS), a ligand-binding domain (LBD), and several transcriptional activation functions (Fig. 1). Their ligands are fat-soluble steroid hormones derived from cholesterol that bind to the LBD of their specific intracellular SHR after diffusing into the cell. After binding of the steroid hormone (K_d between 0.1 and 4 nM), the conformation of the SHR changes, exposing the NLS and the complex of steroid hormone, and SHR gains access to the nucleus (Fig. 2). Utilizing the two zinc fingers of their DBD, SHRs bind as homodimers to unique DNA sequences called hormone response elements (HREs). The HRE is comprised of two half-sites organized as palindrome with a three nucleotide spacer. SHRs regulate the expression of target genes after association with large multisubunit complexes that contain transcriptional co-activators such as histone acetylases and several other proteins that facilitate transcription. Several signaling pathways furthermore influence the activity of SHRs, by modifying either SHRs directly or partner proteins. SHRs can also act without binding to DNA via interaction with other transcription factors, thereby altering their own or their partner's properties.

During the last years, there is increasing evidence that SHR is also associated with the plasma membrane. Rapid, membrane-initiated steroid signals (MISS) were characterized in cell and mouse models for all SHR. Selective pharmacological tools are on the way to dissect the role of genomic and non-genomic signals in vivo.

The physiological and pathophysiological roles of the sex steroid receptors are diverse and will be summarized separately for AR, ERα, ERβs, and PR in the following paragraphs. Estrogen-related receptors (ERRs) comprising ERR-alpha, ERR-beta, and ERR-gamma (gene symbol ESRRA, ESRRB, ESRRG) share structural and functional similarities with ERs. They are orphan receptors indicating that there is no known natural ligand and are therefore not grouped as SHR.



Sex Steroid Receptors: Androgen Receptor, Estrogen Receptors, Progesterone Receptor, Fig. 1 Schematic structures of AR, ERα, ERβ, and PR

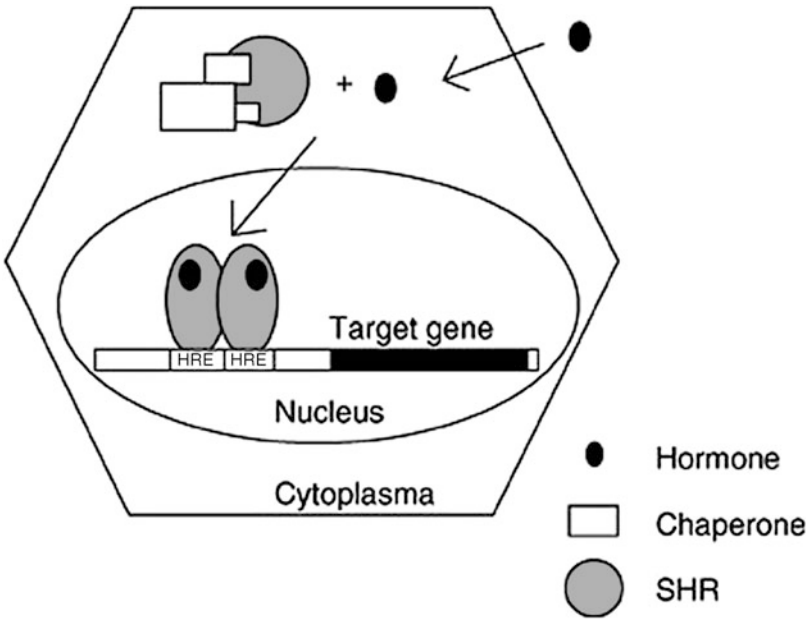
Androgen Receptor

Androgens act via the AR and play an important role in the development and differentiation of the male sexual organ. Furthermore, they are involved in several diseases, the most important being partial and complete androgen insensitivity syndrome (AIS; formerly known as the testicular feminization syndrome), spinal and bulbar muscle atrophy (SBMA; Kennedy's disease), and the neoplastic transformation of the prostate. The two natural occurring androgens are testosterone (T) and the more potent 5α-dihydrotestosterone (DHT). T is mainly produced by the Leydig cells of the testis and can also be produced in most peripheral tissues from the adrenal-produced inactive steroid precursors dehydroepiandrosterone, its sulfate, and androstenedione. T is converted into DHT by the 5α-reductase enzyme expressed in the urogenital tract. Besides positive regulation of target genes by the androgen-loaded AR, there is growing evidence of additional regulation pathways and indirect regulation mechanism of the AR. The expression of specific transcriptional coactivators of the AR in different tissues can fine-tune the transcriptional AR activity. Ligand-independent activation of the AR by protein kinase pathways can circumvent the need for androgens. Non-genomic AR signaling activates c-Src, a tyrosine kinase known to drive prostate metastasis, and p85α the regulatory subunit of PI3K (phosphatidylinositol 3-kinase). In addition, protein-protein interactions of the AR with other transcription factors regulate the transcriptional activity of these partner proteins.

Eight exons of the AR gene encode a protein of around 917 aa depending on two polymorphic regions of polyglutamines (CAG) and polyglycines (GGN) in the N-terminal activation domain. Two isoforms are detected in tissues: the predominant (80%) 110 kD (B isoform) and 87 kD (A isoform). It is under investigation whether the two isoforms also serve different functions.

The structure of the AR comprises an N-terminal transactivation domain of around 500 aa, a DBD of 66–68 aa, and a LBD of 250 aa. The hinge region contains the lysine-rich NLS. The AR possesses two activation functions (AFs): AF-1 in the N-terminal region and the AF-2 core domain in the LBD.

The AF-1 contains two polymorphic regions of CAG and GGN repeats. Normally the number of the 5′ CAG repeats is 11–31 (average 21), whereas up to 50 repeats are found in individuals affected with SBMA. Since the number of glutamines inversely correlates with the transcriptional



Sex Steroid Receptors: Androgen Receptor, Estrogen Receptors, Progesterone Receptor, Fig. 2 The classical activation pathway of SHR. SHRs (gray circle) are associated with chaperones (rectangles). After binding of steroid hormones (black circle), SHRs activate target genes in the nucleus. Additional regulation mechanisms, e.g., phosphorylation, are described in the text. For non-genomic signaling, see Arnal (2017)

activity of the AR, these amplified repeats lead to a reduced activity of the AR. The increased size of a polymorphic tandem CAG repeat is associated with the X-linked spinal and bulbar muscular atrophy and may also be associated with oligospermic infertility in men and with low serum androgens in a subset of anovulatory female patients. On the other hand, a shorter CAG length correlates with a higher risk of more severe and earlier onset of prostate cancer probably resulting from a higher activity of the AR. Experimental evidence also correlates increased or prolonged induction of AR activation with a higher incidence or acceleration of prostate cancer.

In the beginning, prostate cancer cells are largely dependent on androgens for growth and survival. Observations that castration is beneficial in prostate cancer made androgen ablation and antiandrogen therapy a standard treatment for patients with metastatic prostate cancer following surgery of the tumor tissue. Antiandrogen therapy includes inhibition of androgen synthesis by aminoglutethimide or ketoconazole and inhibition of 5α-reductase by finasteride in combination with AR antagonists such as flutamide or cyproterone acetate. Second-generation antiandrogens are currently in clinical trials.

Unfortunately, remaining prostate cancer cells eventually adapt to grow in the low-androgen environment, rendering the tumor growth independent of androgens. Androgen-independent prostate cancer may result from one or more of the following mechanism: increased gene copy number; altered interaction of the AR with coregulatory proteins, e.g., resulting from AR mutations, bypassing of the AR pathway; or ligand-independent activation of AR, e.g., by protein kinase pathways and vice versa.

Estrogen Receptor

Estrogens mainly affect the growth and maturation of the female reproductive system and the maintenance of its reproductive capacity [5]. In addition, estrogens act on several other tissues, e.g., on lipid and bone metabolism. Uterus, placenta, and testis are the principal sites of 17-β-estradiol (E2) production. Agonists of ERs are used for treatment of menopausal symptoms, hormone replacement therapy (HRT), osteoporosis, and cardiovascular diseases, whereas antagonists of ERs such as tamoxifen are used for treatment of breast cancer. Pure antagonists of ER that are as effective as tamoxifen without having tamoxifen's partial agonistic effects on ERs are in different phases of testing. The pure antagonist fulvestrant (ICI 183,780) is licensed as treatment for advanced breast cancer. The main isoforms of the human ERs are ERα and ERβ, which display distinct expression patterns. Additional ER isoforms, generated by alternative mRNA splicing, have been identified in several tissues. A cell-specific localization for each of the ER subtypes is found in the majority of the reproductive organs studied. The role of the different ER isoforms in modulating the estrogen response or in tumorigenesis is not completely understood. In addition, ERRs which were coexpressed with ERs can influence the expression level of ER target genes either directly or by interaction with ERs. ERs bind most ligands with similar affinities and display equal transcriptional activation. However, in some assays ER isoforms respond differently to ligands. The naturally occurring phytoestrogen genistein or antiestrogens, such as tamoxifen or raloxifene, are examples of these selective ER modulators (SERMs). The characterization of SERMs that specifically regulate defined functions promises to increase efficacy and reduce side effects in estrogen-regulated processes (Table 1).

ERα

ERα (also called ESR1 and ESRA) is involved in the differentiation and maintenance of reproductive, neural, skeletal, and cardiovascular tissues. Two separate AFs mediate transcriptional activation, the ligand-dependent AF-2 in the LBD and the ligand-independent AF-1 in the N-terminus. After binding of estrogen to the LBD, ERα activates target genes such as the progesterone receptor gene by binding to the estrogen response elements (EREs). Besides this classical activation, nonestrogen-dependent activation of ERα has been described. Signaling pathways and extracellular signals such as EGF, IGF-I, or insulin

Sex Steroid Receptors: Androgen Receptor, Estrogen Receptors, Progesterone Receptor, Table 1 Basic characteristics of sex steroid receptors

SHR	Gene map locus	cDNA size	Natural agonist	Binding half-site	Main expression
AR	Xq11–q12	Ca. 919 aa (see text)	5α-dihydrotestosterone (DHT), testosterone (T)	AGAACA	Prostate, male urogenital system, muscle
ERα	6q25.1	595 aa	17β-estradiol (E2)	AGGTCA	Ovary, uterus, mammary gland, vagina, testis (Leydig cells), bone
ERβ	14q22–q24	530 aa, 583 aa, further isoforms	17β-estradiol (E2)	AGGTCA	Ovary, testis (Sertoli and Leydig cells, efferent ducts), prostate, bone, thymus, spleen, brain
PR	11q22	Two isoforms:	Progesterone	AGAACA	Uterus, ovary, central nervous system
		PRA 769 aa			
		PRB 933 aa			

SHR Sex Steroid receptors, *AR* Androgen receptor, *ER* Estrogen receptors, *PR* Progesterone receptor

stimulate phosphorylation of the receptor. Phosphorylation of ERα affects all steps of transcriptional activation, such as ligand binding, dimerization, DNA binding, and interaction with cofactors. Heregulin (NRG1) also called Neuregulin 1 is an example of an extracellular signal modulating ER activity. After binding of NRG1 to its receptor ErbB2 (HER-2), ER is rapidly phosphorylated, followed by transcription of the *PR* gene. Since heregulin promotes hormone-independent growth of breast cancer cells, activation of ER by ErbB2 signaling may be involved in the development of E2-independent cancer cell growth.

Besides the classical activation model, ERα is also found associated with the plasma membrane via palmitoylation, from where it initiates rapid, non-nuclear signaling. These nongenomic, rapid, membrane-initiated steroid signals (MISS) are particularly relevant for endothelial effects of estrogen. MISS-selective agents such as estrogen-dendrimer conjugates and mouse models, e.g., carrying mutations for the palmitoylation site of ERα, will help to dissect genomic and membrane-initiated signals of ERα and their jointly actions.

In addition to the major 66 kDa full-length ERα (ESR66), a 46-kD isoform exists that lacks the 173 N-terminal amino acids which results either from alternative splicing, proteolysis, or translational variation. ESR46 can competitively inhibit the ligand-independent transactivation function of the larger receptor and modulate membrane-initiated estrogen actions. The regulation of its expression that is detected in various cell types and a majority of breast tumors is currently not understood.

ERα can also regulate gene expression by interacting with different transcription factors. For example, interaction of ERα with the c-Rel subunit of NF-κB prevents binding to NF-κB response element resulting in reduced interleukin-6 transcription. Here, ERα acts E2-dependently but without directly binding to DNA. However, the complex formation of ER with the transcription factor Sp1 is hormone-independent and enhances Sp1 binding to DNA. ERα as well as ERβ thereby enhances transcription of the retinoic acid receptor *α1* gene. Other partner proteins are fos/jun family members, which regulate gene expression via AP-1 sites. In this situation E2 can either act as agonist in the presence of ERα or as antagonist in the presence of ERβ. Another possibility to modulate ER signaling is the aryl hydrocarbon receptor (AhR). Ligands for AhR mediate antiestrogenic effects by several pathways.

The measurement of ER has become a standard assay in the clinical management of breast cancer. The presence of ERα identifies those breast cancer

patients with a lower risk of relapse and better clinical outcome. Receptor status also provides a guideline for those tumors that may be responsive to hormonal intervention. But only about half of ER-positive patients respond to hormonal therapies. Of those who respond initially, most will eventually develop an estrogen unresponsive disease following a period of treatment even though ERα is often still present. Mutant receptors and constitutively active receptors as well as hormone-independent activation of the ERα are discussed. The involvement of ERβ isoforms is under investigation.

Analysis of ERα-deficient mice showed that both sexes are infertile and display a variety of phenotypic changes associated with the gonads, mammary glands, reproductive tracts, and skeletal tissues. In addition, both hyperplasia and hypertrophy of adipocytes were found in these mice.

In females, ovariectomy or menopause leads to rapid loss of trabecula bone and osteoporosis indicating that E2 maintains bone mass. E2 enhances bone formation by osteoblasts and suppresses bone resorption by osteoclasts by regulating several important growth factors. In mice, ERα seems to induce growth but not maintenance of trabecular bone, whereas ERβ terminates growth during late puberty. However, while in humans the role of ERα and ERβ in bone is not completely clear, ESRA polymorphism is related to bone density and height during late puberty and at attainment of peak bone density in young men.

ERβ

In 1995 the discovery of ERβ (also called ESR2 and ESRB) explained many actions of estrogens in tissues where no ERα was found. ERβ shows high homology to ERα in the DBD and LBD but encodes a distinct transcriptional AF-1 domain. At least five isoforms, designated ERβ-1 through ERβ-5, are described that differ in their C-terminal sequences and tissue expression patterns or have extended N-termini. This new complexity of isoforms is further enhanced by the fact that ERβ isoforms cannot only heterodimerize with each other but also with ERα. The functional consequences for the action of estrogens depending on the expression pattern are only beginning to be evaluated.

ERβ is highly expressed in the ovary, male organs, and parts of the central nervous system (CNS), but also in other organs such as spleen and thymus. The phytoestrogen genistein binds better to ERβ than to ERα, whereas the partial ERα agonists tamoxifen, raloxifene, and ICI-164384 are antagonists for ERβ. It has been postulated that co-factor recruitment is different for the ERs; however knowledge about this interesting field of selective ER regulation is only beginning to accumulate. Since only ERβ is expressed widely in the male urogenital tract of several animals, it is now under evaluation whether the pronounced effects of E2 in men are caused by direct action of E2 on ERβ in these reproductive organs. The view that E2 acts only indirectly by reducing androgen levels via the CNS clearly has to be corrected.

Analysis of ESRB $^{-/-}$ mice showed fewer and smaller litters than wild-type mice as well as abnormal vascular function and hypertension. The reduction in fertility was attributed to reduced ovarian efficiency. Mutant females had normal breast development and lactated normally. Older mutant males displayed signs of prostate and bladder hyperplasia. *Esr2*-deficient mice furthermore display diverse regulatory defects in the function of the brain, lung, and white blood cells. The results indicated that ESRB is essential for normal ovulation efficiency but is not essential for lactation, female or male sexual differentiation, or fertility.

Progesterone Receptor

The PR is involved in diverse functions in female reproduction, such as implantation of the embryo, and in the maintenance of pregnancy. Progesterone is mainly produced in the corpus luteum in the second half of the menstrual cycle and in early pregnancy, later in the placenta. The PR is expressed in the uterus, ovary, and the CNS. In men there is no known function. Estrogens induce expression of the *PR* gene. PR agonists such as medroxyprogesterone or the synthetic R5020 are called progestins or gestagens.

The human PR exists as two functionally distinct isoforms PRA and PRB transcribed from two promoters from a single gene. PRA lacks the N-terminal 164 aa and is a 769 aa protein. PRB functions as a transcriptional activator in most cell and promoter contexts. In contrast, PRA is transcriptionally inactive and functions as a strong ligand-dependent transdominant repressor of SHR transcriptional activity. Different cofactor interactions were demonstrated for PRA and PRB, probably due to an inhibitory domain within the first 140 aa of PRA, which is masked in PRB. Both PR isoforms, however, repress estradiol-induced ER activity when liganded. Several other mRNA isoforms are present in PR-positive tissues such as breast cancer with unknown clinical significance.

In addition to the classical activation pathway, a substantial part of PR action is mediated by non-genomic signaling by interaction of PR with other signaling pathways. For example, rapid, non-genomic cytoplasmic signaling by PR is initiated by binding to c-Src via a specific polyproline motif in the N-terminal domain of PR, leading to downstream MAP kinase pathway activation.

Mice models reveal that both PR forms are physiologically important. Mice lacking the *PR* gene fail to ovulate, are infertile, and have impaired thymic function. Selective PRA-deficient female mice are infertile due to reduced oocyte and uterine deficiency in implantation. However, these mice had normal mammary epithelium proliferation and differentiation and showed normal thymic involution. In mice, PR regulates expression of proteases that degrade the follicular wall, thereby facilitating ovulation.

In breast cancer patients, total PR status is measured for hormonal treatment. The presence of PR is associated with increased survival rates and hormonal responsiveness of mammary tumors. PR agonists are widely used in contraception, hormone replacement therapy (HRT), breast cancer, and endometrial hyperplasia. Antiprogestins such as RU486 are used for blocking ovulation and preventing implantation, and in addition they are in clinical testing for the induction of labor and to control various neoplastic transformations.

Selective progesterone receptor modulators (SPRM) (mesoprogestins) are PR ligands with agonistic and antagonistic activities. Some SPRM show weak antiglucocorticoid or mixed androgenic/antiandrogenic activities. SPRM are currently tested and may be useful, e.g., for the treatment of endometriosis.

Drugs

Pure and partial agonists and antagonists (see individual SHR) are therapeutically used for contraception, hormonal ablation in breast and prostate cancer, and HRT in osteoporosis.

Contraceptives Gluco-Mineralocorticoid Receptors Selective Sex Steroid Receptor Modulators

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Silent Information Regulator 2 (Sir2) Proteins

► Sirtuins

Sirtuins

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Synonyms

Histone deacetylases; Silent information regulator 2 (Sir2) proteins

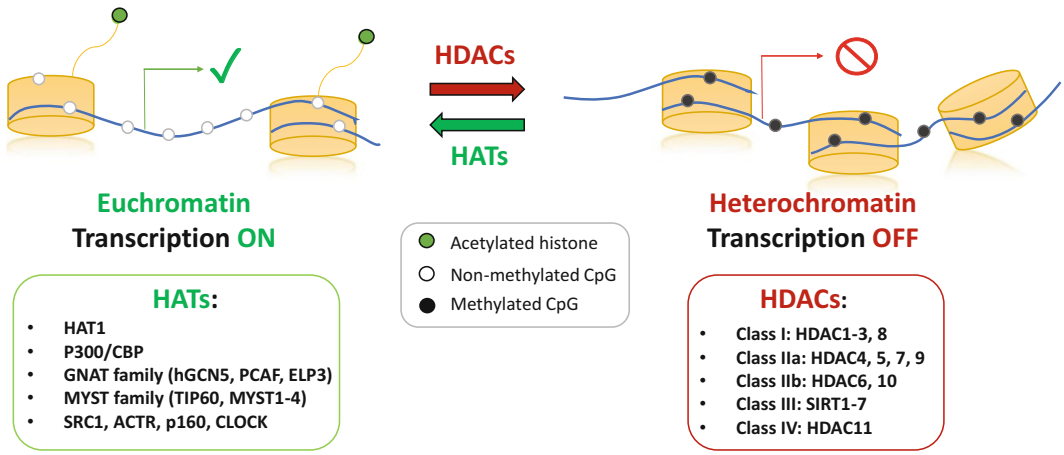
Definition

Histone lysine residues are modulated by post-translational modifications (PTMs) that specifically regulate the three-dimensional structure of chromatin as well as its accessibility to chromatin remodeling complexes and transcriptional factors, thus controlling the entire mechanism of gene expression. Acetylation pattern results from the balanced activities of two classes of epigenetic enzymes known as histone acetyltransferases (HATs) and histone deacetylases (HDACs) that, respectively, add (from acetyl-CoA) and remove the acetyl group from the ε-amino group of specific histone and non-histone lysine residues (Carafa et al. 2016; Schiedel et al. 2018b; Zwergel et al. 2019). HDAC enzymes have been grouped in four different classes, known as I, II, III, and IV, and based on sequence homology to their yeast orthologues Rpd3, HdaI, and Sir2. In particular, to the class III HDACs belong the silent information regulator 2 (Sir2) proteins also known as Sirtuins, which differ from classes I, II, and IV HDACs mainly because of their mechanism of action, which is nicotinamide adenine dinucleotide (NAD^+)- instead of Zn^{2+}-dependent (Carafa et al. 2016; Schiedel et al. 2018b; Zwergel et al. 2019).

► Histone acetylation always correlates with the activation of gene expression and transcription, thanks to the electrostatic change that this process entails at the chromatin level: the introduction of an acetyl group neutralizes the physiologically positive charge of the ε-amino group of histone lysine residues, thus counteracting the electrostatic interaction with the negatively charged DNA phosphate groups (Fig. 1) (Shahbazian and Grunstein 2007).

Sirtuins are involved in numerous biological processes such as cell survival and metabolism, aging, and DNA repair; thus they modulate crucial metabolic pathways.

The seven members of the Sirtuin family are more and more seen as potential targets for the treatment of cancer, metabolic, neurodegenerative, and cardiovascular diseases.



Sirtuins, Fig. 1 Illustration of the euchromatin/heterochromatin switch as a result of the HATs/HDACs balance

Basic Characteristics of Sirtuins

The Sirtuin family consists of seven distinct isoforms showing specific cellular localization, substrate, and catalytic activities which correlate with different physiopathological roles (Table 1) (Kupis et al. 2016).

In fact, despite that Sirtuins are mainly known for their deacetylating activity toward histone and non-histone proteins, such enzymes were firstly identified as ADP-ribosyltransferases, and, subsequently, also the capability to remove acyl moiety different from the simple acetyl group, including lipoyl, malonyl, glutaryl, 3-hydroxy-3-

Sirtuins, Table 1 SIRT1–7 characterization in terms of localization, substrates, catalytic activity, functions, and relative pathological roles

Sirtuin	Localization	Substrates	Catalytic activity	Functions	Pathology
SIRT1	Nucleus, cytosol	PGC1α, eNOS, FOXO, NF-kB, MyoD, PPAR α, H1K26ac, H3K9ac, H4K16ac	NAD^+-dependent protein deacetylation	Cell survival, insulin, signaling, inflammation, metabolism regulation, lifespan regulation, oxidative stress response	Colon, prostate, ovarian, lung, and breast cancer, glioma, melanoma, lung adenocarcinoma, acute myeloid leukemia (AML); neurodegenerative diseases
SIRT2	Nucleus, cytosol	H3K56ac, H4K16ac, α-tubulin, Foxo3a, p53, G6PD, MYC	NAD^+-dependent protein deacetylation, demyristoylation	Cell cycle regulation, nervous system development	Glioma; neurodegenerative diseases
SIRT3	Nucleus, mitochondria	AceC2, ShdhA, SOD2, PDMC1a, IDH2, GOT2, FoxO3a	NAD^+-dependent protein deacetylation	Regulation of mitochondrial energetic metabolism	B-cell chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), breast and gastric cancer; neurodegenerative diseases
SIRT4	Mitochondria	GDH, MCD, PDH	NAD^+-dependent protein deacylation (acyl, lipoyl, and 3-hydroxy-3-methyglutaryl) and ADP-ribosylation	Regulation of mitochondrial energetic metabolism, fatty acid oxidation, and insulin secretion	Breast, colorectal cancer and esophageal squamous cell carcinoma (ESCC)
SIRT5	Mitochondria	Histone H4, CPS1, cyt c	NAD^+-dependent deacylation (malonyl, succinyl, and glutaryl)	Urea cycle and apoptosis regulation	Breast, pancreatic, non-small cell lung carcinoma
SIRT6	Nucleus	H3K9ac, H3K56ac, PARP1	NAD^+-dependent protein deacylation (acyl and long-chain fatty acyl) and ADP-ribosylation	Genome stability, DNA repair, nutrient-dependent metabolism regulation	Breast and colon cancer
SIRT7	Nucleus	RNA pol I, p53, H3K18ac	NAD^+-dependent protein deacetylation	Regulation of rRNA transcription and cell cycle	Liver, spleen, testis, thyroid, and breast cancer

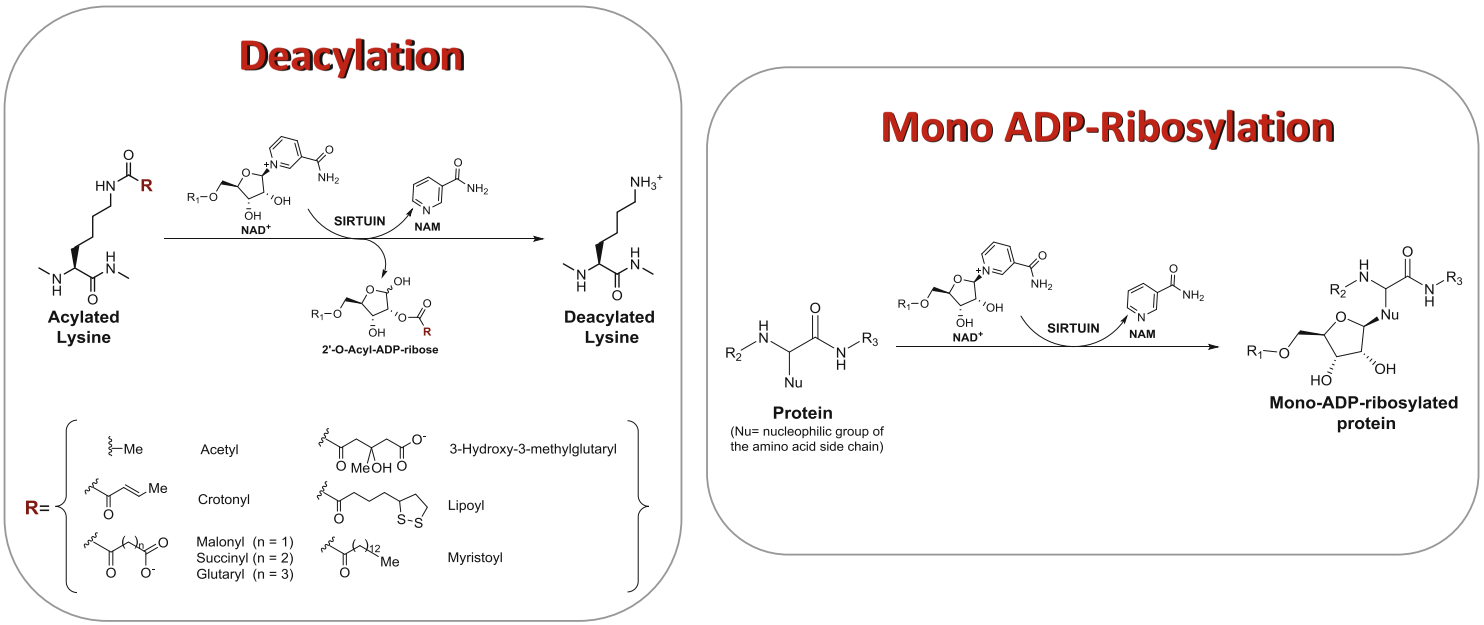
methylglutaryl, succinyl, crotonyl, fatty acyl, etc., has been highlighted (specifically for SIRT2,4–6). Given their mechanism of action (Fig. 2), Sirtuins can be described as metabolism sensors sensitive to cellular NAD^+/nicotinamide (NAM) cellular levels (Kupis et al. 2016). In particular, NAM acts as an endogenous inhibitor of their activity.

All Sirtuins show a conserved catalytic NAD^+-binding site, which consists of about 275 amino acids framed in between an *N*- and *C*-terminal sequences of different length susceptible to post-translational modifications, responsible for variability in their functions and localization (Fig. 3).

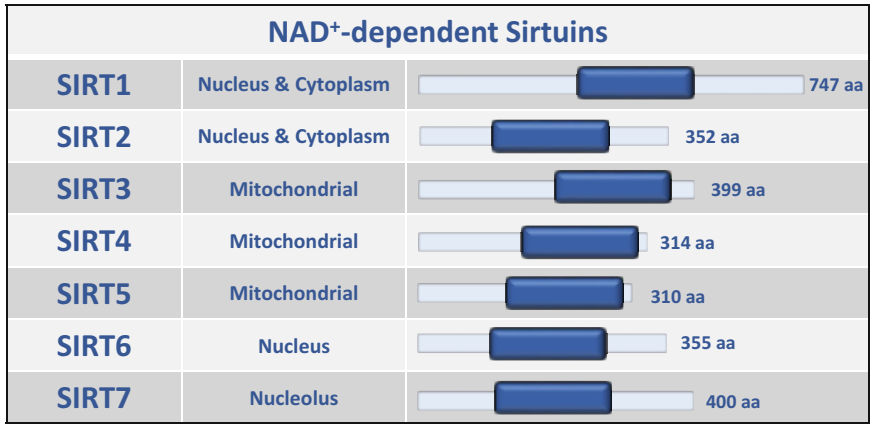
In addition to the essential α/β Rossmann-fold domain, which is typical of NAD^+-binding protein, Sirtuins are also characterized by a Zn^{2+}-binding domain that is composed of three stranded antiparallel β-sheets and a variable α-helical region. The loop responsible for the NAD^+ binding linked the Rossmann-fold with the Zn^{2+}-binding domain and is characterized by four loops, thus generating an extended cleft that forms the enzyme's active site. As previously mentioned, despite that the zinc ion does not take part into the catalysis since it is located too far from the active site, it results crucial for the structural stability of Sirtuins coordinating four different conserved cysteine residues and keeping the three β-strands together (Moniot et al. 2012). Despite that structural rearrangements at the base of the dynamic structure of this class of enzymes have been discovered, individual Sirtuins are characterized by specific preference(s) in sequence related to variables in their electrostatics properties and peptide-binding groove shape. Also, the capability of Sirtuins to recognize a huge number of substrate moieties proves their high adaptability.

Physiopathological Characteristics of Sirtuins

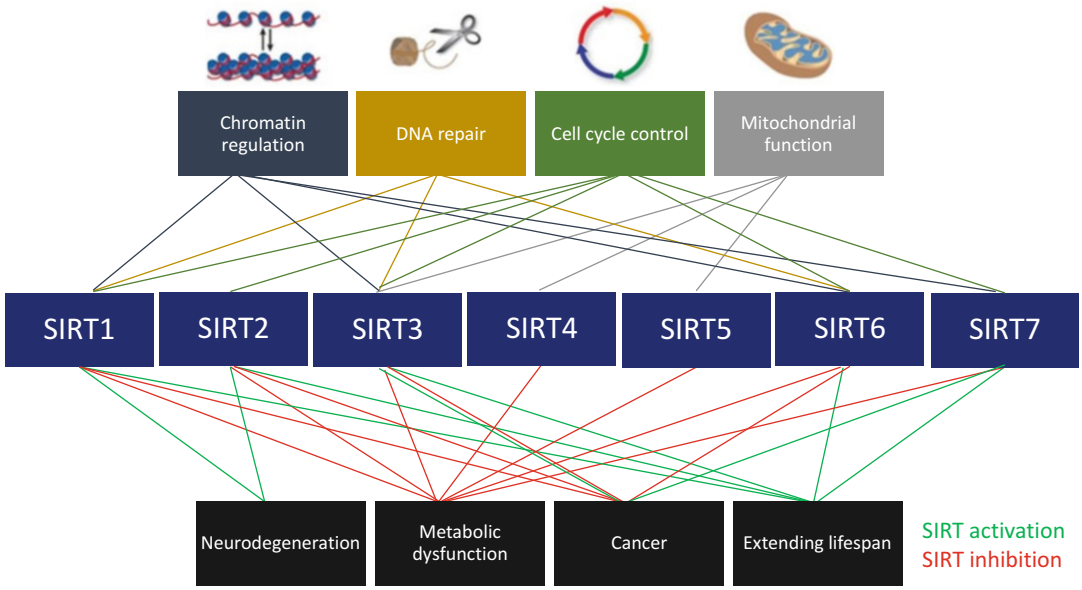
As previously mentioned, Sirtuins activities do not involve only histone substrates but also non-histone targets directly or indirectly related to chromatin regulation, apoptosis, stress response, or differentiation including p53, *c*-Fos, nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB), α-tubulin, BubR1, E2F1, tumor necrosis factor-α (TNFα), *c*-Myc, Forkhead box class O (FOXO) factors, hypoxia-inducible factors (HIFs), as well as other epigenetic proteins such as HATs, including p300 and males absent on the first (MOF), the histone methyltransferase (HMT) Enhancer of zeste homolog 2 EZH2, and DNA methyltransferase 1 (DNMT1) (Martinez-Redondo and Vaquero 2013). Sirtuins are implicated in the regulation of a huge variety of crucial processes including transcription, metabolism, aging, cell homeostasis, senescence, apoptosis, and ► inflammation. Several pieces of evidence proved their connection with the onset and progression of metabolic (obesity and diabetes), neurodegenerative (Alzheimer's (AD), Huntington's (HD), and Parkinson's diseases (PD)), and inflammatory diseases (psoriasis) as well as with both hematological (B-cell chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML)) and solid tumors (breast, colon, lung, prostate cancer, etc.); (see Fig. 4) (Carafa et al. 2016; Schiedel et al. 2018b; Zwergel et al. 2019). SIRT1 is the first SIRT family member to be identified and is the isoform for which the most exhaustive information has been collected to date. Through its deacetylase activity, SIRT1 activates the peroxisome proliferator-activated receptor-γ (PPARγ) coactivator 1α (PGC1α) and FOXO1, thus promoting gluconeogenesis, whereas it inhibits glycolysis via the repression of the glycolytic enzyme phosphoglycerate mutase 1 (PGAM-1). In addition, liver kinase B1 (LKB1) SIRT1-mediated deacetylation in the skeletal muscle activates ► AMP-activated protein kinase (AMPK), thus establishing a reciprocal positive regulating loop in which AMPK in turn activates SIRT1 enhancing the level of NAD^+, thanks to the upregulation of NAD^+ synthetic enzyme nicotinamide phosphoribosyltransferase (NAMPT) encoding genes. While SIRT1 seems to exert a neuroprotective role modulating the expression and survival of brain-derived neurotrophic factor (BDNF), it also correlates with tumorigenesis leading to the inhibition of p35 and p73 via their deacetylation and to the activation of the oncoprotein BCL6, thus favoring an antidifferentiating and anti-apoptotic effect through the deacetylation of different



Sirtuins, Fig. 2 Schematization of Sirtuins mechanisms of action: on the left is represented the diacylation, and on the right the ADP-ribosylation process



Sirtuins, Fig. 3 Schematic representation of human Sirtuins in which the shared NAD^+-catalytic domain is highlighted in blue



Sirtuins, Fig. 4 Schematic representation of the involvement of SIRT isoforms in various diseases and their possible modulation

transcription factors (FOXO3a, Ku70, E2F1, etc.). Despite that an overexpression (OE) of SIRT1 has been registered in different kind of chemotherapy-resistant tumors such as prostate, breast, liver, colorectal, ovarian, melanoma, and epithelial cancer, also a downregulation of such Sirtuin correlates with malignancies (glioblastoma, bladder and colon cancer). SIRT1 functions within the oncological scenario seem to be context specific since SIRT1 modulates the expression of various DNA repair and tumor suppressor genes.

SIRT2 is principally localized in the cytoplasm and is responsible for the deacetylation of the Lys40 of α-tubulin, but several data showed that, during cell cycle progression from phase G_2 to M, it moves toward the nucleus providing the deacetylation of H4K16 which correlates with metaphasic heterochromatin. In addition, also

p300, FOXO1, FOXO3, and glucokinase regulatory protein (GKRP) have been identified as other targets of its deacetylase activity. Glucokinase (GCK) is crucial for retaining the glucose homeostasis modulated by the binding of GKRP, which, in its acetylated state, correlates with diabetes mellitus. In addition, other evidence emphasized the role covered by SIRT2 in other metabolic processes, such as adipogenesis. Unlike SIRT1, SIRT2 possesses a neurodegenerative role in neurological pathologies, and it modulates cell progression and mitotic and apoptotic events displaying both tumor promoter and suppressor capability.

SIRT3, together with SIRT4 and SIRT5, is one of the three mitochondrial Sirtuins. Specifically, SIRT3 takes part in the modulation of mitochondrial activities by providing the deacetylation and consequent activation of various electron transport chain complexes (I and II) components as well as acetyl-CoA synthetase (ACS). SIRT3 is known to be involved in the counteraction of the oxidative stress protecting cells from reactive oxygen species (ROS) in microglia and activating, under calorie restriction condition, the essential mitochondrial ► antioxidants actor superoxide dismutase 2 (SOD2) in cochlea neurons, thus playing an overall neuroprotective role. SIRT3 controls differentiation, proliferation, and survival of cells acting as a tumor suppressor through the deacetylation and activation of pyruvate dehydrogenase (PDH) that correlates with the repression of glycolysis metabolism. However, also the role covered by SIRT3 in tumors still needs to be clarified.

Until recently, SIRT4 was the less-investigated Sirtuin, and it was firstly described for its ADP-ribosyltransferase activity toward glutamate dehydrogenase (GDH). Effects of SIRT4 are a reduced amino-acid-stimulated insulin secretion in pancreatic B-cells and an increased genome stability as a consequence of the SIRT4-mediated inhibition of glutamine anaplerosis, a crucial step for an effective cell cycle arrest after DNA damage. Thus, SIRT4 seems to act as a tumor suppressor, but, in this framework, further investigations are necessary since the delayed cell cycle in DNA-damaging condition mediated by SIRT4 can also reduce the sensitivity of tumors cells to cytotoxic treatment. As proof of this, overexpression of SIRT4 correlates with increased mortality rate during cisplatin treatment (Jeong et al. 2016). SIRT4 inhibits the PDH via its lipoamidation and shows a weak deacetylase activity toward the malonyl Co-A decarboxylase (MCD), which is the main way, in addition to the indirect interaction with ANT2 and AMPK, whereby SIRT4 represses the β-fatty acid oxidation. SIRT4 also mediates the sensitivity to cardiac performance to hypertrophic stress modulating the activity of manganese superoxide dismutase (MnSOD). Recently, Pannek et al. reported the 3-hydroxy-3-methylglutaril (HMG) residue as new acyl toward which SIRT4 shows an efficient enzymatic activity solving also the crystal structure of SIRT4 from *Xenopus tropicalis* (xSIRT4, 81% similarity with human SIRT4 (hSIRT4)) (Pannek et al. 2017).

SIRT5 is mainly expressed in the heart, liver, kidney, and brain and exhibits as main targets the restricting enzyme of the urea cycle carbamoyl synthetase 1 (CPS1) and poly-(ADP-ribose) polymerase 1 (PARP1), which is responsible for the stimulation of DNA repair mechanisms upon oxidative-induced damage, toward which SIRT5 acts as a deacetylase and ADP-ribosyltransferase, respectively. Despite that SIRT5 OE was detected in non-small cell lung carcinoma (NSCLC), overlapping with poor prognosis, further evidence is required to explain in a comprehensive way its role in cancer.

SIRT6 catalyzes the removal of fatty acyl moiety from Lys19 and Lys20 of TNF-α regulating its release. SIRT6 controls metabolism, cellular homeostasis, telomere maintenance, and DNA repair and for these reasons has been defined as "epigenetic guardian of cellular differentiation."

The last component of this family, SIRT7 thanks to its capability to mediate the deacetylation of H3K18ac leading to the malignant transformation marker H3K18, is being considered a promising and challenging target in the context of the epigenetic fight against cancer. OE levels of SIRT7 were typically registered in cancers with poor prognosis, but also its depletion is associated with aggressive tumors. Also, non-

histone interactions have been identified for this protein such as those with HIF-1α and HIF-2α, thus reducing their expression.

Sirtuin Modulators

Sirtuin modulators are today applied alone or in combination with either approved drugs or other epigenetic agents. They have been investigated for the treatments of neurodegeneration or metabolic diseases as well as various types of cancer (Villalba and Alcain 2012; Schiedel et al. 2018b). Table 2 is summarizing all the SIRT modulators discussed herein.

Sirtuin Inhibitors (SIRTi)

Small Molecules

The first SIRTi splitomicin (**1**) appeared in literature in 2001 and was identified via a yeast-based phenotypic screening. This compound was inactive against human Sirtuins but was the initial point for the discovery of numerous human SIRT1/2i (Carafa et al. 2016; Zwergel et al. 2019).

One important splitomicin derivative is the SIRT1i HR-73 (**2**) able to block the HIV transcription via TAT protein deacetylation (Carafa et al. 2016; Zwergel et al. 2019).

EX-527 or selisistat **3**, discovered in 2005, was the first potent and selective cell-permeable SIRT1i. Even if this compound increased p53 acetylation levels, only poor effects were observed in cancer. Compound **3** is now being investigated with promising results in Huntington's disease also in a clinical context. Additionally, **3** blocked via a SIRT1 inhibition-mediated mechanism the viral amplification of human papillomavirus (Carafa et al. 2016; Zwergel et al. 2019).

Cambinol (**4**) is a moderate SIRT1/2i, and its treatment led to hyperacetylated p53, α-tubulin, FOXO3a, and Ku70 in lymphoma and HeLa cancer cells decreasing the tumor burden also in xenograft mice (Carafa et al. 2016; Zwergel et al. 2019). Auspicious anticancer features were also described for hepatocellular cancers and neuroblastoma (Carafa et al. 2016; Zwergel et al. 2019). Following studies resulted in various optimized derivatives with improved potency and/or selectivity for either SIRT1 or SIRT2. One example of such a strategy is MC2141 (**5**) (Carafa et al. 2016; Zwergel et al. 2019).

AGK2 (**6**) possesses a selectivity against SIRT2 over SIRT1/3, being a low micromolar SIRT2i able to maintain the acetylation of α-tubulin in HeLa cells. Compound **6** rescues dopaminergic neurons from α-synuclein toxicity in Parkinson's disease and leads to caspase-3-dependent apoptosis in glioma cells (Carafa et al. 2016; Zwergel et al. 2019).

Tenovin1 (**7a**) and its water-soluble derivative (**7b**) are micromolar SIRT1-3i able to activate p53 in melanoma, leukemia, and gastric cancer in vitro and in vivo (Carafa et al. 2016; Zwergel et al. 2019).

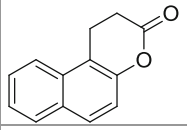
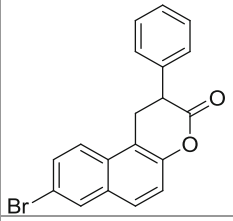
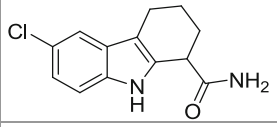
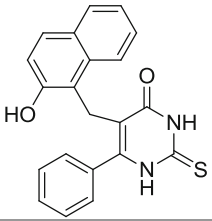
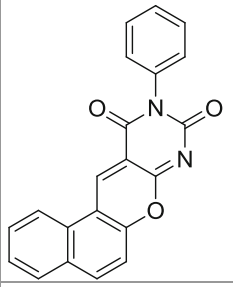
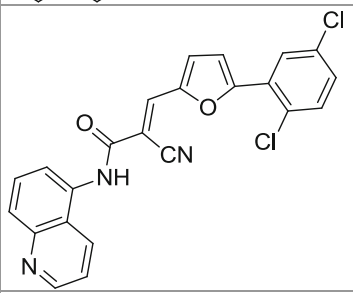
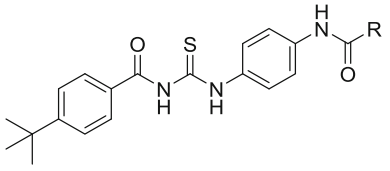
Also, sirtinol (**8**), a SIRT1/2i, identified in 2001 via a high-throughput screening, possesses numerous anticancer effects in human MCF7 and H1299 cells as well as in adult T-cell leukemia/lymphoma (ATL). Furthermore **8** increases the chemosensitivity to cisplatin and camptothecin in PC3 and HeLa cells (Carafa et al. 2016; Zwergel et al. 2019).

Salermide (**9**) is an optimized form of compound **8**. Besides the numerous anticancer effects through SIRT1 inhibition which result in the H4K16ac deacetylation and ultimately in the repression of pro-apoptotic genes such as *CASP8*, *TNF*, *TNFRSF10B*, and *PUMA*, salermide treatment also protects muscle cells in a dystrophy model (Pasco et al. 2010). Interestingly, **9** has beneficial effects upon *Schistosoma mansoni* (Sm) infections via *Sm*SIRT inhibition leading to apoptosis, death of schistosomula, and reduced egg laying (Carafa et al. 2016; Zwergel et al. 2019).

Subsequently, selective SmSIRT2i were identified from a commercial library and afterward optimized. Compound **10** possesses a good efficacy against larval and adult worms without affecting human Sirtuins and human cells (Monaldi et al. 2019).

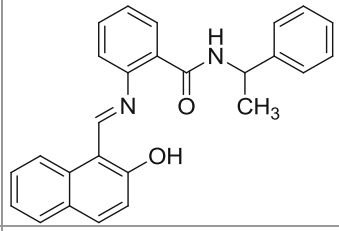
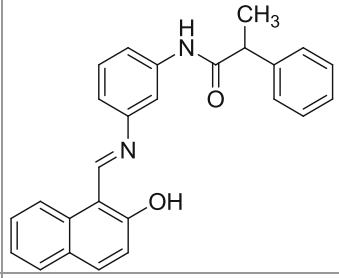
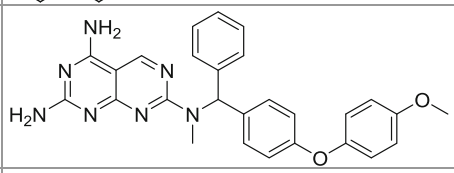
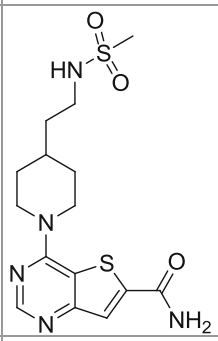
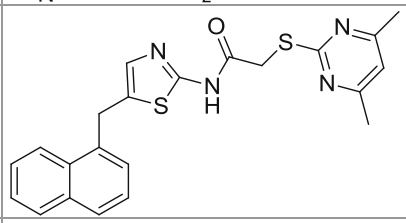
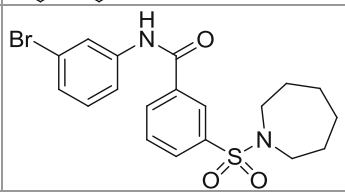
Compound **11** and its analogues are one of the most potent SIRTi discovered so far. Even though the crystal structure has been solved and a

Sirtuins, Table 2 Most relevant sirtuin modulators

Most relevant sirtuin inhibitors		
Compound	Structure	Enzyme activity
1, Splitomicin		ySir2 $IC_{50} = 60\ \mu M$ SIRT1: No inhibition at 500 μM
2, HR-73		SIRT1 $IC_{50} = <5\ \mu M$
3, EX-527, selisistat		SIRT1 $IC_{50} = 0.098\ \mu M$ SIRT2 $IC_{50} = 19.6\ \mu M$ SIRT3 $IC_{50} = 48.7\ \mu M$
4, Cambinol		SIRT1 $IC_{50} = 56\ \mu M$ SIRT2 $IC_{50} = 59\ \mu M$ SIRT3: No inhibition at 300 μM SIRT5: 42% inhibition at 300 μM
5, MC2141		SIRT1 $IC_{50} = 9.8\ \mu M$ SIRT2 $IC_{50} = 12.3\ \mu M$
6, AGK2		SIRT1 $IC_{50} > 50\ \mu M$ SIRT2 $IC_{50} = 3.5\ \mu M$ SIRT3 $IC_{50} > 50\ \mu M$
7a, tenovin-1 7b, tenovin-6	 **7a** R = CH_3 **7b** R = $(CH_2)_4N(CH_3)_2$ HCl	**7a**: IC_{50}s not determined due to lack of water solubility **7b**: SIRT1 $IC_{50} = 21\ \mu M$ SIRT2 $IC_{50} = 10\ \mu M$ SIRT3 $IC_{50} = 67\ \mu M$

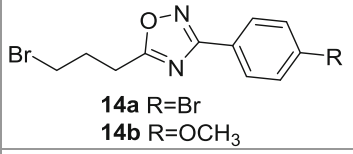
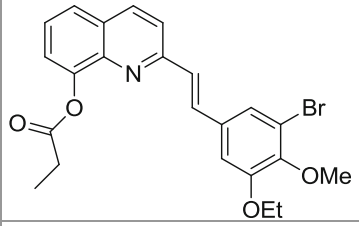
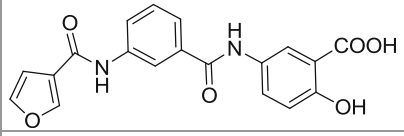
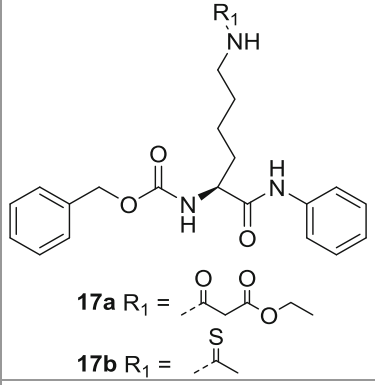
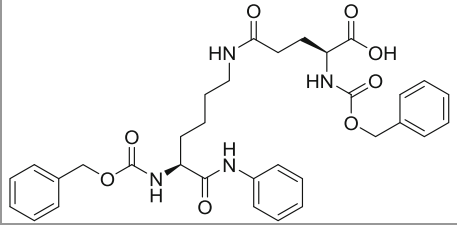
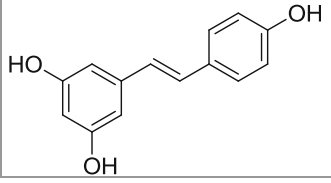
(continued)

Sirtuins, Table 2 (continued)

Most relevant sirtuin inhibitors		
Compound	Structure	Enzyme activity
8, Sirtinol		ySir2 $IC_{50} = 70$ μM SIRT1 $IC_{50} = 131$ μM SIRT2 $IC_{50} = 49$ μM
9, Salermide		SIRT1 $IC_{50} = 43$ μM SIRT2 $IC_{50} = 25$ μM
10		*Sm*SIRT2 $IC_{50} = 2.3$ μM hSIRT2 $IC_{50} = 22.1\%$ at 25 μM
11		SIRT1 $IC_{50} = 4$ nM SIRT2 $IC_{50} = 1$ nM SIRT3 $IC_{50} = 7$ nM
12, SirReal2		SIRT2 $IC_{50} = 0.14$–0.44 μM SIRT1 22% inhibition at 100 μM SIRT3 no inhibition at 100 μM SIRT4 no inhibition at 200 μM SIRT5 no inhibition at 200 μM SIRT6 19% inhibition at 200 μM
13, AK-7		SIRT2 $IC_{50} = 15.5$ μM SIRT1/3 no inhibition at 50 μM

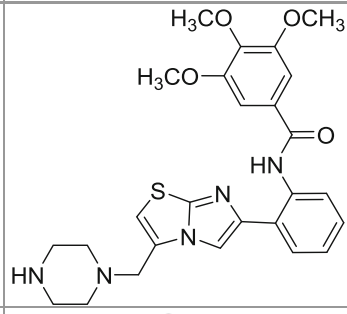
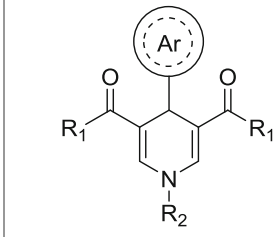
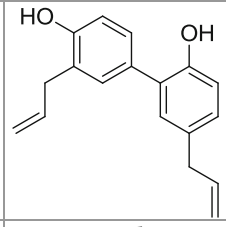
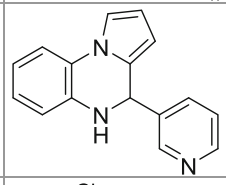
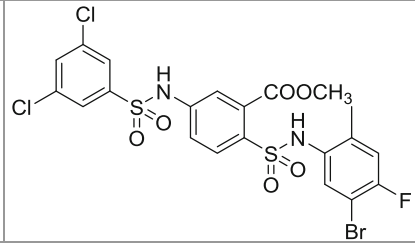
(continued)

Sirtuins, Table 2 (continued)

Most relevant sirtuin inhibitors		
Compound	Structure	Enzyme activity
14a, 14b	 **14a** R=Br **14b** R=OCH_3	**14a**, SIRT2 IC_{50} = 1.5 μM **14b**, SIRT2 IC_{50} = 10.4 μM Inactive at 100 μM against SIRT1, SIRT3, and SIRT5
15, SDX-437		SIRT3 IC_{50} = 700 nM SIRT1 no inhibition at 100 μM
16		SIRT6 IC_{50} = 89 μM SIRT1 IC_{50} = 1578 μM SIRT2 IC_{50} = 751 μM
17a, 17b		**17a** SIRT1 IC_{50} = 3.9 μM **17b** SIRT1 IC_{50} = 2.7 μM SIRT2 IC_{50} = 23 μM SIRT3 IC_{50} > 100 μM
18, MC3482		SIRT5 42% at 50 μM No inhibition for other isoforms at 50 μM
Most relevant Sirtuin activators		
19, Resveratrol		SIRT1 $EC_{1.5}$ (effective concentration able to increase the enzyme activity of 150%) = 46.2 μM

(continued)

Sirtuins, Table 2 (continued)

Most relevant sirtuin inhibitors		
Compound	Structure	Enzyme activity
20, SRT1460		SIRT1 $EC_{1.5} = 2.9$ μM SIRT1 max act. 447%
21, DHPs	 Ar= aryl, heteroaryl R_1= OEt, OH, NH_2 R_2= benzyl, benzoyl, acyl, etc.	For Ar = Ph, R_1 = OEt, R_2 = benzyl: SIRT1 $EC_{1.5} \approx 1$ μM SIRT2 $EC_{1.5} = 25$ μM SIRT3 $EC_{1.5} \approx 50$ μM
22, Honokiol		Increases SIRT3 levels by nearly twofold at 5 μM and 10 μM in cardiomyocytes after 24-h treatment In vitro directly binds to SIRT3 and increases the affinity of SIRT3 for NAD^+
23, MC3154		Increases SIRT6 levels in a dose-dependent manner (max. Twofold) $EC_{50} = 38$ μM
24, MDL-800		Increase of SIRT6 deacetylation activity by >22-fold at 100 μM $EC_{50} = 10.3$ μM

nanomolar biochemical activity against SIRT1–3 has been observed, no biological activity has yet been described (Carafa et al. 2016; Zwergel et al. 2019).

SirReal2 (**12**) is a highly selective sub-micromolar SIRT2i (>1000 fold over all SIRT isoforms) (Carafa et al. 2016; Zwergel et al. 2019).

Crystallography studies revealed a previously unexploited SIRT2-binding mode inducing a structural rearrangement in the active site of SIRT2. Hyperacetylation of α-tubulin and destabilization of the SIRT2 substrate BubR1 upon SirReal2 treatment confirmed the involvement of SIRT2 inhibition in HeLa cells. Recently the SirReal hybrid compounds with thalidomide were created to apply the proteolysis-targeting chimeras (PROTACs) approach, being the first example of chemical induction of SIRT2 degradation (Schiedel et al. 2018a).

Another micromolar, selective, and brain-penetrating SIRT2i, AK-7 (**13**), possesses SIRT2-dependent neuroprotective properties in neurodegenerative diseases such as HD and PD; furthermore it is capable to downregulate the neuronal cholesterol biosynthesis, confirming the crucial role of SIRT2 as a target in neurodegenerative disorders (Carafa et al. 2016; Zwergel et al. 2019).

SIRT2i can be applied for cancer treatment as well. The compounds **14a** and **14b** out of a 1,2,3-oxadiazole series are selective single-digit micromolar SIRT2i (inactive at 100 μM against Sirt1, Sirt3, and Sirt5) able to induce pro-apoptotic and antiproliferative effects in diverse leukemia cell lines (Carafa et al. 2016; Zwergel et al. 2019).

An example of a selective submicromolar SIRT3i has been described SDX-437 (**15**); however biological data are not yet available (Carafa et al. 2016; Zwergel et al. 2019).

The first SIRT6i **16** was found through an in silico high-throughput screening approach, a greater than eightfold selectivity over SIRT1/2 (Carafa et al. 2016; Zwergel et al. 2019). This compound might be a promising first step toward optimized SIRT6i and an eventual therapeutic application, since it hyperacetylates the SIRT6 substrate H3K9, and increases glucose uptake via GLUT-1 upregulation

Peptides and Pseudopeptides

Besides small molecules, also peptide-based SIRTi are described in literature. In the first one, the N^{ε}-acetyl-lysine residue was substituted with a thioacetylated moiety, and the *C*-terminal was prolonged with a p53 protein tail (Schiedel et al. 2018b), resulting in a SIRT1/2i with also a moderate effect on SIRT3. Thereafter other quite potent thioacetylated peptide-based inhibitors as well as other lysine N^{ε}-modifications (such as propionyl, α-hydroxyacetyl, homocitrulline, or homoarginine residues) were synthesized and tested resulting in the inhibition of SIRT1/2/3 using different SIRT substrates such as human α-tubulin or acetyl-coenzyme A synthetase 2 (AceCS2) (Schiedel et al. 2018b).

Most of the peptidic or pseudopeptidic SIRTi (**17a, 17b**) reported to date are SIRT1/2/3i; nevertheless some of them were slight active also toward SIRT6 (Carafa et al. 2016; Zwergel et al. 2019) or SIRT5 (Rajabi et al. 2017; Kalbas et al. 2018).

A specific peptide-based SIRT5i MC3482 (**18**) has been shown to increase ammonia production in non-liver cells leading to autophagy via modulation of the glutamine metabolism (Zwergel et al. 2019).

SIRT Activators

Besides the above presented SIRTi, also SIRT activators (SIRTa) have been described in literature (Schiedel et al. 2018b). The first and most prominent example is the natural polyphenol resveratrol (**19**), a SIRT1a. This compound can extend the lifespan of rather simple organisms such as yeasts, worms, fishes, and insects. Later on these promising outcomes, **19** was also published to improve mitochondrial functions in mammals such as normal and obese rodents (Carafa et al. 2016; Zwergel et al. 2019).

The selective SIRT1a SRT1460 (**20**) was identified via a high-throughput screening. Interestingly, it shows similar effects like **19** in vivo however associated with a higher potency (Carafa et al. 2016; Zwergel et al. 2019). Compound **20** and its analogues exhibited encouraging results in preclinical studies for age-related diseases; thus some of them are now evaluated in clinical trials (Carafa et al. 2016; Zwergel et al. 2019).

In the past, SIRT1 activation was much debated due to the fact that the initial studies had positive outcome only with artificial substrates such as carboxytetramethylrhodamine (TAMRA)-

labeled ones, whereas SIRT1 activation was only recently confirmed by the delicate structural and positional requirements of SIRT1 in the presence of its natural substrates such as FOXO3a and PGC-1α (Carafa et al. 2016; Zwergel et al. 2019) and by the crystal structure of an engineered human SIRT1-sirtuin-activating compound (STAC) complex, which revealed the STAC-binding site within the *N*-terminal domain of hSIRT1 (Dai et al. 2015).

1,4-Dihydropyridines (DHPs) of the general structure **21** are SIRT1a able to decrease the acetylation state of α-tubulin in U937 cells, to increase the NO release in HaCat cells, and to improve wound healing and skin repair in a mouse model via the activation of the SIRT1/AMPK pathway. Additionally, a water-soluble DHP analogue increased H4K16ac deacetylation resulting in antiproliferative effects in diverse cancer cell lines at a micromolar level (Carafa et al. 2016; Zwergel et al. 2019).

SIRT3 can be directly activated and overexpressed by the natural compound honokiol (**22**) resulting in reduced acetylation levels of the mitochondrial SIRT3 substrates MnSod or oligomycin-sensitivity conferring protein (OSCP). The compound **22** showed beneficial properties in a mouse model of hypertrophy via SIRT3 activation (Carafa et al. 2016; Zwergel et al. 2019).

Compound **23** is the first small molecule identified as SIRT6a. The biochemical and crystallographic data revealed a direct, specific, and substrate-independent binding mode of **23** within the catalytic site of SIRT6 and its subsequent potent activation which results in the deacetylation of peptide substrates and entire nucleosomes (Zwergel et al. 2019). The possibility of SIRT6 activation by small molecules has been recently further confirmed by a more potent compound **24** (Huang et al. 2018).

Cross-References

- ▶ AMP-Activated Protein Kinase
- ▶ Antioxidants
- ▶ Histone Acetylation
- ▶ Inflammation

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Skeletal Metabolism

► Bone Metabolism

Skeletal Turnover

► Bone Metabolism

Small G Proteins

► Small GTPases

Small GTPases

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Synonyms

Low molecular mass GTPases; Small G proteins

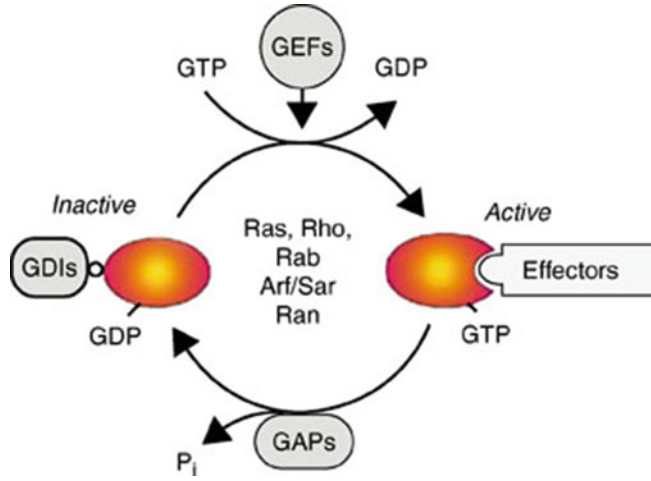
Definition

Small GTPases are monomeric 20–40 kD GTP-binding proteins that interconvert between an active (GTP-bound) and an inactive (GDP-bound) state. As molecular switches they are involved in the regulation of complex cellular processes.

Basic Characteristics

Regulation

Activation of small GTPases occurs by GDP/GTP exchange catalyzed by guanine nucleotide exchange factors (GEFs) (Fig. 1). They stimulate the dissociation of GDP in response to an upstream signal which results in binding of GTP. In the GTP-bound form the GTPases are active, and bind to and activate a number of effector molecules. The small G proteins are able to hydrolyze the bound nucleotide to GDP. This inactivation step is accelerated by GTPase activating proteins (GAPs). In the GDP-bound form the GTPases are inactive. Some GTPases bind to guanine nucleotide dissociation inhibitors (GDIs) that stabilize the inactive form and cover the lipid modification of the GTPase forming a cytosolic complex. Novel roles of GDI in GTPase regulation like the delivery of GTPases to specific sites within the cell are discussed. Moreover, local synthesis and degradation of GTPases seem to



Small GTPases, Fig. 1 The GTPase cycle: GTPases are inactive in the GDP-bound form. In a complex with guanine nucleotide dissociation inhibitors (GDIs) the inactive form is stabilized (note: not all small GTPases are regulated by typical GDIs, e.g., not Ras, Ran, and Arf subfamily proteins). Guanine nucleotide exchange factors (GEFs) cause the release of GDP and binding of GTP, and thereby the activation of the proteins. The active state of the GTPases is turned off by GTP hydrolysis catalyzed by GTPase activating proteins (GAPs)

play additional roles in the spatial regulation of small GTPases. In mammalian cells, each family of the regulating proteins GAPs, GEFs, and GDIs comprise numerous members that are more or less specific for individual GTPases, cell types, GTPase functions, and signaling pathways.

General Structural Properties

All small GTPases are folded in a similar way. They possess four consensus amino acid sequences in common, which are involved in nucleotide binding and hydrolysis: GXXXXGK, DXXG, NKXD, and EXSAX. Two highly flexible regions (Switch I and Switch II regions) determine the nucleotide-dependent activation state of the GTPases and the protein–protein interactions with effectors and regulatory proteins.

Posttranslational Modification

All small GTPases (except Ran) are posttranslationally modified. Most important is the isoprenylation of the C-terminus. The type of modification is determined by the COOH-terminal amino acid sequence. GTPases with a C-terminal CAAX-box (A = aliphatic amino acid, X = any amino acid) are farnesylated at the cysteine residue followed by the proteolytic degradation of the last three amino acids and subsequent methylation of the carboxy-terminus. In the case of CAAL or CAC, the cysteines are modified by geranylgeranylation. In some cases an additional cysteine is palmitoylated or N-terminal myristoylation occurs. All these post-translational modifications allow the interaction of GTPases with the phospholipid bilayer. Lipid modification of the GTPases is required for membrane localization and GDI binding.

Families

The superfamily of small GTPases consists of more than 100 members from yeast to human with more than 80 members expressed in mammalian cells. Based on structural and functional similarities the GTPases are subdivided into five major classes.

Ras GTPases

The mammalian family of Ras GTPases consists of more than 15 members, which share high homology to each other and include Ha-Ras, Ki-Ras, N-Ras, R-Ras, Rap, Ral, Rheb, Rin, and Rit proteins (Hancock 2003). Ras proteins have achieved attention with the discovery that they contain point mutations in 15% of all human

tumors (more than 90% in pancreatic tumors), leading to the exchange of conserved amino acids, for example, at positions 12 and 61. Amino acid exchanges at these positions block the GTP hydrolyzing capacity of the GTPases, resulting in constitutive activation. Ras GTPases are involved in proliferation and/or differentiation. They couple receptor tyrosine kinases with a cascade of cytosolic kinases termed Raf/ERK kinase pathway (also known as MAP kinase cascade). Activation of this pathway leads to phosphorylation and activation of transcription factors like Elk-1, and stimulate gene expression. Activated Ras has been shown to transform culture cells and to produce tumors in nude mice. Besides the Raf kinase, also RalGDS, which is an activator of the Ral subfamily proteins, and the PI3 kinase involved in inositol signaling are important effectors of Ras signaling. Ral GTPases (<50% identical with Ras) control cell proliferation, Ras-mediated cell transformation, vesicle traffic, phospholipase D, and cytoskeleton organization. Rap GTPases have been identified in a screen for cDNAs that are able to revert the transforming phenotype of Ki-Ras (Kirsten Ras) and, therefore, were also termed K-rev proteins.

Rho GTPases

Members (>20) of the Rho family of GTPases, including RhoA, B and C, Cdc42 and Rac1, 2 and 3, share more than 50% sequence identity. These GTPases are important regulators of the actin cytoskeleton (Etienne-Manneville and Hall 2002). RhoA regulates the formation of actin stress fibers, whereas Cdc42 is known to induce filopodia. Rac is involved in the formation of lamellipodia and membrane ruffles. Rho GTPases are involved in migration, phagocytosis, endo- and exocytosis, and cell–cell and cell–matrix contact. Rac regulates NADPH oxidase. Furthermore, Rho GTPases are involved in transcriptional activation, cell transformation, and apoptosis. Considering their diverse functions, Rho GTPases are regulated by a large number of GEFs and GAPs (>60 members of each family have been identified), suggesting spatiotemporal, function-specific regulation.

A subfamily of Rho proteins, the Rnd proteins are always GTP-bound and seem to be regulated by expression and localization rather than by nucleotide exchange and hydrolysis. Many Rho GTPase effectors have been identified, including protein and lipid kinases, phospholipase D, and numerous adaptor proteins. One of the best characterized effector of RhoA is Rho kinase, which phosphorylates and inactivates myosin phosphatase; thereby RhoA causes activation of actomyosin complexes. Rho proteins are preferred targets of bacterial protein toxins (► Bacterial Toxins).

Rab GTPases

The largest family of small GTPases with more than 40 members identified is the family of Rab GTPases (Zerial and McBride 2001). Rab proteins are important regulators of specific steps of intracellular vesicle trafficking, including budding, targeting, docking, and fusion with acceptor membranes. Each Rab protein has an organelle-specific subcellular localization and seems to be functionally specialized. Rab1A and Rab1B are two of the most extensively studied members of the Rab family. Both proteins are found in membranes of the ER, Golgi apparatus, and intermediate vesicles between these compartments. They appear to function in the anterograde trafficking of proteins from the ER to the Golgi compartment. Rab4 and Rab5 are present on early endosomes and are involved in the endocytic process, whereas Rab6 is localized at the Golgi apparatus regulating processes of the secretory pathway. One of the best studied members of the Rab protein family is Rab3a. This GTPase is a key regulator of Ca^{2+}-induced exocytosis, particularly in nerve terminals. Several effectors of Rab proteins like Rabphilin, Rabaptin, and Rim have been identified and characterized as essential for vesicle trafficking. Recently, the Rab effector Rabkinesin6 has been identified that links Rab proteins to the microtubule cytoskeleton. Rabkinesin6 may be the motor driving vesicles along microtubules from the Golgi apparatus to the periphery.

Arf/Sar1 GTPases

The name Arf (ADP-ribosylation factor) stems from its discovery as a cytosolic factor with the

ability to enhance the ADP-ribosylation of the α-subunit of the G protein G_S by cholera toxin. Arf is known to regulate phospholipid metabolism. Studies with dominant active or dominant negative mutants of Arf proteins in mammalian cells suggest the involvement of these GTPases in the trafficking of coated vesicles, and it is now known that Arf1 regulates the formation of COPI-coated vesicles for retrograde transport between Golgi apparatus and endoplasmic reticulum (Souza-Schorey and Chavrier 2006). Sar1, which is 37% identical to Arf1, is needed for the assembly of COPII proteins for vesicle transport in the opposite direction. Taken together, Arf and Sar proteins play crucial roles in the recruitment of COP components to vesicles thereby regulating vesicle budding. In contrast to the other small GTPases, Arf/Sar1 proteins are not regulated by GDI proteins, whereas different GEF and GAP proteins have been identified. Myristoylation of Arf proteins at the N-terminus is required for its membrane localization.

Ran GTPases

In mammalian cells there is only one *Ran* gene, which was discovered as a Ras-like gene (Ran: Ras-related nuclear protein) (Dasso 2002). In contrast, in yeast more than one related *Ran* genes have been identified. The predominant nuclear localization of the GTPase was the first hint that Ran is involved in nucleocytoplasmic transport processes. Interestingly, the only Ran GEF present in mammalian cells, RCC1 (regulator of chromatin condensation), is localized exclusively in the nucleus, whereas the single Ran GAP (Ran GAP1) is in the cytoplasm. This specialized localization of the regulators is the prerequisite for the asymmetric distribution of the GDP- and GTP-bound form of Ran and for its role as a nucleocytoplasmic transporter. In contrast to other GTPases, the activity of Ran is dependent on the gradient of the GTP-bound GTPase from cytoplasm to nucleoplasm that allows the transport of cargo proteins. Ran is involved in nuclear import as well as in export of proteins through the nuclear pore complex. Both processes require the formation of protein complexes, including Ran, the cargo protein, and Ran-binding proteins like importins or exportins. In addition to its transporter function, Ran has been shown to participate in microtubule organization during the M phase of the cell cycle.

Cascades and Cross-Talk

Small GTPases are not isolated molecular switches. Signaling cascades within one subfamily and cross-talk between members of different subfamilies are known. For example, Cdc42/Rac/Rho is sequentially activated after extracellular stimuli in quiescent Swiss 3T3 cells. Moreover, reciprocal modulation between Rho GTPases has been described. Ras and Rho proteins act in a cooperative manner in Ras-induced transformation. A further example of cross-talk between GTPase families is the cooperative function of Rho and Rab proteins during cell migration, with Rho proteins controlling the actin cytoskeleton, and Rab proteins regulating vesicular traffic for the recruitment of membrane material, and the recycling of proteins like integrins. Arfaptin connects signaling via Arf and Rac in regulating fundamental processes like endocytosis and secretion.

Drugs

Small GTPases, among other activities, regulate cell growth, neurite outgrowth, and signaling of immune cells involved in inflammation. Pharmacological modulation of the activity of small GTPases is thus a useful aim in cancer and anti-inflammatory therapies. Despite many years of scientific work, specific inhibition of Ras is not yet possible; however, the prenyl binding protein PDEdelta sequesters oncogenic Ras within the cytosol to suppress its membrane localization and signaling. Moreover, inhibitors for an activating mutation of the Ras effector Raf are in clinical use. Farnesyltransferase inhibitors block the posttranslational modification of several GTPases, to block correct cellular localization and therefore the transforming activity of Ras or Rho GTPases. Moreover, inhibitors of Rho effectors like Rho kinase inhibitors have been generated.

Cross-References

- ▶ Bacterial Toxins
- ▶ Growth Factors

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Small Molecules

- ▶ Rheumatoid Arthritis
- ▶ Small-Molecule Screens

Small-Molecule Screens

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Synonyms

Chemical biology; Drug screening; High-throughput screening (HTS); Small molecules

Definition

Methods to search for compounds of low molecular weight which modulate biological experiments, typically using libraries of several thousands to millions of small molecules. Robotic automation is typically used for a precise and simultaneous transfer of reagents and biologically active probes such as enzymes or living cells to microtiter test plates. Automated detection systems measure biological responses, such as a decrease of fluorescence through the inhibition of an enzyme by a small molecule. Other studies examine the effects of compounds on proteins or other purified components to measure their interactions or functions. More complex experiments analyze the effects of small molecules on living cells, such as cancer cells from patients, or pluripotent stem cells as they develop into other cell types or organ-like structures. Screens may also be based on animal models such as C. *elegans* or zebrafish embryos. Identifying small molecules that modulate biological functions has become crucial to studying molecular mechanisms in health and disease, validating novel drug targets, and developing novel diagnostics and drugs.

Description

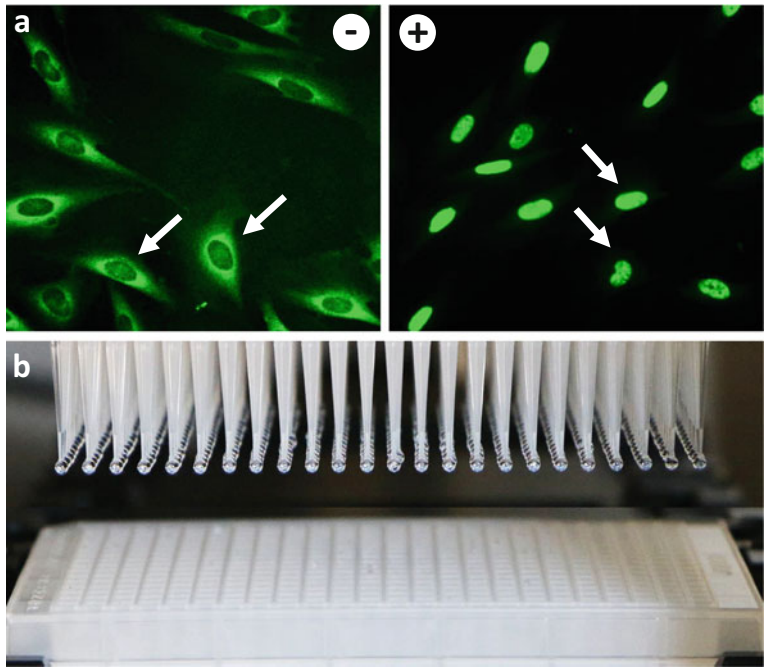
Small Molecule

A compound of natural or synthetic origin, defined by a low molecular mass of less than 1,000 daltons. Common small molecules include nucleotides in DNA, which have molecular masses of about 330 daltons; amino acids lie between 89 and 204 daltons.

Small-Molecule Screens

The process of using a library to search for small molecules that affect a biological system or experiment. Screens are often carried out using high-throughput screening (HTS), based on robotic systems and high-speed detection systems to ensure the precise transfer of reagents and accurate measurements.

The screening process comprises several steps, starting with development of a robust biological assay (Fig. 1a, b). Depending on the complexity of the biological test system, which may involve purified enzymes, cancer cells (Fig. 1a), or animal models, developing an assay for image-based screen may take up to one year. The next steps include optimizing the assay so that it can be used for moderate- to high-throughput screening in a



Small-Molecule Screens, Fig. 1 The figure summarizes the process from the development of the biological assay for high-throughput screening of compound libraries to the identification of small molecules that modulate biological functions. (**a**) *Cellular assay development:* An appropriate test is set up, with positive and negative controls such as reference drugs or an inducible cellular response. In inducible biological responses, a fluorescent protein (green color) may be sequestered in the cell cytoplasm (−); after the addition of an inducer (+), the protein may translocate to the nucleus. This example shows a transcription factor translocation to the nucleus in cancer cells, after induction and labeling by a green fluorescent protein-specific antibody. Nuclei are marked by arrows. (**b**) *Assay miniaturization:* For HTS, biological assays must be downscaled to microtiter plates containing either 1536 (48 × 32), 384 (24 × 16) or 96wells (12 × 8) per single plate and typical assay volumes ranging from 1–100 µl. Plates have a standardized footprint for robotic assay automation and are labeled by machine-readable barcodes encoding for library probes on a given test plate. Robotic grippers transport test plates from climate-controlled incubators to pipetting positions of pipetting heads with up to 1536 tips (example shows 384 disposable tips mounted at a 384-channel head pipetting liquid to a 384-well plate) or to detection systems such as microscopes or plate readers. Data are often collected and analyzed in parallel. Detection systems are either sequentially analyzing plate wells or support whole-plate imaging, for faster testing. The analysis of hits from primary screening combines different types of information, including a clustering of structurally related hits (structure-activity relationships), measurements made to detect interference with the test system, dosage-dependent interference, data regarding the purity and integrity of hits, and the frequency of hit detection in distinct tests (frequent hitters). Results are compared to public databases to assess the already known activity of structurally similar molecules

microtiter plate format with robotic automation (Fig. 1b). It needs to pass an assay acceptance test, plus a pilot screen in which a few thousand compounds are used to control the robustness of the assay in the presence of compounds and their solvent. Next comes the primary screen against the whole library of molecules. Often a counter-screen is needed to identify false positive hits which interfere with the detection reagents, coupled enzyme reactions or a cellular reporter system in an unspecific manner. Finally, primary hits are validated by testing for dosage-dependent modes of action, to exclude compounds which catalyze chemical reactions and have toxic effects. These steps depend on computational support in data documentation, the automated analysis of huge datasets, and the conversion of images into multiparameter tables. The selection of hits for further characterization and chemical optimization typically requires automatically searching

for structurally related small molecules and their known effects in public databases such as PubChem or ChEMBL, and patent databases such as Espacenet (for Europe) or USPTO (USA).

Assay development for small-molecule screens: Biological tests based on purified proteins such as enzymes, transcription factors, or scaffolding proteins provide rather simple and robust systems for screening. Often these targets play key roles in diseases due to mutations in their corresponding genes that cause hyperactivation or an impairment of their functions. Enzyme assays often use synthetic substrates whose fluorescence is altered when the enzyme is activated or inhibited. Interactions between two distinct proteins can be studied using protein-specific antibodies that only transfer a fluorescent signal if they are brought into close proximity through an interaction of the proteins. Small molecules that interfere with their interaction decrease the fluorescence, as measured with plate readers and multiwell microtiter plates. Tests with purified components permit inhibitors to be chemically optimized, altering substructures that affect their affinity and specificity for proteins and enzymes. Further tests in cells and animal models are essential to testing the bioactivity of small-molecule hits and their roles in diseases.

Cellular systems represent a next level of complexity in identifying bioactive compounds, to determine whether they can cross the cellular membrane (membrane permeability), reach proper concentrations in the cytoplasm (biostability) and act specifically on the target protein (specificity). Such tests often use cancer cells in which the endogenous target protein has become permanently activated by a mutation. The cells are genetically manipulated through the stable integration of a reporter gene into the genome whose expression depends on the activity of the protein. This reporter may encode a fluorescent protein (GFP, YFP) or an enzyme (luciferase) that produces luminescence in the presence of its substrate. Small-molecule inhibitors reduce the fluorescence or luminescence. These experiments require a counter-screen using a second, unspecific reporter cell line to identify compounds which target the reporter enzymes or fluorescent proteins themselves. Plate readers or automated microscopes are used to quantify fluorescent signals, to image the translocation of fluorescent proteins within living cells or to document cellular signal transduction. Induced pluripotent stem cells (IPSCs) have become a major source for cellular assays for screens involving heart muscle, neurons, and many other types of cells. They can be also used to generate small organ-like structures (heart or brain organoids) for screens.

Animal models for disease like nematodes or zebrafish embryos present the next level of complexity, to determine whether small molecules enrich in specific tissues and maintain sufficient concentrations over time to affect pathological processes despite the activity of secretory and metabolic clearance processes. Such screens may rely on analyses of reporter genes that encode fluorescent proteins or morphological analyses of tissues and organs.

Assay optimization: Screens of tens of thousands to a million small molecules require robust biological assays in which negative and positive controls should generate consistent readouts. Potential counteracting factors include variability between biological sample preparations, systematic variations in the measurement process, and unsystematic noise due to random influences. Biological tests need to be optimized to obtain an optimal signal-to-noise ratio and exclude an overlap due to weak signals and strong variations. Optimization affects buffer conditions for enzyme tests, enzyme and substrate concentrations, and also an optimal choice of test plates and enzyme stability. Requirements for cellular testing include an optimal density of cells and the best time window for measurements; the significant parameters for cellular responses that are to be detected with automated microscopes and image recognition software need to be identified and optimized. Assays using animal models must also be optimized for the number of animals per test and the well sizes on test plates. Furthermore, the number of repeats for compound addition and amount of compounds that are applied must be optimized for appropriate dosage using known references.

Pilot screen: After an assay passes the acceptance test, a sublibrary of a few thousand

compounds is used to test the robustness of the assay in the presence of small molecules solubilized in DMSO as a solvent. This tests for interference that arises through the physical properties of compounds (fluorescence, colored compounds) and hit rates. The pilot and primary screen may use either single point or duplicate tests and measurements at single or multiple concentrations for each compound.

Primary screen: Batches of compound plates are selected for automated processing in robotic liquid handling systems with incubators and detection systems like plate readers or automated microscopes within a single day. Depending on incubation and processing times, 10,000–50,000 tests can be finished per day. Biological responses to compound addition are analyzed on the fly to immediately identify systematic variations. Measurements of the responses are presented on a colored plate map, generally using shades of red to represent the intensity of inhibition and blue colors for activation. Each plate contains wells with compounds and negative and positive control wells to normalize the signal output for each plate over the course of the screen. This is necessary to normalize results because enzymes or reagents may become less active over time. Primary hits (activators or inhibitors) are selected and tested for dosage-dependent activity.

Primary hit validation: Depending on the complexity of the test, a counter-screen may be applied to remove primary hits that act nonspecifically. This requires controls in which signal is produced by alternative pathways or regulatory elements, rather than those specifically targeted by the test compound. Generally, selecting hits for further characterization depends on dosage-dependent effects. They are applied at a range of dosages, from those that achieve complete inhibition to low concentrations in which activity remains at 100%. Analysis reveals an IC50-value for each small-molecule inhibitor: a concentration at which the system is inhibited to 50% of its maximal activity. Selected hits are either repurchased from vendors or resynthesized to test for impurities to cause biological effects. The structures of validated small molecules are then used to search for similar molecules and select drugs for continued experiments, drawing on publicly available data on known bioactivity and patents.

Small-molecule screening libraries: Libraries contain compounds which conform to certain rules on physiochemical and structural properties that correlate with the oral availability and the success rate in drug development (Lipinski et al. 2001). These rules limit the size of drug-like compounds to a molecular mass of less than 500 daltons, the number of hydrogen bond donors and acceptors to less than 5 and 10, respectively, and the water solubility to a log P value of less than 5.

Pharmacological Relevance

The majority of drugs currently used in treatments or diagnosis are small molecules of natural or synthetic origin. This toolbox is complemented by biopharmaceuticals generated by genetically modified cells, including antibodies, peptide hormones, DNA, and RNA. In academia, small-molecule screens are mainly used in the context of chemical biology to identify bioactive small molecules for use as research tools. As dosage-dependent modulators of biological functions which can be applied and withdrawn, bioactive compounds are important complements to methods such as gene ablation or CRISPR-Cas9 gene editing.

The use of cellular screens as disease models is based on the work of Rudolf Virchow, whose theory of cellular pathology states that diseases are based on changes at the cellular level (Morrison and Weiss 2006; Virchow 1855). Paul Ehrlich established the principle of chemotherapy and launched the first small-molecule screen to search for chemicals that were toxic to pathogenic bacteria causing Syphilis (Ehrlich and Bechhold 1906). His work resulted in the development of the first effective drug against Syphilis, Salvarsan (1910–1970). The success of this systematic approach combining chemical synthesis with biological screening made this technology a standard for drug identification and development in the pharmaceutical industry.

In academia, its systematic use started with the roadmap published in 2004 by the NIH (National Institutes of Health, USA), which provided

funding for small-molecule libraries and specialized technology centers for chemical biology. European academic platforms were launched a few years later. Academic platforms and research projects are not restricted to cellular targets for modulation – the "druggable genome" which represents about 10% of human genes. They can also explore diverse other targets, such as the motor proteins that separate chromosomes during cell proliferation as anticancer targets (Overington et al. 2006; Mayer et al. 1999). There has been a steady increase in the impact of academic chemical biology projects on the validation of novel targets (first in class drugs), the development of novel technologies for screening, combinations of drug interference with genome-wide interference via RNA interference (specific degradation of gene transcripts and depletion of encoded proteins), and CRISPR-Cas9 editing (gene-specific modification or deletion of coding sequences) due to their systematic use in research.

Common Screening Technologies

Fluorescence intensity (FI): In HTS, radioactive labeling has largely given way to fluorescent labeling, thanks to progress in the development of synthetic enzyme substrates that generate fluorescence quenching and emitting side chains in one molecule, only becoming fluorescent after cleavage. Fluorescently labeled antibodies detect proteins and their interactions, exposing biochemical pathways that are activated in cells or animal models, in health or disease. Changes of the intensity of fluorescence may indicate a modulation of an enzyme's activity or the translocation of a protein in a cell. Fluorescent proteins such as GFP or YFP and others can be fused with cellular proteins to permit live-cell analyses of their locations and quantities and how they change through the activation of cellular pathways or addition of pathogens. Generally, FI detection methods provide an ideal readout for small-molecule screens (Janzen 2014).

Fluorescence polarization (FP): Exciting fixed fluorophores with polarized light results in emissions that can be detected through polarization filters. The FP technique requires a fluorescently labeled small molecule or peptide which binds to a larger binding partner such as a protein. Without the binding partner, the small-molecule label is free for dynamic rotation and emits fluorescence into all directions. Specifically binding to a protein, either as a substrate of an enzyme or as a peptide hormone to a receptor, restricts the tumbling of the labels. Applying fluorescence polarization filters during excitation and detection permits quantifying the change in rotation, which correlates to the proportion of small-molecule or protein peptide complexes that have formed. This method permits screening for small molecules which interfere with the binding of labeled ligands such as hormones or enzyme substrates. Components are only added to start the reaction, which makes the method well suited for HTS.

Fluorescence resonance energy transfer (FRET): In principle a pair of fluorophores is selected for which the first (donor) – after excitation by light –emits fluorescence at a wavelength which excites the second fluorophore (acceptor) to emit fluorescence. This only happens efficiently if the fluorophores are brought into extremely close proximity. In a FRET experiment, the two fluorophores are attached to specific proteins using, for example, antibodies which are each linked with either the donor- or acceptor-fluorophore. If two proteins bind to each other, in an in vitro or cellular assay, the energy transfer occurs after the addition of antibodies and this results in generation of a measurable fluorescent signal at the site of interaction.

Time-resolved fluorescence (TRF): Related to FRET screens, this method uses a long-wavelength donor fluorophore with a long decay time (μsec). The purpose is to avoid interference by fluorescent screening molecules, whose fluorescence generally decays within nanoseconds. Introducing a time gap to measure the signal from the second fluorophore eliminates this type of interference. This technique is widely used in very high-throughput screens of biochemical and cellular assays.

Luminescence: Isolating enzymes which use small-molecule substrates and oxygen to produce

light (bioluminescence) from a range of organisms has provided key tools to study cellular gene expression. These methods combine regulatory sequences with genes encoding these enzymes to create reporter genes. An example of their use is to monitor the binding of transcription factors to regulatory DNA elements. Permanently activated oncogenes in cancer cells, for example, can be constructed which generate luminescence if their activation is not inhibited by small molecules. In contrast to the expression of fluorescent proteins, enzymes strongly amplify transcription factor–mediated activation and thus provide a more sensitive detection of oncogenic activities. Other luminescence methods couple enzyme activity to protein interactions by fusing the enzyme and luminescence substrate to different proteins. Luminescence requires close proximity, so only sites of interaction will produce a signal. Luciferase reporter systems are also widely used in HTS.

Alpha screen: This technology delivers a chemiluminescent quantification of interactions. Two distinct proteins or interacting factors are coupled to donor or acceptor beads. The surface of each bead class is constructed to specifically bind only one type of factor. If the factors interact, this puts the beads in close proximity, triggering a laser-induced release of reactive oxygen from donor beads to acceptor beads and a chemiluminescent reaction. If small molecules inhibit binding, the beads are not coupled and the reactive oxygen becomes neutralized before reaching the acceptors. This method delivers a high number of false positive compounds which interfere with the release of reactive oxygen, which strip coupled factors from beads or interfere directly with the chemical luminescence reaction. Due to its high sensitivity, it is used, for example, to quantify levels of second messengers such as cAMP in cellular assays set up to detect G-protein-coupled receptor signaling.

Surface plasmon resonance: A protein is immobilized on a detection surface in a microfluidic chamber and a ligand such as a second protein or a small-molecule compound is added. Their binding and stability can be analyzed by measuring increases and the persistence of reflected light from the detection surface. This method permits a determination of binding constants for affinity and stability, and to detect irreversible binding or denaturation of proteins.

Capillary electrophoresis (microfluidic chips): This technology is based on hand-sized chips with up to 12 microfluidic channels and sippers which aspirate simultaneously a few nanolitres of 12 enzyme test reactions. They are capable of separating enzyme substrates and products by electrophoresis in high throughput that differ in charge – for example, after phosphorylation by kinases (which increases the negative charge of the product) or dephosphorylation by phosphatases. The enzymatic incubation is stopped after 50% of peptide substrate has been converted to product, resulting in two bands of equal amount after separation, if no inhibitor is present. The substrate is labeled by a fluorophore. This permits laser-mediated excitation and detection with a charge-coupled device camera (CCD). Applying an inhibitor decreases the fluorescence of the product band. The system permits profiling about 20.000 reactions per day and provides high-quality data, because detection is not affected by fluorescence quenching or unspecific physical properties of compounds.

Flow cytometry: This method is used to analyze cells in suspension, not adhered to a surface. Cells are marked by fluorescent dyes or antibodies of biological relevance, indicating, for example, their proliferative state or developmental stage, then excited by a laser. This permits very sensitive detection and readouts. Tens of thousands of cells in one test well can be sorted by size and fluorescence intensity, which means, for example, that a cancer marker can be detected on different cell types in a single experiment.

High-content screening and machine learning: Automated microscopes with 2D or 3D functions through confocal imaging are currently widely used to profile morphological alterations of cells derived, for instance, from patients. This broad data collection is termed high-content screening, and it is currently being used in conjunction with iPSC technologies to study 3D organoids and other structures. Applying drugs to organoids for colon cancer, for example, has been shown to

produce results that more closely resemble those of clinical trials than other current methods.

High-content screening requires the automated, software-guided identification of cellular objects such as nuclei, filaments, and membrane compartments. These structures are automatically recognized and converted to parameters representing size, fluorescence intensity, etc. Sometimes, small changes in specific parameters may provide a more significant readout for cellular responses than larger ones which may be more obvious in a visual examination. Therefore, machine learning is combined with a human visual sorting of experimental outcomes to develop computer-guided, positive, and negative correlations and filter a training set of data. Afterwards this artificial intelligence can be used to analyze image-based HTS data.

Impedance (label free): This method makes use of microtiter plates with golden electrodes at the bottom. A low electric current is applied to analyze changes in the electric resistance of cells adjacent to the electrodes. The impedance may change, for example, through signaling processes which cause a release of calcium from endogenous stores, or induce a stronger attachment to electrodes. In contrast to other methods, no chemical fixation of cells and membrane permeabilization for antibody detection is required, resulting in dead cells, instead living cells can be analyzed over time without further treatment (label free).

Automated data documentation and analysis: The high-throughput screening of large small-molecule libraries requires the automated documentation and analysis of the data that is acquired. This can be achieved by adapting commercial and free open-source software. Biological and chemical data such as compound identifiers and structure information must be linked in the documentation. Scripts that are appropriate to the screening technology must be written to filter and analyze hits and to set curves to fit kinetic or end-point data collections. A conversion of measurements to colored heat maps reflecting biological activities permits a rapid detection and correction of systematic errors. Researching structural similarities and known bioactivities in public databases is essential in designing follow-up tests and selecting candidates for further study. Small-molecule screening represents a dynamic marriage of technologies with biological, chemical, and computational expertise in an inherently interdisciplinary enterprise that holds great promise for the future identification and development of potent new drugs.

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Smooth Muscle Tone Regulation

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Synonyms

Regulation of smooth muscle contractility

Definition

The following organs contain as major functional part smooth muscle layers: arterial and venous

vessels; lung and bronchia; oesophagus, stomach, and small and large intestine; urinary tract and bladder; and uterus. Hormones, locally released transmitters and shear stress or pressure regulate the tonus of these organs. Each organ has a slightly different regulation of its contractility, but the basis for this regulation, i.e. the intracellular signaling pathways, is very similar or identical. This article will focus on major findings that may be identical in all smooth muscles.

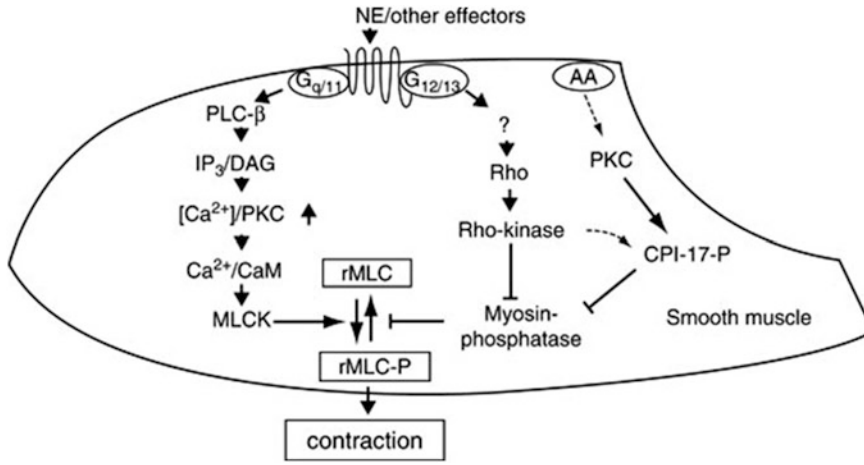
Basic Mechanisms

Key mechanism of smooth muscle tone regulation is the phosphorylation of Ser-19 of the regulatory myosin light chain II (rMLC) (Pfitzer 2001). Phosphorylation and dephosphorylation are catalysed by myosin light chain kinase (MLCK) and the type 1 myosin phosphatase (MLCP), respectively. Calcium-dependent and calcium-independent signal pathways regulate the activity of both enzymes and thereby the phosphorylation status of rMLC. An increase in the cytosolic calcium concentration leads to phosphorylation of the rMLC and contraction within 4 s. The correlation between percent phosphorylated rMLC and developed force is quite variable. Maximal force can be attained at 0.2–0.3 mol phosphate per mol rMLC. Phosphorylation can decline during maintenance of tension suggesting that even dephosphorylated cross-bridges can contribute to force maintenance.

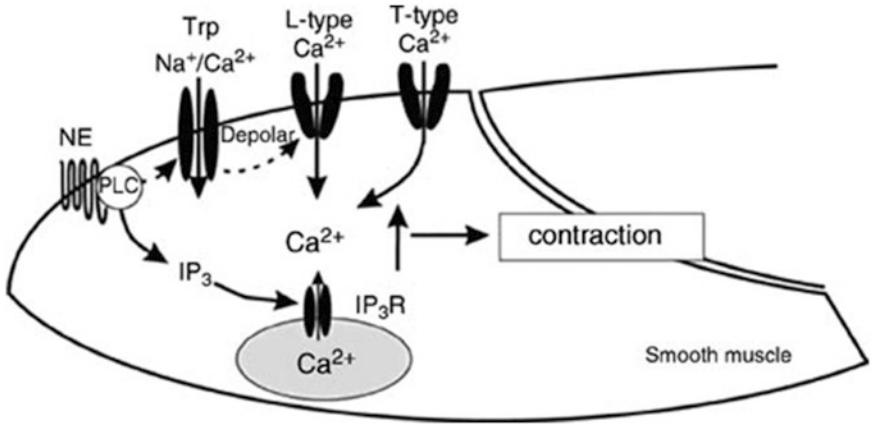
Calcium-dependent regulation involves the calcium-calmodulin complex that activates smooth muscle MLCK, a monomer of approximately 135 kDa. Dephosphorylation is initiated by MLCP. MLCP is a complex of three proteins: a 110–130 kDa myosin phosphatase targeting and regulatory subunit (MYPT1), a 37 kDa catalytic subunit (PP-1C) and a 20 kDa subunit of unknown function. In most cases, calcium-independent regulation of smooth muscle tone is achieved by inhibition of MLCP activity at constant calcium level inducing an increase in phospho-rMLC and contraction (Fig. 1).

Calcium-Dependent Contraction

Different agonists such as norepinephrine, acetylcholine or angiotensin II activate smooth muscle contraction by binding to a heptahelical receptor, i.e. α-adrenergic, muscarinergic or AT-1 receptors, followed by an increase in cytosolic calcium (Figs. 1 and 2). The activation of the trimeric G proteins G_q or G_{11} increases the activity of phospholipase Cβ (PLC) generating inositol trisphosphate (IP_3) and diacylglycerol (DAG) and other fatty acid-derived compounds. Classical



Smooth Muscle Tone Regulation, Fig. 1 Mechanisms leading to agonist-stimulated calcium-dependent and calcium-independent contraction of smooth muscle. NE, norepinephrine. See text for the other abbreviations



Smooth Muscle Tone Regulation, Fig. 2 Membrane mechanisms leading to an increase in cytosolic calcium concentration. Depolar, depolarization of the membrane; see text for abbreviations

findings suggested that IP_3 stimulates calcium release from intracellular stores that binds to calmodulin and activates MLCK. This simple scheme is not in line with the fact that a block of the L-type Ca^{2+} channel inhibits contraction. The importance of the L-type calcium channel is further supported by genetic deletion of the corresponding $Ca_v1.2$ gene. Mice lacking the smooth muscle Cav1.2 channel have severe difficulties to contract intestinal and other smooth muscle. It is therefore likely that activation of a heptahelical receptor leads to depolarization of the membrane and activation of the L-type calcium channels (Fig. 2). Possible candidates are TRP channels (most likely TRPC6) activated either by DAG or by interaction with empty IP_3 stores. The inflowing cations may depolarize the membrane to potentials that activate T- and thereafter L-type calcium channels or directly L-type channels. A second channel depolarizing the membrane is the calcium-activated chloride channel present in many smooth muscle cells. Calcium released from IP_3 stores or flowing in through TRP channels could activate this chloride channel and depolarize thereby the membrane. The L-type calcium channel provides calcium to trigger calcium release from ryanodine receptor-controlled calcium stores and for refilling various intracellular calcium stores (Jaggar et al. 2000). The mechanism behind pressure- or shear stress-induced contraction is unsolved but may again involve TRP channels and mechanosensitive cation channel Piezo1. Piezo1 is mainly expressed in endothelial cells. Shear stress activates the endothelial Piezo1 and contributes thereby to endothelial-dependent hyperpolarization (EDH) and relaxation of arterial resistance vessels (Douguet et al. 2019).

Calcium-Independent Contraction

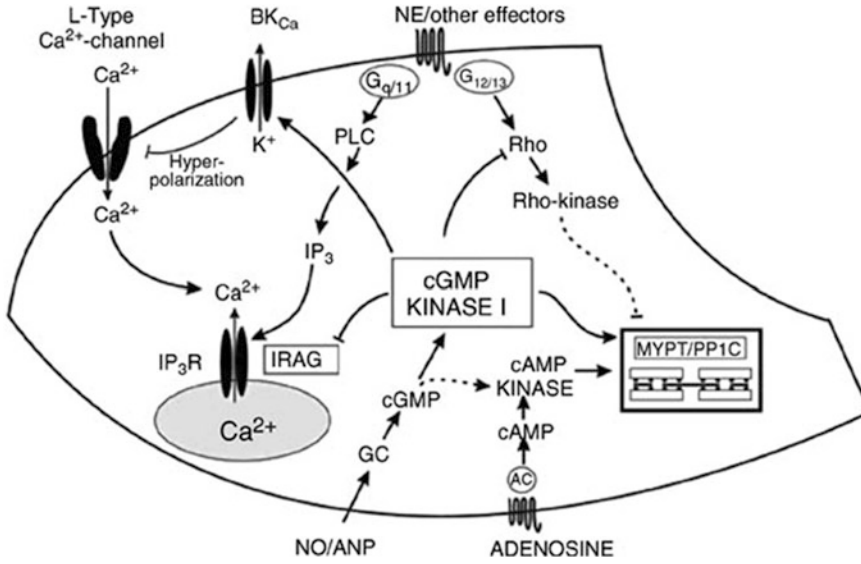
Agonist-activated receptors can induce contraction at a constant intracellular calcium concentration (Somlyo and Somlyo 2000), if the receptor activates the G proteins G_{12} or G_{13} (Fig. 1). Activation of these G proteins recruits the monomeric GTPase Rho to the membrane, where Rho exchanges GDP against GTP and activates Rho kinase. By a still unsolved cascade eventually involving ZIP kinase, the MYPT1 subunit of MLCP is phosphorylated at Thr-697 and Thr-854 (rat MYPT1) which reaction inhibits MLCP activity. Since the activity of MLCK is not affected by this cascade, rMLC is phosphorylated to a higher level. Phosphorylation and inhibition of MLCP activity are only observed, if a central exon of MYPT1 is present. These results could explain the old finding that certain agonists induce calcium sensitization of the contractile machinery in most but not all smooth muscles. MLCP activity is also affected by a smooth muscle-specific

inhibitor protein of PP-1C, named CPI-17. Protein kinase C (PKC) phosphorylates CPI-17, which becomes then a high affinity inhibitor of the catalytic subunit of MLCP. The nature of the PKC subtype is not clear. It is possible that it is one of the atypical PKC enzymes that is activated directly (?) by arachidonic acid (AA). Rho kinase which phosphorylated CPI-17 in vitro apparently may affect directly in vivo the phosphorylation status of CPI-17 (Dimopoulos et al. 2007). Arachidonic acid inhibits dephosphorylation of MLCs, i.e. by a second mechanism by dissociating the MLCP holoenzyme.

Relaxation of Smooth Muscle

The major relaxing transmitters are those that elevate the cAMP or cGMP concentration (Fig. 3). Adenosine stimulates the adenosine receptor A_2, increases cAMP and activates thereby the activity of cAMP kinase. The next step is not clear, but evidence has been accumulated that cAMP kinase decreases the calcium sensitivity of the contractile machinery. In vitro, cAMP kinase phosphorylated MLCK and decreased thereby the affinity of MLCK for calcium-calmodulin. However, this regulation does not occur in intact smooth muscle. Possible other substrate candidates for cAMP kinase are the heat-stable protein HSP 20, a heat stable protein of 20 kDa that is phosphorylated by cGMP kinase. It has been postulated that phospho-HSP 20 interferes with the interaction between actin and myosin allowing thereby smooth muscle relaxation without dephosphorylation of the rMLC. Rho A and MLCP are phosphorylated also by cGMP kinase I (Fig. 3).

A major relaxing factor is NO, a signal molecule synthetized by three different NO synthases (NOS). NO synthetized in the endothelial layer of the vessels diffuses into the smooth muscle layer, where NO activates soluble guanylate cyclase (GC) and generates high concentrations of cGMP. In non-vascular systems such as the intestinal smooth muscle, NO is released from non-adrenergic, non-cholinergic neurons. An alternative pathway for the production of cGMP is the stimulation of particulate GC by the natriuretic peptides ANF, BNF and CNF. ANF and BNF are released from cardiac atrial and ventricular muscle, respectively, and lower blood pressure. These effects of the natriuretic peptides are mediated through cGMP and cGMP kinase I. NO has additional effects which



Smooth Muscle Tone Regulation, Fig. 3 Major mechanisms leading to relaxation of smooth muscle. See text for the abbreviations

are not mediated by cGMP kinase I. CNP is released from the vascular endothelial layer and exerts its relaxing effects either through activation of the particulate guanylyl cyclase B identical with the NPR-B or through the NPR-C receptor. The endothelial layer of the vessels initiates a number of diverse signaling pathways that lead to endothelium-dependent hyperpolarization (EDH) and relaxation of vascular smooth muscle (Garland and Dora 2017).

Smooth muscle contains the two cGMP kinase isozymes Iα and Iβ and a number of identified substrates (Hofmann et al. 2006). The NO/cGMP/cGMP kinase pathway interferes with the calcium-dependent and the calcium-independent contraction. A number of researchers have shown that cGMP-dependent phosphorylation of the BK_{Ca} channel increases its open probability resulting in hyperpolarization of the membrane potential and closure of voltage-dependent calcium channels. The activity of the BK_{Ca} channel is upregulated by the intracellular calcium concentration establishing a negative feedback loop. It is well established that cGMP kinase decreases the release of calcium from intracellular stores. Recently, it was found that cGMP kinase Iβ is associated with the IP_3 receptor type 1 and the 130 kDa protein IRAG. Phosphorylation of IRAG inhibited the release of calcium from IP_3-sensitive stores in COS cells. However, isozyme-specific reconstitution of cGMP kinase I-deficient mice suggested that, in murine aortic smooth muscle cells, cGMP kinase Iα and Iβ lowered norepinephrine-stimulated increases in the cytosolic calcium concentrations. This result is in line with the recent notion, which calcium-dependent contraction of smooth muscle requires membrane depolarization and calcium influx through membrane localized ion channels (see above). It is possible that the IP_3-sensitive calcium pool associated with IRAG and cGKIβ controls other smooth muscle functions such as phenotype changes and smooth muscle growth.

It was demonstrated that cGMP kinase I inhibits also smooth muscle contraction due to the calcium-insensitive pathway. A possible mechanism could be phosphorylation of Rho by cGMP kinase I. The phosphorylation site is identical with a cAMP kinase site identified in Rho from non-smooth muscle cells. It was reported that phosphorylation of Rho by cGMP kinase I prevents membrane association of Rho that is required to stimulate the GDP/GTP exchange. Alternatively, phosphorylation of telokin may interfere with the calcium sensitization of contraction. cGMP kinase Iα interacts specifically with a leucine zipper present at the C-terminus of MYPT1. Depending on the tissue, this leucine zipper is present or not. MLCP activity increased when MYPT1 is phosphorylated by cGMP kinase Iα at Ser-696.

Pharmacological Intervention

A large number of drugs interfere with the smooth muscle contraction. These compounds lower blood pressure and are referred to as antihypertensive. In this section, only those compounds will be mentioned that have a direct effect on smooth muscle tone. Phenylephrine is an agonist on most smooth muscles and activates α_1 adrenoceptors. Carbachol is an agonist on some smooth muscles and activates contraction through muscarinic receptors. Blockers of the α_1-adrenoceptors such as prazosin and urapidil are competitive inhibitors of the α_1-receptor in vascular and bladder smooth muscle. Phenoxybenzamine is an irreversible blocker of α_1 receptors, and phentolamine blocks α_1 and α_2 receptors. Ca^{2+} channel blockers such as the dihydropyridines, phenylalkylamines and benzothiazepines lower smooth muscle tone by blocking the L-type calcium channel.

Nitrates (glyceryl trinitrate, isosorbide dinitrate, pentaerythritol tetranitrate, molsidomine, sodium nitroprusside) that generate NO increase cGMP concentrations and lower smooth muscle tone by activation of cGMP kinase I. Nitrates relax in vivo mainly the capacitive part of the circulation system, i.e. the venous part. Sildenafil, a specific inhibitor of phosphodiesterase 5, a cGMP hydrolysing enzyme, increases cGMP in the corpus cavernosum and lowers together with nitrates the blood pressure by the combined effect on cGMP level. An additional potentially important drug family is compounds that stimulate the soluble guanylyl cyclase independent of NO (Evgenov

et al. 2006) and lower elevated blood pressure. The inhibitor of Rho-kinase, Y-27632, lowers elevated blood pressure in hypertensive animals, without affecting significantly the blood pressure of non-hypertensive animals (Uehata et al. 1997).

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S-Nitrosation

▶ Protein S-Nitrosylation

Sodium- and Potassium-Activated Adenosine 5′-Triphosphatase (EC 7.2.2.13)

▶ Na^+/K^+-ATPase

Sodium Calcium Exchanger

▶ Na^+/Ca^{2+} Exchangers

Sodium Pump

▶ Na^+/K^+-ATPase

Sodium/Proton Antiporter

▶ Na^+/H^+ Exchangers

Sodium/Proton Exchanger

▶ Na^+/H^+ Exchangers

Soluble Epoxide Hydrolase

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Synonyms

EPHX2; Epoxide hydrolase 2; sEH

Definition

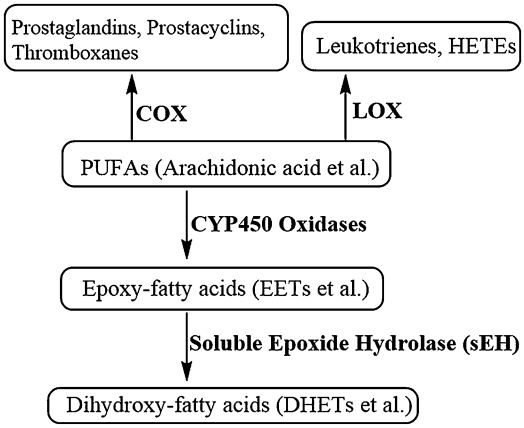
The epoxide hydrolase (EH) family of enzymes is responsible for converting the epoxide residue of various endogenous and exogenous compounds to the corresponding vicinal diols (Morisseau and ammock 2005). Soluble epoxide hydrolase (sEH) is an enzyme in the α-/β-hydrolase fold protein family, primarily found in the cytosolic and peroxisomal fractions of cells (Chiamvimonvat et al.

2007). It is encoded by the EPHX2 gene. It is a bifunctional homodimer that has EH activity at the C-terminal domain and phosphatase activity at the N-terminal domain (He et al. 2016). sEH is abundantly expressed in the liver, but it is also present in most organs including the kidney, brain, smooth muscle, intestines, and lungs (Morisseau and Hammock 2005). Its substrate selectivity is largely restricted to noncyclic 1,2-disubstituted epoxides, and hence, unlike the microsomal epoxide hydrolase (mEH), it is generally not involved in the metabolism or detoxification of xenobiotics (Morisseau and Hammock 2005). On the other hand, polyunsaturated fatty acid (PUFA)-derived bioactive lipid mediators are usually good sEH substrates and are subject to hydrolysis. These epoxy fatty acids (EpFAs) have demonstrated numerous tissue healing and organ-protective effects. sEH inhibitors (sEHI) help stabilize endogenous levels of EpFAs, thus enhancing their bioavailability and facilitating their biological action. Hence, targeting sEH with novel and potent small-molecule inhibitors is a pharmacological tool employed to capitalize on the beneficial effects of EpFAs and potentially treat several disease conditions (Morisseau and Hammock 2005). Alternative approaches involve (i) administration of EpFAs themselves, (ii) synthesis and application of EpFA mimics, and (iii) stimulation of EpFA biosynthesis.

Basic Characteristics

Metabolism of PUFAs

Arachidonic acid (20:4, ω-6) is a PUFA derived from the phospholipids of cell membranes and is metabolized by three main enzymatic pathways (Fig. 1) (Imig and Hammock 2009). The cyclooxygenase (COX) and lipoxygenase (LOX) routes generate largely pro-inflammatory mediators and are the target of several pharmaceuticals currently available on the market (e.g., nonsteroidal anti-inflammatory drugs a.k.a. NSAIDs and selective COX-2 inhibitors a.k.a. COXIBs). The third pathway primarily involves cytochrome P450-mediated epoxidation to epoxy metabolites known as epoxyeicosatrienoic acids (EETs). Other ω-3



Soluble Epoxide Hydrolase, Fig. 1 Schematic depicting major pathways of polyunsaturated fatty acid (PUFA) metabolism

PUFAs such as docosahexaenoic acid (DHA, 22:6) and eicosapentaenoic acid (EPA, 20:5) are similarly metabolized, and the CYP450 branch generates epoxydocosapentaenoic acids (EDPs) and epoxyeicosatetraenoic acids (EEQs), respectively (He et al. 2016). EETs and other EpFAs are mono-epoxides that are chemical mediators and act in both autocrine and paracrine manners. However, the chemically stable EpFAs have transient metabolic half-lives due to rapid sEH-mediated degradation. Recently, epoxides of omega-3-derived endocannabinoids with anti-inflammatory, anti-tumorigenic, and analgesic function have been reported, suggesting a still broader role of sEH in regulatory biology (Das et al. 2019).

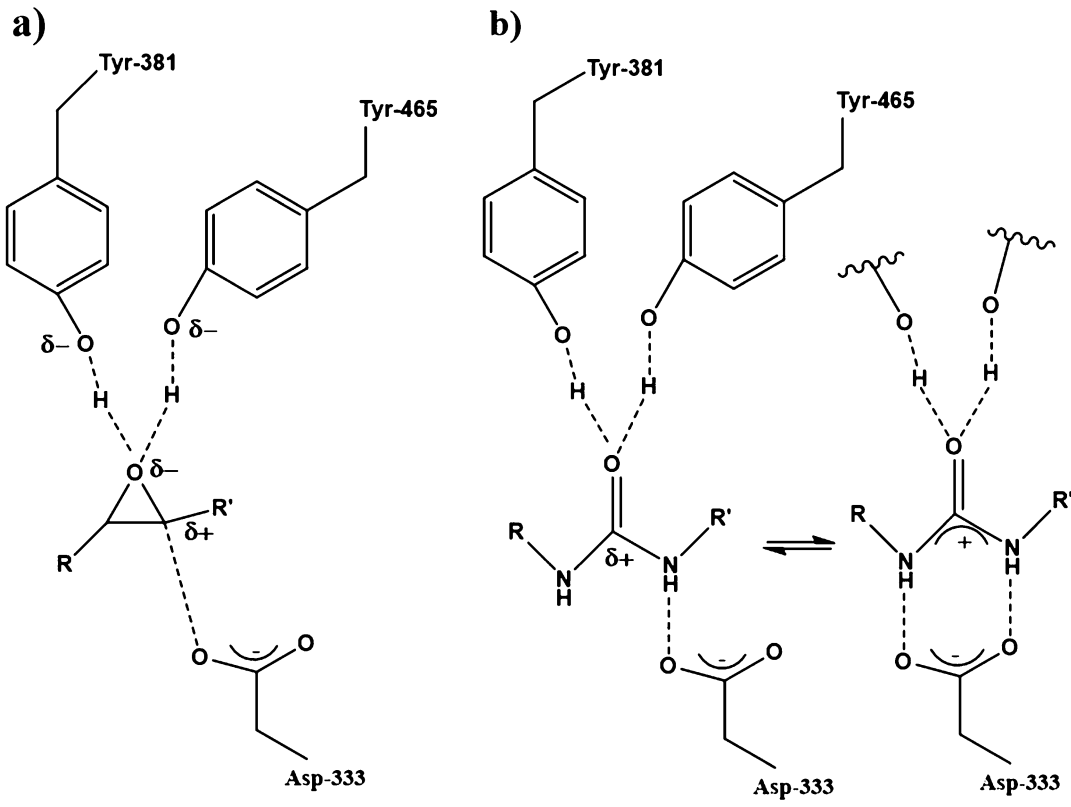
Hydrolysis of EpFAs

sEH hydrolyzes EETs and other EpFAs by an exothermic, two-step base-catalyzed mechanism (Morisseau and Hammock 2005). It involves the formation of a covalent hydroxyl-alkyl-enzyme ester intermediate via a catalytic aspartic acid, followed by the attack of water on the enzyme ester carbon that releases the 1,2-diol product. Within the hydrophobic active site, two tyrosine residues hydrogen bond to the epoxide oxygen, polarizing it. A nucleophilic aspartate acid conducts a backside attack on the epoxide, forming the covalent intermediate, while an

additional aspartate residue acts as orienting and activating acid for the histidine general base. This activates a water molecule which conducts the described hydrolysis step. sEH metabolism of EpFAs is regioselective and stereoselective (Chiamvimonvat et al. 2007). For example, the EET regioisomer with the epoxide furthest from the carboxylic acid end (14,15-EET) is hydrolyzed the fastest, while the one closest is hydrolyzed most slowly (5,6-EET).

Mechanism of sEH Inhibition

Enzyme inhibitors acting to mimic reaction intermediates or transition states have been found as potent inhibitors in many α-/β-hydrolase fold enzymes (Morisseau and Hammock 2005). Based on the catalytic mechanism of sEH, the transition states of epoxide ring opening are effectively mimicked by the urea functional group (Fig. 2). The C=O moiety accepts hydrogen bonds from the two tyrosine residues and hence acts as a surrogate for the epoxide oxygen. One mechanism suggests that one of the urea NH groups donates a hydrogen bond to the nucleophilic aspartate, mimicking the electrophilic center (Fig. 2). Alternatively, both NH groups donate hydrogen bonds to the aspartate, stabilizing a partial salt bridge with the enzyme, and thus mimic the interaction between the enzyme and substrate during the transition states (Fig. 2). Accordingly, a series of small-molecule 1,3-disubstituted ureas as well as amides, carbamates, and heterocycles have been developed which act as slow, tight-binding, competitive, and highly potent (low nanomolar to picomolar IC_{50}) inhibitors of sEH (Morisseau and Hammock 2005). The functional groups attached to the primary urea pharmacophore are generally hydrophobic to allow effective interaction with



Soluble Epoxide Hydrolase, Fig. 2 (**a**) Transition state of epoxide ring opening within the sEH active site. (**b**) Two possible binding modes of 1,3-disubstituted urea inhibitors mimicking multiple transition states and reaction intermediates along the reaction coordinate

the sEH active site, with a smaller group on one side and a typically larger group on the other. A polar moiety (e.g., ether, ester, alcohol, or ketone) added to the side with the larger group can act as a secondary pharmacophore and improve water solubility, pharmacokinetic parameters, and drug formulation properties, without lowering its potency (Imig and Hammock 2009).

Biological Action of EETs and EpFAs

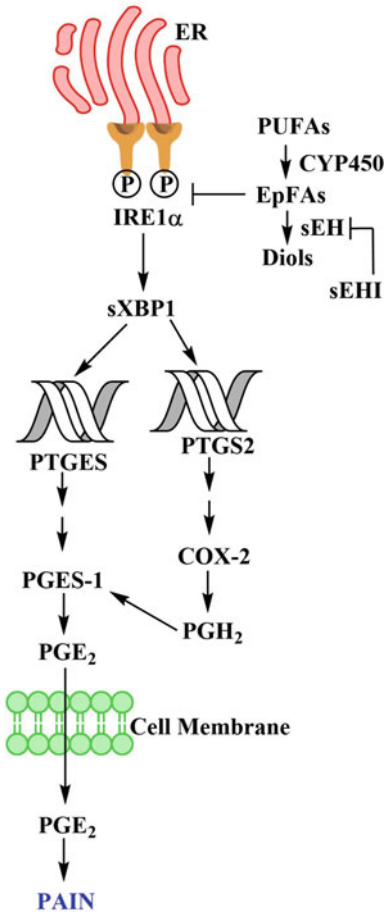
The inhibition of sEH has yielded therapeutic potential in models for various inflammatory, hypertensive, metabolic, fibrotic, and neurodegenerative diseases. The physiological effects of sEH inhibitors stem from the upregulation and subsequent action of EpFAs. The lack of a defined receptor for EETs and other EpFAs makes the assessment of the specific mode of action more challenging. Nevertheless, EpFAs are known to exert several biological effects and are hypothesized to act through multiple pathways.

Inhibition of sEH attenuates and resolves chronic inflammation through multiple pathways (Imig and Hammock 2009). Certain EETs such as the 11,12-regioisomer prevent nuclear translation of NF-κB and thereby block transcription of genes that encode pro-inflammatory enzymes (e.g., iNOS, LOX-5, COX-2, PGE2 synthase). 11,12-EET reduces IKK activity, which decreases phosphorylation and degradation of IκB, allowing it to sequester NF-κB in the cytosol. Other pathways include STAT3 tyrosine-705 phosphorylation by 14,15-EET, PPAR-alpha and PPAR-gamma agonism, inhibition of cytokine-induced VCAM-1, reduced TNFα secretion and, as discussed later, the endoplasmic reticulum (ER) stress response.

sEH inhibition has also been associated with smooth muscle relaxation and vasodilatory function (Imig and Hammock 2009). EETs, specifically the 11,12- and 14,15-regioisomers, are endothelium-derived hyperpolarizing factors (EDHFs). They stimulate calcium-activated potassium channels (K_{Ca}^{+}), triggering an efflux of K^{+} from vascular smooth muscle cells. This results in membrane hyperpolarization and subsequent dilation of blood vessels. cAMP activation of protein kinase A (PKA) and ADP ribosylation of the alpha subunit of G_s are mechanisms by which EETs have been demonstrated to activate K_{Ca}^{+} channels and promote vascular relaxation (Imig and Hammock 2009).

Disruptions in endoplasmic reticulum (ER) homeostasis caused by reactive oxygen species (ROS), high glucose, or xenobiotic perturbations result in ER stress. The ability of EpFAs to attenuate ER stress and downregulate the triggered unfolded protein response (UPR) pathways has been shown to underlie the amelioration of multiple metabolic disorders including fibrosis, inflammatory, and neuropathic pain (Fig. 3) (Inceoglu et al. 2017). sEHI modulation of the PERK/eif2α branch of the UPR enhances systemic insulin sensitivity and improves glucose tolerance (Inceoglu et al. 2017). It is also considered to play a role in ensuring proper pancreatic exocrine functionality and preventing pancreatitis. Mitigation of the IRE1α and ATF6 UPR pathways by sEH inhibition decreases the TGF-β-induced myofibroblastic differentiation of lung fibroblasts and helps prevent the development of lung fibrosis. The inhibition of sEH has also exhibited a promising analgesic action toward diabetic neuropathy and other types of chronic pain. One proposed mechanism suggests downregulation of the three UPR arms in peripheral nerves, which reduces predisposition to aberrant axonal firing and decreases apoptotic signaling and oxidative stress (Inceoglu et al. 2017).

The inhibition of sEH and resulting elevated EpFA levels in the brain is considered to increase brain-derived neurotrophic factor (BDNF) levels in the prefrontal cortex and hippocampus (Hashimoto 2019). BDNF binding to tropomyosin receptor kinase B (TrkB) and enhanced BDNF-TrkB signaling promotes synaptogenesis and corrects dopaminergic dysfunction, leading to an improvement in depressive and schizophrenic symptoms. EpFAs also seem to play a role in preventing phosphorylation of α-synuclein and sequentially reducing the aggregation of α-synuclein in multiple brain regions (Hashimoto 2019). By blocking the deposition of phosphorylated α-synuclein aggregates (a.k.a. Lewy bodies), the loss of dopaminergic neurons from the



Soluble Epoxide Hydrolase, Fig. 3 Epoxy fatty acids (EpFAs) preserved by sEH inhibitors (sEHI) stabilize mitochondria against disruption, reduce reactive oxygen species (ROS), and shift the endoplasmic reticulum (ER) stress response away from initiating cell damage and inflammation and back toward maintaining homeostasis (Inceoglu et al. 2017). Downstream from mitochondrial stabilization and reduction in ROS, EpFAs alter ER stress-induced unfolded protein response (UPR) pathways. For one pathway acting through IRE1α (shown above), sEHI reduce protein and message for COX-2 (Schmelzer et al. 2005) and PGES-1 (Chopra et al. 2019), thus dramatically reducing PGE_2 and other inflammatory and pain-initiating eicosanoids

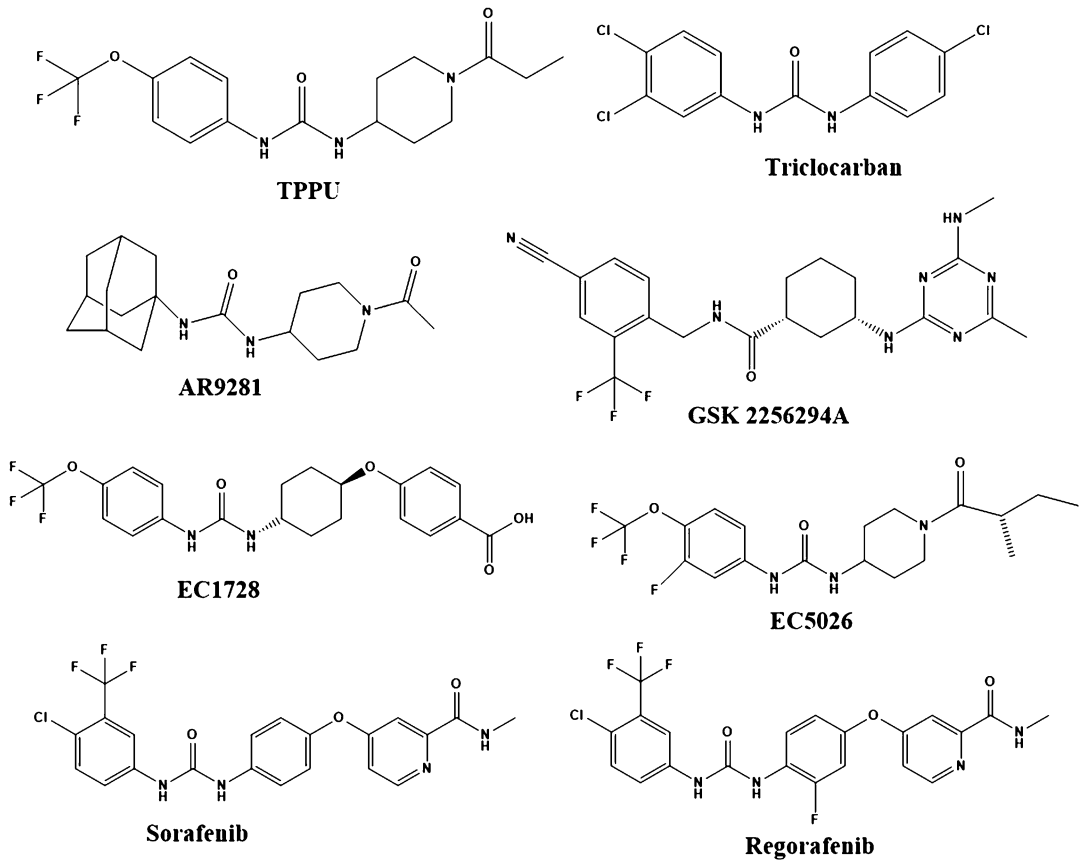
substantia nigra is potentially mitigated, and the pathogenesis of Parkinson's disease and dementia with Lewy bodies (DLB) may be controlled.

For pain as an endpoint in animal studies, the inhibition of sEH forgoes the common adverse side effects of NSAIDs and COXIBs (e.g., GI ulceration, cardiovascular issues) (Wagner et al. 2017). Additionally, it displays none of the reward-seeking addictive potential associated with the use of opioid narcotics (Wagner et al. 2017). EETs can be angiogenic at very high doses, and sEHI upregulation of EETs is considered to slightly promote tumor growth. This phenomenon has been attributed to downstream COX metabolites of EETs (Rand et al. 2017). Combining sEH inhibitors and COX inhibitors has been shown to have significant anti-angiogenic effects and blunt tumor growth, as has the use of sEHI in animals with an enhanced omega-3 and depleted omega-6 diet.

Drugs

Representative sEH Inhibitors

TPPU (i.e., UC1770) is extensively used in animal disease models because it is orally bioavailable and has favorable pharmacokinetic characteristics in addition to high potency (Fig. 4). Currently, no clinical drug on the market is used as an sEH inhibitor. However, antineoplastic agents sorafenib and regorafenib, inhibitors of vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), and Raf family kinases are powerful sEH inhibitors (Fig. 4) (Liu 2019). This activity likely reduces severe toxicity associated with the use of protein kinase inhibitors. Triclocarban, a commonly used antibacterial, has also displayed sEH inhibitory potential (Fig. 4) (Liu 2019). These compounds contain the ideal disubstituted urea substructure which indicates the diversity of structures that can inhibit the sEH. Several pharmaceutical companies have been or are in the process of discovering and developing potent sEH inhibitors in order to treat several clinical indications. These include Arête Therapeutics, Boehringer Ingelheim, Taisho, Merck, GlaxoSmithKline (GSK), and EicOsis. One compound, AR9281 (i.e., UC1153 or APAU) from Arête, was designed to target moderate hypertension and impaired glucose tolerance (Fig. 4). It completed Phase I and Phase IIA clinical trials. AR9281 was well tolerated for both a single oral dose and multiple doses.



Soluble Epoxide Hydrolase, Fig. 4 Chemical structures of representative sEH inhibitors

However, as expected from the adamantane in the structure, AR9281 had poor pharmacokinetic and pharmacodynamic parameters suggesting at least a twice- or thrice-daily dosing regimen for effective sEH inhibition (Liu 2019). Not only was the inhibition of the human enzyme weak, but the target occupancy was also poor. Despite no safety concerns in clinical trials, AR9281 failed to show efficacy in early-stage hypertension indicating hypertension might not be a suitable primary clinical indication for sEHI and AR9281 was not appropriate for sustained inhibition of the target sEH. Another inhibitor, GSK2256294A from GSK, was targeted to treat chronic pulmonary obstructive disease (CPOD) and completed Phase I clinical trials (Fig. 4). It has shown no adverse effects and a dose-dependent increase in plasma drug levels and sEH inhibition (Liu 2019). EicOsis designs pain therapeutics, and the compound EC1728 (i.e., *t*-TUCB) has been shown to successfully treat equine laminitis as well as inflammatory pain in dogs and cats (Fig. 4). Currently, EC1728 is pending an FDA review to begin Phase 1 trials in horses in 2020. The inhibitor EC5026 has been selected for treating diabetic neuropathy and other forms of neuropathic pain in patients while promoting opioid-sparing effects (Fig. 4). It has displayed considerable efficacy in preclinical studies as well as ideal pharmacokinetic properties and stability. The IND has been filled, and it entered Phase 1 clinical trials in December of 2019.

So far, sEHI have generally demonstrated few to no adverse side effects even at relatively high doses, and they have an excellent therapeutic

index. However, the broad range of biological effects observed with the use of sEHI, like other drugs acting on the arachidonate cascade, suggest they should be utilized with caution.

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Solute Carrier 9 Family

▶ Na^+/H^+ Exchangers

Somatostatin

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Definition

Somatostatin, also known as somatotropin-release inhibitor factor (SRIF), is a cyclic neuroendocrine peptide that was first isolated and identified as a hypothalamic peptide that inhibited growth hormone (GH) secretion from anterior pituitary cells (Brazeau et al. 1973). SRIF is expressed in the central nervous system (CNS) and in peripheral tissues and exerts potent inhibitory actions on neuronal excitability, hormone secretion, and exocrine secretion. SRIF also negatively affects smooth muscle cell contraction and cell proliferation. Its physiological functions are mediated by five G protein-coupled receptors (GPCRs) called somatostatin receptors ($SST_{1–5}$).

Basic Characteristics

Somatostatin and Somatostatin-Related Peptides

SRIF exists in two main isoforms: the tetradecapeptide SRIF-14 and the 28-amino-acid peptide SRIF-28. The two isoforms are generated from the same precursor through post-translational processing at a distinct cleavage site and are expressed at variable amounts in the same tissues. The SRIF-14 primary structure and cleavage sites generating the two isoforms have been highly conserved

during evolution in vertebrates (Conlon et al. 1997). In humans, the somatostatin precursor (pre-proSRIF) is encoded by the SS1 gene, which is localized on chromosome 3q27.3. Two additional products of the SRIF precursor have been isolated in mammals. Neuronostatin is a 13-amino-acid noncyclic amidated peptide whose sequence immediately follows the signal peptide (Samson et al. 2008; Yosten et al. 2015). A second peptide expressed in enteric neurons comprises the N-terminal 13 amino acids of SRIF-28 (Ensinck et al. 2002, 2003). In 1996, a neuropeptide showing high similarity to SRIF was identified and termed cortistatin (CST) (de Lecea et al. 1996). CST is encoded by the *CORT* gene (Liu et al. 2010). Cleavage of preproCST gives rise to two mature products, CST-17 and CST-29, in humans. CST is also structurally and functionally related to the urotensin II peptide family. The CST precursor is mainly expressed in the cerebral cortex and hippocampus. It is also expressed in the periphery but at lower levels than SRIF.

Tissue Distribution

In the CNS, SRIF has been localized in the mediobasal hypothalamus and median eminence, amygdala, preoptic area, hippocampus, striatum, cerebral cortex, and the brainstem (Johansson et al. 1984). SRIFergic neurons can be distinguished in hypophysiotropic neurons, long-projecting GABAergic neurons, and GABAergic interneurons (Viollet et al. 2008; Urban-Ciecko and Barth 2016). In the periphery, SRIF is expressed in secretory cells in gastrointestinal mucosa, δ-cells in pancreatic islets, and a subpopulation of C cells within the thyroid gland. SRIF is also produced by inflammatory and immune cells (Patel 1999) and has been localized in human epidermis (Merkel and dendritic cells) (Vockel et al. 2010, 2011). Finally, SRIF is believed to function as a neurotransmitter, neuromodulator, or trophic factor in the retina (Cervia et al. 2008). Plasma levels of SRIF are very low because of rapid degradation by ubiquitous peptidases.

Physiology

SRIF is a neurotransmitter, neuromodulator, and paracrine factor acting in the same tissues where it is expressed. Hypothalamic SRIF is the main inhibitory factor involved in the control of GH secretion and reduces the release of prolactin and thyroid-stimulating hormone (TSH) (Müller et al. 1999). In the brain, SRIF actions are mediated by presynaptic or postsynaptic mechanisms. SRIF modulates neuronal excitability, and in the hippocampus, cortex, and hypothalamus, it causes presynaptic inhibition of excitatory neurotransmission (Peineau et al. 2003). Finally, it is co-released with GABA from axonal terminals in different brain areas (Olias et al. 2004). SRIF has a role in cognitive functions, learning, memory, control of locomotor activity, food intake, nociception, and autonomic functions. SRIF is highly expressed in regions associated with seizures and has been suggested to act as an endogenous antiepilectic (Stengel et al. 2015). In the gastrointestinal tract, SRIF exerts a generalized inhibitory effect on release of gut hormones and exocrine secretion (gastric acid, digestive enzymes, bile, and colonic fluid). SRIF also negatively affects gallbladder contraction, small intestinal segmentation, and gastric emptying. In pancreatic islets, release of SRIF inhibits secretion of insulin, glucagon, and other peptides. SRIF may affect key cellular processes in diverse tissues by regulating the release of growth factors and cytokines as well as cellular responses to these stimuli. SRIF can contribute to control of lymphocyte and inflammatory cell proliferation and activity, tumor cell growth, and normal tissue plasticity (Patel 1999; Rai et al. 2015). In the human skin, SRIF has been suggested as a negative regulator of epidermal wound healing (Vockel et al. 2011). Cortistatin binds to all SRIF receptors with similar affinity (Siehler et al. 2008) than SRIF and shares many functional properties with SRIF in the brain, within the hypothalamus-pituitary system and in the periphery (Spier and de Lecea 2000). Actually, cortistatin may activate GPCRs other than SSTs, i.e., the ghrelin receptor 1a (GHS-R1a) (Callaghan and Furness 2014).

Somatostatin Receptor Subtypes

Between 1990 and 1997, five distinct SRIF receptor subtypes were cloned in mice, rats, and humans. According to the International Union of Basic and Clinical Pharmacology (IUPHAR)

guidelines, these receptors are now referred to as SST_1 to SST_5 (Gunther et al. 2018) (Table 1). In the early 2000s, it was shown that some SSTs were capable of forming homodimers as well as heterodimers with other subtypes of the same family or with other GPCRs (Rocheville et al. 2000a, b; Pfeiffer et al. 2001, 2002). This kind of interaction between cell surface receptors may further enlarge the number of distinct receptor sites with differential functional properties for the same ligand. SSTs can be separated into two groups on the basis of their structural homologies and pharmacological properties. The first class comprises SST_2, SST_3, and SST_5. The second class comprises SST_1 and SST_4. All the SSTs are class A GPCRs that belong to the rhodopsin-like family of receptors (Table 1). They all possess seven transmembrane domains according to the structure of a prototypical GPCR. There is high sequence similarity between different subtypes and for a given subtype across different species. Moreover, all the five subtypes share some primary structural features:

- The conserved YANSCANPILY sequence in the seventh transmembrane domain is regarded as a mammalian SST signature.
- The DRY motif in the second intracellular loop is involved in coupling to G proteins.
- The consensus motif X-[S/T]-X-Φ at the end of the carboxyl-terminal tail is a potential PDZ domain binding site and is crucial for interaction with scaffolding proteins.
- N-glycosylation sites.

In recent years, variants of SST5 mRNA formed by splicing of non-canonical donor and acceptor splice sites have been identified in humans, pigs, and rodents. The human SST_5 variants may generate truncated receptors containing five or four transmembrane domains (SST_5TMD5 and SST_5TMD4, respectively) and distinct carboxyl termini (Durán-Prado et al. 2009), which display distinct tissue distribution and subcellular localization, compared with canonical full-length SST_5 (Córdoba-Chacón et al. 2011). The truncated receptors are highly expressed in tumor tissues and can interact with full-length SST_5 and SST_2 to retain them in intracellular vesicles. Hence, they may act as functional dominant-negative partners for the full-length receptors.

Canonical SSTs share many structural characteristics and also the main intracellular signaling pathways they are coupled to. All SSTs mediate the inhibitory actions of SRIF on endocrine and exocrine secretion and neuronal excitability by recruitment of G_i/G_o proteins, members of heterotrimeric, pertussis toxin (PTX)-sensitive guanine-nucleotide-binding proteins. The activation of G_i/G_o proteins by SSTs is coupled to the suppression of the second messenger cyclic AMP (cAMP) by inhibition of adenylyl cyclase activity and the decrease of cytosolic Ca^{2+} caused by inhibition of Ca^{2+} influx. To this end, SRIF can inhibit calcium channels both directly and indirectly by opening G-protein-activated-inward-rectifier K^+ channels (GIRK) to induce membrane hyperpolarization that leads to inhibition of Ca^{2+} influx via voltage-operated calcium channels (VOCC). The signaling events mediating the inhibitory effects of SRIF on cell proliferation are debated. The activation of protein tyrosine phosphatases (i.e., Src homology region 2 domain-containing phosphatase SHP-1 and SHP-2) may counteract growth factor-stimulated tyrosine kinase activity and inhibit multiple mitogenic pathways. The modulation of the extracellular-signal-regulated kinase (ERK) pathway may also play a role. Finally, the second class of SSTs and SST_3 is able to exert an inhibitory activity on sodium/hydrogen exchanger-1 (NHE1) via a PTX-insensitive mechanism. A decrease in NHE1 activity results in extracellular medium acidification that may be involved in inhibition of cell migration by SRIF (Buchan et al. 2002). SSTs can be better differentiated according to their expression in normal and tumor tissues, cellular localization, functional and pharmacological properties, and differential modes of activity regulation.

Agonist-Dependent Receptor Regulation

SRIF binding to its receptors results in desensitization, followed by internalization of receptor-

Somatostatin, Table 1 Class II and Class I somatostatin receptor subtypes: tissue distribution and role in physiology

Receptor	Expression in normal tissues	Expression in human tumors	Phenotype of mice lacking receptor	Receptor function
SST_1	Brain and spinal cord, anterior pituitary, pancreatic islets, GI tract Subcellular localization: when expressed in transfected HEK293 cells, localized to intracellular vesicles In primary cells and in sections from diverse human tumors, localized both at the plasma membrane and in the cytoplasm	GH adenomas, NET, prostate cancer	Altered insulin homeostasis	Presynaptic localization, autoreceptor in the CNS Within hypothalamus, inhibition of GHRH release and SRIF release Control of gastric emptying and colonic mobility Control of inflammatory response and nociception transmission Control of GH release from pituitary and insulin from beta-cells
SST_4	Brain, retina, dorsal root ganglia, placenta		Increased seizure susceptibility Increased anxiety Increased inflammatory and nociceptive response	Post-synaptic localization in the CNS Expressed in areas involved in learning and memory processes Involved in the modulation of behavioral and neuroendocrine response to acute and chronic stress (related to anxiety and depression-like behavior) Anticonvulsant effects Therapeutic target for development of anti-inflammatory and/or analgesic drugs without endocrine side effects (airway inflammatory diseases and bronchoconstriction)
SST_2	Brain (including cerebellum), spinal cord, retina, dorsal root ganglia, pituitary, GI tract, pancreatic islets, lymphatic tissue, adrenals (reticular zone), kidney	Brain tumors, GH adenomas, TSH adenomas, pheochromo-cytomas, NET	High basal acid secretion, inhibition of glucagon release, impaired motor coordination	Inhibitory neuromodulation in the CNS, main role in transduction of SRIF action in hippocampal formation Antiepileptic activity Antinociceptive activity Control of extrapyramidal motor system Increase of food intake and rapid-onset water consumption Control of stress response (autonomic and behavioral) Control of GH and TSH release from pituitary, glucagon, and insulin release from pancreatic islets Retinal neuroprotection

(continued)

Somatostatin, Table 1 (continued)

Receptor	Expression in normal tissues	Expression in human tumors	Phenotype of mice lacking receptor	Receptor function
SST_3	Brain, anterior pituitary, pancreatic islets, GI tract, lymphatic tissue, adrenal cortex and medulla, kidney Subcellular localization: primary cilia (but not restricted to) in neurons and endocrine cells	GH adenomas, ACTH adenomas, non-functioning adenomas	Impaired novel object recognition	Involved in object recognition memory mediated by primary neuronal cilia (rodent brain) Anticonvulsant activity Control of GH from pituitary, insulin release from pancreatic islets
SST_5	Anterior pituitary, pancreatic islets, GI tract, lymphatic tissue, adrenal cortex and medulla, kidney, parotid glands, thyroid (C cells) Subcellular localization: both at the plasma membrane and in the cytoplasm	GH adenomas ACTH adenomas Thyroid carcinomas, NET, breast, cervical, ovarian, and prostate carcinomas, differentiated colorectal cancers	Increased insulin secretion, basal hypoglycemia	Physiological control of pituitary function (GH, ACTH, TSH secretion) and endocrine pancreas activity (insulin secretion) SST5 displays constitutive activity (tonic inhibition of cAMP and ERK1/2 signaling) contributing to the control of pituitary hormone secretion

ligand complexes, a critical process that leads to receptor recycling to plasma membrane and resensitization or, alternatively, to receptor degradation in intracellular vesicle compartments. Regulation of GPCRs depends upon molecular events starting from phosphorylation at the cytoplasmic C-terminal tail by different GPCR kinases (GRKs), followed by recruitment of β-arrestins and receptor endocytosis. β-Arrestins may also act as scaffolding proteins involved in intracellular signaling cascades. Studying SST phosphorylation and trafficking has received great attention because the fate of internalized receptors following agonist exposure may affect cell responsiveness to endogenous ligands and drugs of therapeutic interest. The distinct SST subtypes undergo differential events upon agonist binding. Moreover, as to a given subtype, internalization and trafficking may differ across species (Tulipano and Schulz 2007).

SST_1 In heterologous cell system, rat SST_1 undergoes endocytosis and recycling upon SRIF binding, whereas human SST_1 displays very slow internalization despite rapid desensitization and uncoupling of adenylyl cyclase. One amino acid change at a putative phosphate-acceptor site (Thr383-Cys384-Thr385-Ser386) in the rat versus human receptor C-terminal tail (human Ser386 has been replaced by alanine in rat SST_1) may explain the differential interaction with β-arrestin1 and differential trafficking.

SST_2 This receptor subtype is almost completely confined to the plasma membrane. Agonist binding (SRIF, octreotide) causes multiple phosphorylation events within the C-terminal tail by GRK_2 and GRK_3 followed by receptor internalization. The following phosphorylation sites have been identified in both rat and human SST_2: S341, S343, T353, T354, T356, and T359. Unlike SRIF and octreotide, pasireotide (also known as SOM230) stimulates the phosphorylation of S341 and S343 residues only and causes partial receptor internalization (Lesche et al. 2009; Lehmann et al. 2014). The activation of SST_2 results in marked recruitment of both β-arrestin-1 and β-arrestin-2. The β-arrestin-dependent trafficking of the SST_2 somatostatin

receptor resembles that of a class B GPCR receptor (Oakley et al. 2000) in that upon receptor activation, β-arrestin and the receptor form stable complexes and internalize together into the same endocytic vesicles. Unlike other class B receptors, however, the SST_2 receptor undergoes rapid resensitization and recycling to the plasma membrane after activation by SRIF-14. Actually, receptor recycling is dependent on endosomal acidification and endothelin-converting enzyme-1 (ECE-1)-dependent degradation of the ligand. SRIF-28 and octreotide are resistant to cleavage by ECE-1. Hence, upon activation by these ligands, SST_2 remains in the intracellular vesicular compartment for a prolonged period of time (Zhao et al. 2013).

SST_3 A unique feature of this subtype is its long carboxyl-terminal tail. Upon agonist binding, the receptor is phosphorylated by GRK_2 and GRK_3. Phosphorylation sites have been identified in human and rat SST_3. The human SST_3 receptor is phosphorylated at S337, T341, T348, and S361 (Lehmann et al. 2016). The rat SST_3 receptor is phosphorylated at S341, S346, S351, and T357 (Roth et al. 1997). Receptor phosphorylation triggers β-arrestin recruitment and internalization. The β-arrestin mobilization of the SST_3 resembles that of a class A receptor in that upon receptor activation, β-arrestin and the receptor form relatively unstable complexes that dissociate at or near the plasma membrane. Unlike other class A receptors, SST_3 receptor undergoes substantial downregulation upon agonist exposure by ubiquitin-dependent lysosomal degradation and does not rapidly recycle to the plasma membrane (Tulipano et al. 2004; Lesche et al. 2009; Lehmann et al. 2016). Finally, synthetic analogs such as pasireotide and octreotide have been reported to act as partial agonists with regard to SST_3 phosphorylation and internalization compared to SRIF-14 (Lehmann et al. 2016).

SST_4 In heterologous cells, rat SST_4 is not subject to SRIF-induced phosphorylation or internalization and does not recruit β-arrestin. The absence of internalization was also shown in brain tissue after in vivo treatment with SRIF (Kreienkamp et al. 1998; Schreff et al. 2000). A single amino acid residue (Thr331) in the rat SST_4 sequence confers resistance to agonist-induced internalization (Kreienkamp et al. 1998). As to human SST_4, poor internalization occurs in transfected cells. A rapid dissociation of the SRIF-receptor complex and rapid recycling of the receptor to the plasma membrane has been suggested. Actually, in contrast to the rat receptor, human SST_4 signaling (NHE1 activation and ERK phosphorylation) was shown to be subject to sustained desensitization. Hence, this event can be regarded as not linked to receptor sequestration (Smalley et al. 2001).

SST_5 Two phosphorylation sites have been mapped in the SST_5 carboxyl-terminal tail. T347 is phosphorylated even in the absence of ligand, whereas T333 is phosphorylated by GRK2 in response to agonist binding and is involved in β-arrestin recruitment and receptor internalization. The third intracellular loop plays a key role in the interaction between the receptor and β-arrestin (Peverelli et al. 2008; Petrich et al. 2013; Schulz et al. 2014). Like SST_3, SST_5 forms relatively unstable complexes with β-arrestin, which rapidly dissociate before SST_5 trafficking to early endosomal vesicles. In contrast to SST_3 and SST_2, the proportion of SST_5 internalized after exposure to SRIF is rather low. The 36 terminal residues of the carboxyl-terminal tail are believed to contribute to the inhibition of receptor internalization. As to the internalized receptors, in heterologous cells, it has been shown that ligand-receptor dissociation leads to receptor recycling to the cell surface, accompanied by recruitment of receptors residing in an intracellular pool to the plasma membrane (Stroh et al. 2000). Finally, it should be noted that the phosphorylation and trafficking following to agonist binding to SST_5, are ligand- and cell context-dependent. SRIF-14 induces rapid phosphorylation, whereas octreotide does not. Pasireotide

has been shown to induce SST5 phosphorylation to a lesser extent than SRIF-14.

Drugs

Ligands

Receptor	Endogenous agonists	Synthetic agonists	Synthetic antagonists
SST_1	SRIF-14, SRIF-28; CST-17, CST-19	L-797,591 BIM-23926 Pasireotide	SRA880
SST_2	SRIF-14, SRIF-28; CST-17, CST-19	L-779,976 BIM-23120 Octreotide Lanreotide Pasireotide (partial agonist) Veldoreotide (somatoprim)	JR-11
SST_3	SRIF-14, SRIF-28; CST-17, CST-19	L-796,776 Octreotide Pasireotide	ACQ090 sst3-ODN-8 MK-4256
SST_4	SRIF-14, SRIF-28; CST-17, CST-19	NNC26-9100 L-803,087 J-2156 Veldoreotide (somatoprim)	
SST_5	SRIF-14, SRIF-28; CST-17, CST-19	L-817,818 BIM-23268 Octreotide Lanreotide Pasireotide (higher affinity vs other SSTs) Veldoreotide (somatoprim)	S5A1

SRIF Analogs in Clinical Practice

Octreotide and **lanreotide** are the two SRIF analogs still most widely used in clinical practice. Increased metabolic stability, potent suppressing activity on GH secretion, and low insulin suppressing activity have made them useful for treating acromegaly (Bauer et al. 1982; Heiman et al. 1987). SRIF analogs were also shown to reduce GH-secreting pituitary tumor size (Giustina et al. 2012). The partial dissociation of the inhibitory effects on GH and insulin secretion compared to native SRIF was explained with the identification of distinct SRIF receptor subtypes which can be differentiated in relation to their expression levels in normal and tumor tissues. Indeed, octreotide and lanreotide have greater affinity (subnanomolar) for SST_2 and moderate affinity for SST_5. Both receptors are involved in the control of GH secretion in humans. On the other hand, SST_5 plays a main role in the control of insulin secretion from ß-cells. If the rationale for developing SRIF analogs showing subtype selectivity to limit side effects is still valid, the concept of targeting multiple SSTs by a multisubtype-selective analog or targeting complexes formed by multiple receptors (heterodimers) is regarded as attractive for possible enhanced efficacy. Indeed, octreotide and lanreotide do not allow to achieve normalization of GH and IGF-1 levels in a significant percentage of acromegalic patients, and there is pre-clinical evidence that greater SST_5 activation may lead to a better control of GH hypersecretion in patients which are only partially responsive to these analogs. BIM-23244 can be regarded as a bi-selective analog targeting SST_2 and SST_5. Actually, its high activity at SST_5 discouraged its development for the risk of unwanted pancreatic side effects. Veldoreotide is a ligand with moderate affinity for multiple subtypes (SST_2, SST_5, and SST_4) and is in phase 2 trials for the treatment of acromegaly. Finally, the concept of targeting the interaction between receptors has been extended to multi-ligands able to interact with a given SST subtype and receptors outside of the SST family, i.e., dopamine receptor subtype-2 (D_2). So-called chimeric dopastatins are no longer in development.

Pasireotide is a cyclohexapeptide with high affinity for SST_1, SST_2, and SST_3 and subnanomolar affinity for SST_5. Pasireotide is called a pan-receptor-specific ligand. Pre-clinical and clinical data suggest that pasireotide may be helpful to achieve control of GH secretion in a subgroup (15–20%) of patients resistant to octreotide or lanreotide. In detail, pasireotide can be more effective than octreotide in tumors showing lower expression of SST_2 and lower SST_2/SST_5 ratio

(Gadelha et al. 2014; Gatto et al. 2017). Moreover, in contrast to lanreotide and octreotide, pasireotide has shown potential efficacy in a subset of patients with Cushing's disease. However, in keeping with its high affinity at SST_5, pasireotide may worsen glucose control related to insulin resistance in both acromegaly patients and in Cushing's disease, and monitoring blood glucose and electrocardiogram is important.

Disease	Analog	Therapeutic effects
Acromegaly	Lanreotide Octreotide Pasireotide (long-acting formulations for i.m. or s.c. administration)	Biochemical control (decreased GH and IGF-1 levels) Decreased tumor size Beneficial impact on disease comorbidities (determined by age, degree of achieved biochemical control, duration of the disease)
Cushing's disease	Pasireotide	Decreased urinary-free cortisol (UFC) Decreased mean tumor size Beneficial impact on symptoms related to hypercorticolism (however, blood glucose and glycated hemoglobin levels may increase)
Neuroendocrine tumors (carcinoids, GI and pancreatic NETs)	Lanreotide Octreotide (long-acting formulations)	Symptomatic improvement due to suppression of hormone release Decreased or stabilized tumor mass Improved survival

The efficacy rates of therapies with SRIF analogs vary depending on tumor type and individual patient. As to GH-secreting pituitary tumors, some tumors are resistant to SRIF analogs. Moreover, SRIF analogs do not enable GH and IGF-1 normalization in a subset of responsive patients. To this end, a personalized approach to acromegaly classification and management has been proposed. Adverse determinants of SRIF analog responsiveness include advanced age, tumor size, levels of GH and IGF-1, and a series of molecular markers in pituitary tumor cells (Cuevas-Ramos et al. 2015). Corticotroph tumors are usually resistant to octreotide and lanreotide. About 20% of patients with Cushing's disease achieve biochemical normalization with pasireotide (Colao et al. 2012). Tachyphylaxis has not been observed in acromegaly, but it has been described at variable time intervals in patients harboring NETs.

SRIF-Based Radiopharmaceuticals

Radiolabelled somatostatin analogs are used for tumor imaging (peptide receptor-targeted scintigraphy) and peptide receptor radionuclide therapy (PRRT) of neuroendocrine tumors. A radioiodinated analog of octreotide ($[^{123}I]Tyr^3$-octreotide) was synthetized in 1989 (Krenning et al. 1989) and allowed the first successful non-invasive imaging of SST-rich tumors. This compound can be regarded as a starting point for the development of radiolabeled ligands for SST-targeted imaging and radionuclide therapy. Current ligands include a chelator conjugated to the N-terminus of a peptide structure derived from SST-targeting drugs (i.e., octreotide or lanreotide). The chelator (DTPA, DOTA, NOGADA) ensures the stable complexation of radiometals (^{68}Ga, ^{111}In, ^{177}Lu, ^{90}Y). These compounds exhibited agonistic behavior and high affinity for SST_2 (^{111}In-DTPA-OC, ^{68}Ga-DOTA-TOC, ^{68}Ga-DOTA-TATE, ^{177}Lu-DOTA-TATE). Radiolabelled ligands that bind with high affinity to multiple SST receptor subtypes are in development (^{68}Ga-DOTA-NOC). Metabolic stability, low lipophilicity, renal clearance, and absence of accumulation in the upper abdominal region due to hepatobiliary excretion are favorable properties of currently applied radiolabeled SRIF analogs compared to radioiodinated octreotide. The high sensitivity of SST-targeted has been assumed to be dependent on internalization of the receptor after

radioligand binding that leads to retention of radiometals in target cells. Nevertheless, in the last decade, evidence has accumulated that SST-targeted imaging with radiolabelled antagonists, which poorly internalize, can be more sensitive and effective than that employing corresponding receptor agonists that are highly internalized. The molecular mechanism underlying tumor imaging performance using SST antagonists needs to be elucidated. Actually, it has been suggested that a full antagonist can label receptors in all states (active or inactive), whereas a corresponding agonist binds to an active conformation, only.

Cross-References

► Peptides and Peptidomimetics as Foundations for Drug Discovery

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Specialized Pro-resolving Lipid Mediators

► Pro-resolving Mediators

Sphingosine-1-Phosphate

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Definition

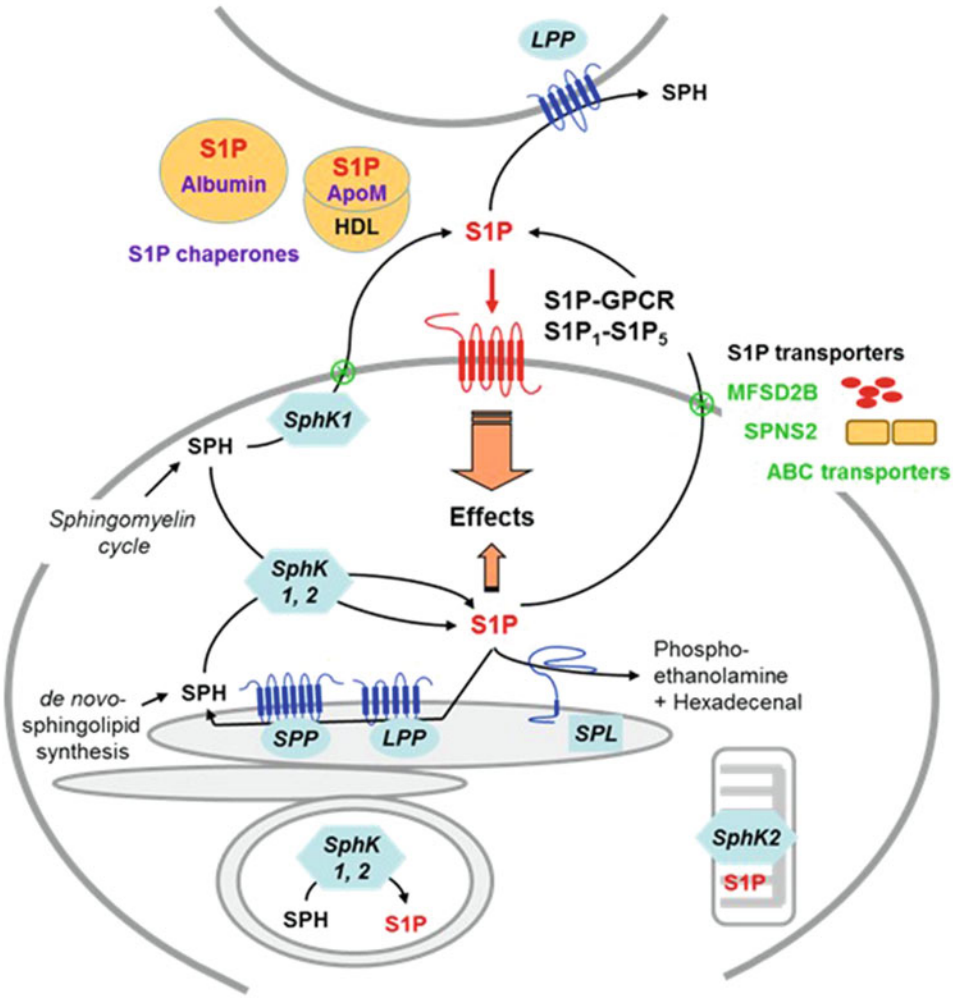
Sphingosine-1-phosphate is a lysophospholipid, and product of sphingolipid metabolism.

Basic Characteristics

Metabolism and Occurrence

Sphingosine-1-phosphate (S1P) is formed by phosphorylation of sphingosine by sphingosine kinases (SphK1,2; human gene names, *SPHK1,2*) (Chan and Pitson 2013; Pyne et al. 2016). Prototypical S1P d18:1 contains the (E,2S,3R)-2-aminooctadec-4-ene-1,3-diol sphingoid base backbone (D-erythro-C18-sphingosine). This structure results from sequential reactions starting with condensation of serine and palmitoyl-CoA by serine palmitoyltransferase (SPT; human gene names, *SPTLC1-3*). The double bond is introduced by delta-4-desaturases (human gene names, *DEGS1,2*) (Maceyka and Spiegel 2014; Obinata and Hla 2019). Under certain conditions, SPT may also use other amino or fatty acids and thereby produce sphingoid base backbones with other chain lengths and substitutions. Degradation of S1P occurs reversibly by non-selective lipid phosphate phosphatases, $LPP_{1–3}$, or by selective S1P phosphatases ($SPP_{1,2}$). S1P lyase (human gene name, *SGPL1*) cleaves S1P irreversibly (see Lysophospholipids, Fig. 1b).

S1P is usually generated intracellularly and needs to be exported for interaction with its specific G-protein-coupled receptors (S1P-GPCR; Fig. 1). SphK1 and SphK2 differ in tissue expression and subcellular localization, regulation and function. Their expression and activity are regulated in diverse ways, for example, by growth factors, cytokines, or GPCR agonists (Chan and Pitson 2013; Pyne et al. 2016). Some stimuli induce a translocation of SphK1 from the cytosol to the plasma membrane, enabling S1P secretion and so-called inside-out signalling. Both SphK isoforms can occur in cytosol and nucleus; however, SphK2 is more abundant in the nucleus and also found at the ER and in mitochondria. Nuclear and mitochondrial SphK2 address intracellular effectors of S1P. These may be, for example, histone deacetylases or prohibitin-2 (Pyne et al. 2016; Spiegel et al. 2019), but these targets still await independent confirmation. S1P excretion is mediated by sphingolipid transporter-2 (SPNS2), major facilitator superfamily domain containing 2B (MFSD2B) and several ATP-binding cassette



Sphingosine-1-Phosphate, Fig. 1 The S1P signalling system. *ApoM* apolipoprotein M, *HDL* high-density lipoprotein, *LPP* lipid phosphate phosphatase, *MFSD2B* major facilitator superfamily domain containing 2B, *SPH* sphingosine, *SphK* sphingosine kinase, *SPL* S1P lyase, *SPNS2* spinster homologue-2, sphingolipid transporter-2, *SPP* S1P phosphatase

(ABC) transporters (Spiegel et al. 2019; Riboni et al. 2020). S1P occurs in plasma at high concentrations (~0.2–0.4 μM). Main sources of circulating S1P are erythrocytes expressing MFSD2B and endothelial cells expressing SPNS2 (Obinata and Hla 2019). S1P is furthermore stored in platelets, released via MFSD2B upon platelet activation, and occurs in serum at ~0.5–0.8 μM. About 65% of plasma S1P is bound to apolipoprotein M (ApoM) within high-density lipoproteins (HDL); the remainder is bound to albumin. These S1P chaperones fine-tune S1P's interaction with S1P-GPCR, with ApoM producing a sustained S1P release (Obinata and Hla 2019). Of note, tissue S1P levels are very low, and there are S1P gradients between tissues and circulation and within tissues. These chemotactic gradients are essential for trafficking of immune cells and positioning of cells within tissues (Dixit et al. 2019).

S1P Receptors

An overview of G protein-coupled S1P receptors as listed in the IUPHAR/BPS Guide to Pharmacology is presented in Table 1. These receptors

Sphingosine-1-Phosphate, Table 1 G protein-coupled S1P receptors

Receptor (previous names)	Signalling	Main biological effects
$S1P_1$ (EDG1)	$G_{i/o}$	Cell migration ↑, cell proliferation, survival, cell-cell contacts, lymphocyte trafficking, angiogenesis, vascular barrier ↑, heart rate and atrioventricular conduction ↓ in humans
		-/- mice: embryonic lethality, defective vascular maturation
$S1P_2$ (EDG5, H218, AGR16)	$G_{i/o}$, $G_{q/11}$, $G_{12/13}$	Cell migration ↓, vascular barrier ↓, vascular development
		-/- mice: hearing loss, B cell lymphoma at high age, neuronal hyperexcitability
		Human mutations: hearing impairment
$S1P_3$ (EDG3)	$G_{i/o}$, $G_{q/11}$, $G_{12/13}$	Vascular development, NO-dependent vasorelaxation, heart rate ↓ in mice
		-/- mice: no obvious abnormalities
$S1P_4$ (EDG6)	$G_{i/o}$, $G_{12/13}$	Expression limited to haematopoietic tissues. Anti-inflammatory activity, proliferation and cytokine secretion of T-cells ↓, 5-lipoxygenase ↓, terminal megakaryocyte differentiation
		-/- mice: delayed recovery from thrombocytopenia
$S1P_5$ (EDG8, NGR-1)	$G_{i/o}$, $G_{12/13}$	Cell proliferation, mitotic progression, cell rounding, oligodendrocyte functions, natural killer cell trafficking
		-/- mice: altered natural killer cell homing and tissue invasion

comprise the class A GPCR and $S1P_{1–5}$ (human gene names, *S1PR1-5*) (see the IUPHAR GPCR database). S1P-GPCR differentially couples to $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$ proteins (Table 1). Thus, these receptors regulate classical signalling pathways such as ▶ adenylyl cyclase, phospholipase C $[Ca^{2+}]_i$, protein kinases C, mitogen-activated protein kinases, Akt, Ras, Rac and Rho. Thereby, they modulate cell growth, survival, re-arrangement of the cytoskeleton, cell migration, Ca^{2+} signalling and other responses. The $G_{12/13}$-coupled receptors also activate the Hippo pathway, which controls cell growth, immunity, fibrosis and cancer (Noguchi et al. 2018). $S1P_{1-3}$ are widely expressed, while $S1P_4$ is rather restricted to haematopoietic tissues and $S1P_5$ is preferentially expressed in the brain, immune cells, skin and other tissues. Functional studies have demonstrated that also S1P can act independently of the known S1P-GPCR. Several intracellular targets for S1P have been described but still await independent confirmation (Pyne et al. 2016; Spiegel et al. 2019).

Biological Actions

The components of the S1P signalling system are widely expressed. They regulate tissue homoeostasis in many organs, including the heart, kidney, liver, intestine, pancreas, adipose tissue, bone, skin and immune and nervous systems.

Development The S1P signalling system plays a role in angiogenesis, neurogenesis, and limb development in mice and regulates cardiogenesis in zebrafish (Mendelson et al. 2014). Mice lacking *S1pr1* die during embryonic development from haemorrhages caused by a failure of pericytes to migrate around newly formed capillaries. Additional deletion of *S1pr2* or *S1pr3* leads to more severe vascular defects. Mice lacking both SphK isoforms do not have measurable S1P concentrations and die during embryonic development from haemorrhages and defective vasculogenesis. Furthermore, they exhibit disturbed neurogenesis and impaired neural tube closure. Defective neurogenesis is also observed in *S1pr1* knockouts, indicating that the *Sphk/S1pr1* axis is important for angio- and neurogenesis (Mendelson et al. 2014). Both the $S1P_2$ receptor and SPNS2 are essential for function of the inner ear. Mice lacking *S1pr2* or *Spns2* suffer from hearing loss early after birth, and mutations of *S1PR2* in humans are associated with deafness (Romero-Guevara et al. 2015). Furthermore, both the $S1P_2$ receptor and SPNS2 are required for migration of cardiac precursors to the midline in zebrafish (Mendelson et al. 2014). Finally, deletion of *Sgpl1*, which represents the only exit point of sphingolipid metabolism, leads to multiple organ defects and early death in mice. Similarly, loss-of-function mutations in *SGPL1*

cause S1P lyase insufficiency syndrome (SPLIS) in humans, with steroid-resistant nephrotic syndrome, damage of the central and/or peripheral nervous systems, adrenal insufficiency and other symptoms (Choi and Saba 2019).

S1P Gradients and the Immune System SphK, LPP, S1P lyase and SPNS2 contribute to formation of S1P concentration gradients between tissues and circulation and within organs (Dixit et al. 2019). S1P-GPCRs regulate cell migration and positioning within these gradients, with $S1P_1$ and $S1P_2$ acting antagonistically. $S1P_1$ activates Rac and stimulates migration, while $S1P_2$ activates Rho, induces stress fibre formation and inhibits migration. This is of particular importance for immune cell trafficking: $S1P_1$ is required for lymphocyte egress from lymphatic tissues, while $S1P_2$ mediates confinement of B cells and follicular T helper cells to lymph node germinal centres. The $S1P_5$ receptor is essential for natural killer cell trafficking. While S1P regulates migration of many types of immune cells, the S1P signalling system also regulates development, differentiation and polarization of these cells (Blaho and Hla 2014; Weigert et al. 2019).

Cardiovascular System S1P is an important mediator in the cardiovascular system. Not only angio- and vasculogenesis but also vascular permeability is regulated by S1P: $S1P_1$ stimulates endothelial cell adherens junction assembly and improves the vascular barrier, while $S1P_2$ disrupts adherens junctions and induces vascular leak (Obinata and Hla 2019). The $S1P_2$ receptor induces contraction of diverse types of smooth muscle, including vascular, bronchial, intestinal and bladder smooth muscle, via $[Ca^{2+}]_i$ increases and activation of Rho/Rho kinase (Blankenbach et al. 2016). Endothelial $S1P_1$ and $S1P_3$ receptors, on the other hand, cause NO-dependent vasorelaxation. S1P has both pro- and anti-atherosclerotic effects (Kurano and Yatomi 2018). $S1P_2$ retains macrophages in atherosclerotic plaques, and knockout of this receptor was beneficial in models of atherosclerosis. $S1P_1$ acts anti-atherosclerotic via activation of NO synthase and suppression of endothelial adhesion molecules and is preferentially activated by ApoM-bound S1P (Kurano and Yatomi 2018). $S1P_3$ in mice and $S1P_1$ in humans, respectively, activate $I_{K(ACh)}$ in atrial myocytes, leading to bradycardia and delay of atrioventricular conduction (Dyckman 2017).

Inflammation, Fibrosis and Cancer The S1P signalling system also affects **inflammation, fibrosis and cancer**. This is due to S1P's role in cell proliferation, cell survival, migration and invasion and its eminent effects on cells of the innate and adaptive immune systems. In particular, the importance of $S1P_1$ for lymphocyte trafficking makes it a target in many inflammatory and autoimmune diseases, such as multiple sclerosis, inflammatory bowel disease, psoriasis, lupus erythematosus and others (see Drugs). Key pro-inflammatory mediators, for example, interleukin-1β and tumour necrosis factor-α, activate and induce SphK (Chan and Pitson 2013), which can lead to either intracellular S1P formation or S1P export and cross-activation of S1P-GPCR. Extracellular S1P can act both pro- and anti-inflammatory (Maceyka and Spiegel 2014; Nagahashi et al. 2018). For example, the $S1P_2$ receptor activates NF-κB and/or induces cyclooxygenase-2 and prostaglandin production in several cell types (Blankenbach et al. 2016). Also the key mediator of fibrosis, transforming growth factor-β (TGF-β), induces SphK1. In inflammatory kidney diseases, SphK1 is often upregulated and appears to act as a brake in kidney fibrosis (Huwiler and Pfeilschifter 2018). Extracellular S1P, on the other hand, can cross-activate the TGF-β/Smad signalling cascade and increase the production of extracellular matrix (Huwiler and Pfeilschifter 2018). In cancer, S1P affects both tumour cells and cells of the tumour microenvironment (Pyne et al. 2018; Riboni et al. 2020).

Drugs

There are currently three drugs approved that target the S1P signalling system: fingolimod and ozanimod for relapsing-remitting multiple sclerosis and siponimod for secondary progressive multiple sclerosis. Fingolimod (FTY720), which structurally resembles sphingosine, is phosphorylated by SphK2

Sphingosine-1-Phosphate, Table 2 Drugs targeting the S1P signalling system

Drug	Target	Indication	Stage
Fingolimod	S1P receptor modulator	Relapsing-remitting multiple sclerosis	Approved
	$S1P_{1/3/4/5}$		
Siponimod (BAF-312)	S1P receptor modulator	Secondary progressive multiple sclerosis	Approved
	$S1P_{1/5}$		
Ozanimod (RPC1063)	S1P receptor modulator	Relapsing-remitting multiple sclerosis	Approved
	$S1P_{1/5}$	Ulcerative colitis	Phase III
Etrasimod (APD334)	S1P receptor modulator	Ulcerative colitis	Phase III
	$S1P_1$ ($S1P_4$, $S1P_5$)	Atopic dermatitis	Phase II
Ponesimod (ACT-128800)	S1P receptor modulator	Multiple sclerosis	Phase II
	$S1P_1$ ($S1P_3$, $S1P_5$)	Chronic plaque psoriasis	Phase II
Cenerimod (ACT-334441)	S1P receptor modulator	Systemic lupus erythematosus	Phase II
	$S1P_1$ (selectivity unknown)		
Amiselimod (MT-1303)	S1P receptor modulator	Relapsing-remitting multiple sclerosis, Crohn's disease, psoriasis, systemic lupus erythematosus	Phase II
	$S1P_1$ ($S1P_5$)		
Ceralifimod (ONO-4641)	S1P receptor modulator	Relapsing-remitting multiple sclerosis	Phase II
	$S1P_{1/5}$		
Mocravimod (KRP-203)	S1P receptor modulator	Ulcerative colitis, cutaneous lupus erythematosus	Phase II
	$S1P_{1/5}$		
Opaganib (ABC294640)	SphK2 inhibitor	Pancreatic cancer, unspecified adult solid tumour	Phase I

and then acts as agonist at all S1P-GPCR except $S1P_2$. The immunosuppressive activity of fingolimod is caused by super-agonistic activation and subsequent downregulation of the $S1P_1$ receptor which is required for lymphocyte egress from lymphatic tissues (Huwiler and Zangemeister-Wittke 2018). Lymphopenia induced by loss of $S1P_1$ represents fingolimod's principle mechanism of action in multiple sclerosis. Undesired effects include bradycardia and atrioventricular block induced by $S1P_1$-mediated activation of cardiac G protein-coupled inwardly rectifying potassium channels, macular oedema caused by disturbance of the vascular barrier, mild increase in arterial blood pressure, bronchoconstriction, increases in liver enzymes and elevated risk for infections (Huwiler and Zangemeister-Wittke 2018). The risk for bradycardia is high during the initial phase of therapy and declines with $S1P_1$ desensitization. Siponimod has a high selectivity for $S1P_1$ and $S1P_5$ and a shorter half-life compared to fingolimod. Despite sparing $S1P_3$, it still causes adverse effects in the cardiovascular system (Al-Salama 2019). Several other S1P receptor modulators with greater S1P-GPCR subtype selectivity are in clinical development (Dyckman 2017) (Stepanovska and Huwiler 2020); some of them are shown in Table 2. All act as super-agonists/functional antagonists at the $S1P_1$ receptor and thereby induce immunosuppression. Consequently, these compounds are developed for diverse autoimmune diseases (Table 2). While pharmaceutical development thus strongly focuses on $S1P_1$, other S1P-GPCR and SphK1/2 might as well be worthwhile targets. However, the only SphK inhibitor in clinical development is ABC294640, a SphK2 inhibitor with its K_i in the micromolar range, which is being evaluated as anticancer agent (Table 2).

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SPMs

▶ Pro-resolving Mediators

Statins

▶ Lipid-Lowering Drugs

Steroids

▶ Glucocorticoids

Stimulant Drugs

▶ Psychostimulants

Stimulants

▶ Psychostimulants

Stress Proteins

▶ Chaperones

Stroke

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Definition

Traditionally, stroke has been defined according to its clinical presentation as a focal neurological deficit with a sudden onset that probably has a vascular cause. The advent of computer tomography, magnetic resonance imaging (MRI), and other imaging techniques has allowed a stronger focus on the cerebrovascular pathology and has led to a broadening of the definition. Now, it includes ischemic infarcts and intracerebral hemorrhages, both with and without symptoms, transient ischemic attacks, subarachnoid hemorrhages, and cerebral venous thrombosis (Sacco et al. 2013). Global cerebral ischemia is usually excluded from the definition of stroke as it differs in clinical presentation and is caused by conditions, such as cardiac arrest or hypotensive shock, that lie outside of the cerebrovascular system.

Its high incidence and mortality make stroke one of the most important causes of death worldwide. The most common form of stroke is an ischemic infarction that is about four times as frequent as intraparenchymal hemorrhage. Based on the vascular origin, ischemic strokes are classified as cardioembolic, atherosclerotic, microvascular, and cryptogenic, each of which represents approximately a quarter of cases (Saver 2016). Additional classification builds on the involved vascular territory or the area of the CNS, including retina and spinal cord. Hypertensive intracerebral hemorrhages occur at preferred sites, such as the basal ganglia and the pons. Outside of these predilection sites, hemorrhages often have another aetiology. In the case of subarachnoid hemorrhage and cerebral venous thrombosis, the identification of an aneurysm or the site of thrombosis is important for the exact definition of the disease.

Basic Mechanisms

Ischemic Penumbra

All forms of stroke are associated with focal ischemia in the brain, spinal cord, or retina. Even in hemorrhagic strokes, compression of vessels or vasospasms leads to secondary ischemia of the tissue contributing to the neurological deficits. However, in contrast to global ischemia, focal ischemia is associated with some residual blood flow through collateral vessels, which provides a route for drugs to reach the lesion. Low perfusion in the core of the ischemia triggers rapid tissue loss. In the border zone between core and healthy tissue, blood flow gradually increases. The tissue, in which blood flow is high enough for acute survival but still so low to put it at risk for subacute demise, is called penumbra. Salvaging the penumbra is the target of stroke therapy. Size and distribution of the penumbra are dynamic and depend on individual factors, such as collateral blood vessels. For patient care, it is therefore important that MRI techniques are available that allow determining a proxy of the penumbra.

Pathomechanisms of Ischemic Brain Damage

Ischemia has a profound impact on the CNS. Four complexes in the pathophysiology explain the high vulnerability to ischemia: periinfarct depolarization, excitotoxicity, regulated cell death, and inflammation.

The CNS spends a lot of energy to maintain the polarization of neurons. Due to the loss of cellular energy in cerebral ischemia, neurons depolarize. A wave of **depolarization** spreads into the penumbra (Dreier 2011). There, the attempt of cells to repolarize strains the energy balance of the tissue that is already at risk due to the reduced perfusion. Depolarization of cells is associated with a shift of extracellular water into cells. This phenomenon is the basis of diffusion-weighted MR imaging that is used in clinical practice to detect early signs of cerebral ischemia. Lesions identified in diffusion-weighted MRI represent the core infarct area in which tissue usually does not recover.

Due to the energy loss in the ischemic tissue, excitatory neurotransmitters, mainly glutamate, are released and their re-uptake is impaired. This

is the basis of **excitotoxicity**. Activation of glutamate receptors, particularly those outside of synapses, triggers a detrimental cascade of events. Accordingly, the inhibition of glutamate receptors is neuroprotective in experimental stroke models, but clinical trials did not show an effect (Lai et al. 2014). A key problem in translating this and other findings seems to be the therapeutic time window in preclinical studies that is often too short for clinical practice. Targeting the events downstream of glutamate receptor activation might help to extend the therapeutic window. The stimulation of glutamate receptors leads to a calcium overload of cells and mitochondria. This damages mitochondria and enhances the production of reactive oxygen species. Another important consequence of glutamate receptor activation is the synthesis of NO by the neuronal NO synthase. This enzyme is coupled by the adaptor protein PSD-95 to glutamate receptors and is activated by elevated intracellular calcium levels. NO and superoxide anions form the highly reactive peroxynitrite that damages numerous macromolecules.

The death of neural cells denotes an irreversible step in the ischemic cascade. Evidence that many cells succumb to some form of **regulated cell death** raised hope that an intervention is possible. Apoptosis, necroptosis, autophagy, ferroptosis, and pyroptosis have all been reported in stroke models. Variants of cell death are often interrelated and blocking one form promotes another. For example, caspase inhibitors reduce apoptosis and ameliorate ischemic damage, but the effect seems to be dampened by enhanced necroptosis. Also, necroptosis inhibitors are protective in cerebral ischemia suggesting that a cocktail of cell death inhibitors may have a superior effect.

Dying cells release damage-associated molecular patterns, such as HMGB1 or ATP, that elicit a sterile **inflammation** in the CNS. Damage-associated molecular patterns act on microglia and other neural cells. In all cells of the CNS including neurons, cerebral ischemia stimulates pro-inflammatory signalling, such as the NF-κB pathway. In addition, neutrophils, monocytes, and lymphocytes are allured to the ischemic tissue. The complex tissue response has both detrimental and beneficial effects. On the damage side, inflammatory cytokines contribute to the demise of neurons. They also open the blood-brain barrier and promote vascular brain edema. In its worst form, brain edema critically elevates the intracranial pressure lowering cerebral perfusion and causing herniation and death of patients. On the protection side, activated microglia or brain macrophages reduce the damage by counteracting excitotoxicity, releasing anti-inflammatory mediators and removing tissue debris. The line between damage and protection seems to be fluid. Local properties including perfusion or systemic factors, such as body temperature, have a profound influence. In addition, timing is very important after stroke. Anti-inflammatory interventions may protect in the acute phase but impair recovery when administered too late (Lo 2008).

Pharmacological Intervention/ Pharmacotherapy

When treating patients with ischemic stroke, the first aim is to **reinstall perfusion**. Since 1996, tissue plasminogen (tPA, alteplase) has been used for thrombolysis and has recently been supplemented by mechanical intravascular thrombectomy. Often intravenous thrombolysis and intravascular thrombectomy are combined in individual patients. tPA variants with enhanced fibrin selectivity and prolonged half-life, such as tenecteplase, may be superior, but in 2020, alteplase is still the only approved agent for thrombolysis within 4.5 h after symptom onset. Imaging of the ischemic territory allows extending the therapeutic window in selected cases (Thomalla et al. 2018). However, with increasing time since onset of ischemia, the efficacy of thrombolysis decreases while the risk for intracerebral hemorrhage rises. Despite successful recanalization of occluded arteries, cerebral perfusion may remain low. This phenomenon, called no reflow, is attributed to a disturbance of the microvasculature. Apparently, small vessels are obstructed by constricting pericytes, swollen

astrocytes, or clogging immune cells (Bai and Lyden 2015). A disturbance of the microvasculature is also a likely cause of intracerebral hemorrhage that can be life-threatening after thrombolysis. Depending on local settings, up to 20% of patients with acute ischemic stroke are eligible for thrombolysis.

Besides acute recanalization therapy, **secondary prevention** is the most successful treatment option for stroke patients. After a stroke or transient ischemic attack, the risk for another event is elevated for several weeks. If cardiac embolism is not the cause of stroke, inhibitors of platelet aggregation significantly lower the risk of recurrence. The standard therapy is aspirin. Clopidogrel or aspirin plus dipyridamole are slightly more effective. Aspirin is also transiently combined with clopidogrel to further enhance its efficacy. Whether other P_2Y_{12} antagonists, like prasugrel or ticagrelor, could provide advantages is the subject of ongoing trials. In patients with cardioembolic stroke, anticoagulant drugs are administered to prevent a secondary event. In the very acute phase of ischemic strokes, anticoagulants are associated with an increased risk for intracerebral hemorrhages. Therefore, a lag time of 4–14 days between stroke onset and start of the anticoagulant therapy is recommended. In the treatment of patients with intracerebral hemorrhage, lowering high blood pressure is beneficial to reduce further bleeding (Moullaali et al. 2019). This is also the rationale why the blood pressure should be below 185 mmHg systolic and 110 mmHg diastolic before recanalization therapy in ischemic stroke. For the acute control of hypertension, the competitive α1 and β adrenergic receptor antagonist labetalol and the dihydropyridine calcium channel blocker nicardipine are used. After the acute phase, a strict control of hypertension has proven to be most effective in lowering the stroke rate. Finally, lipid-lowering drugs, mainly potent HMG-CoA reductase inhibitors, have an established place in the secondary prevention of ischemic stroke. Statins are afflicted with a low risk of causing intracerebral hemorrhages that is, however, outweighed by their beneficial effects in the presence of atherosclerotic diseases (Goldstein et al. 2008).

Future Directions

Stroke has served as a model disease for neurodegenerative disorders. Its pathophysiology has been intensively studied leading to an impressive armamentarium of neuroprotective drugs that work in preclinical experiments. However, translation of neuroprotection into clinical treatments proved to be difficult. In contrast, thrombolysis was more successful and shows that pharmacotherapy of ischemic stroke is possible. Therefore, an obvious aim is to improve thrombolysis further. Optimizing the pharmacokinetics or the fibrin selectivity of thrombolytics as well as limiting its inhibition by the plasma proteins PAI-1 and TAFI may increase the recanalization rate in acute stroke. In addition to plasminogen, alteplase cleaves NMDA receptors and basement membrane proteins which may contribute to its adverse effects. Limiting off-target cleavage may improve its safety profile (Thiebaut et al. 2018).

Blood coagulation is closely linked to inflammation and cell death. Along these lines, it may be possible to extend the success of anticoagulants and thrombolytics and to develop agents that manipulate cell death and inflammation in addition to blood coagulation. Activated protein C and thrombomodulin are endogenous factors that have this potential dual activity. Many inflammatory factors proved to be Janus-faced with both beneficial and detrimental actions. However, this is unlikely to be a universal principle and further anti-inflammatory strategies await to be tested. Other appealing targets are proteins that induce both apoptosis and necroptosis, a possible example being RIPK1.

To evaluate the potential of these and other targets, preclinical models should investigate the **long-term functional outcome** that is essential for clinical efficacy. Clinical trials usually evaluate the neurological deficit over 90 days. In contrast, this aspect has often been neglected in basic research. Long-term recovery is determined by post-insult plasticity and regeneration. The pharmacology of these processes is still largely unexplored but may be worthwhile to study.

In the future, progress may come from a **precision medicine** approach towards stroke. So far, clinical studies have often lumped all stroke patients into heterogeneous groups, irrespective of the aetiology, the size of the penumbra, or the presence of inflammation. New imaging techniques or biomarkers may help identify stroke patients that benefit most from specific therapies. NMR- and mass spectrometry-based lipidomics and metabolomics provide unprecedented insight into the molecular events underlying injury in individual patients and may increase therapeutic precision in the future.

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Structural Bioinformatics

▶ Molecular Modelling

Strychnine-Sensitive Glycine Receptor

▶ Glycine Receptors

sUA

▶ Physiology and Treatment of Hyperuricemia and Gout

Substance P

▶ Peptides and Peptidomimetics as Foundations for Drug Discovery

Substance P Receptor

▶ Neurokinin/Tachykinin Receptors

Substantia Nigra

▶ Dopamine System

Synapse

▶ GABAergic System

Synaptic Transmission

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Synonyms

Neurotransmission

Definition

Synaptic transmission is the transfer of biological information between two neurons (Fig. 1) (Chua et al. 2010). Major events during synaptic transmission are as follows:

(1) An action potential arrives at the presynaptic axon terminal.
(2) The ensuing depolarization triggers the opening of voltage-dependent Ca^{2+} channels (VGCCs).
(3) The increase of Ca^{2+} concentration in the axon terminals triggers the exocytosis of synaptic vesicles.
(4) The neurotransmitter released from the vesicles diffuses through the synaptic cleft.
(5) The neurotransmitter activates receptors in the plasma membrane of the postsynaptic neuron.
(6) Activated postsynaptic receptors mediate ion fluxes through the plasma membrane of the postsynaptic neuron, leading to changes in the membrane potential. The postsynaptic receptors may also mediate manifold biochemical changes within the postsynaptic neuron.

There are numerous transmitter substances. They include the amino acids glutamate, GABA, and glycine; acetylcholine; the monoamines dopamine, noradrenaline, and serotonin; the neuropeptides; and ATP. Nitrergic transmission, i.e., transmission by NO, differs from transmission by other transmitters and is not covered in this essay. Chemical synaptic transmission is by far the dominating kind of synaptic transmission in the human body. However, in a few regions of the nervous system, electrical synapses, based on gap junctions, can mediate bidirectional communication between adjacent neurons (Alcami and Pereda 2019).

Drugs that influence synaptic transmission play an eminent role in therapy, for two reasons. First, the nervous system controls all tissues. Second, with few exceptions synaptic transmission is chemical, operating by means of transmitter substances, and synapses therefore provide a large number of drug targets, for example, transporters and enzymes that remove transmitters from the synaptic cleft. Poisons like sarin and cocaine also target synaptic transmission.

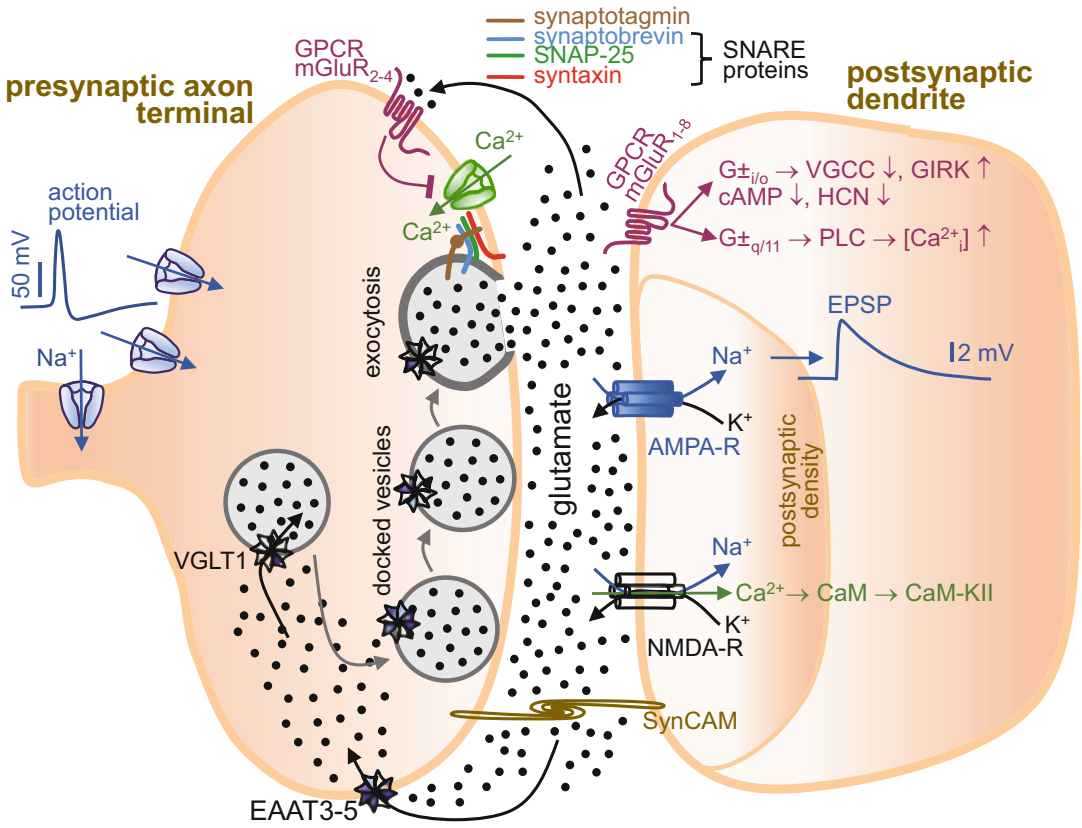
Basic Mechanisms

Transmitter Synthesis and Vesicular Storage

All non-peptide neurotransmitters are produced in the axon terminals, which possess the necessary enzymatic machinery. After synthesis, most of the transmitters are brought into synaptic vesicles by transporters belonging to the solute carrier transporter family (SLC transporters) (Fig. 1). The vesicular SLC transporters mostly utilize the proton electrochemical gradient across the vesicle membrane.

Glutamate and GABA

In neurons glutamine is transformed into glutamate by the enzyme glutaminase. After synthesis glutamate is transported into synaptic vesicles mostly by the vesicular glutamate transporter 1 (VGLT1; SLC17A7). Glutamate itself is the source for the synthesis of GABA: Glutamic acid decarboxylases (GAD1 and GAD2) convert the main excitatory neurotransmitter glutamate into the main inhibitory neurotransmitter GABA. GABA is then carried into the synaptic vesicle by the vesicular inhibitory amino acid transporter (VIAAT; SLC32).



Synaptic Transmission, Fig. 1 Glutamatergic synaptic transmission. The presynaptic terminal contains voltage-dependent Na^+ and Ca^{2+} channels, docked vesicles, a vesicle during exocytosis, the Ca^{2+} sensor synaptotagmin, and the SNARE proteins which are involved in the exocytosis. The vesicular glutamate transporter VGLT1, a group of plasmalemmal glutamate transporters (EAAT3–EAAT5), and a group of presynaptic GPCRs ($mGluR_{2-4}$) are also shown. The postsynaptic cell contains two ligand-gated glutamate receptors (AMPA-R and NMDA-R) and a group of GPRCs ($mGluR_{1-8}$). The axon terminal and the postsynaptic density are held together by the synaptic cell adhesion molecule SynCAM

Acetylcholine

Cholinergic axon terminals contain choline acetyltransferase, which acetylates choline to acetylcholine. In these axon terminals, the synaptic vesicles are filled with acetylcholine by the vesicular acetylcholine transporter (VAChT, SLC18A3).

Monoamines

Dopaminergic axon terminals contain tyrosine hydroxylase, which converts tyrosine into levodopa, and aromatic l-amino acid decarboxylase, which decarboxylates levodopa to dopamine. Noradrenergic terminals contain in addition dopamine β-hydroxylase, which oxidizes dopamine to noradrenaline. The synthesis of serotonin starts with the essential amino acid tryptophan. Tryptophan is at first hydroxylated by tryptophan hydroxylase and then decarboxylated by aromatic l-amino acid decarboxylase to yield serotonin. The vesicular monoamine transporter 2 (VMAT2, SLC18A2) is responsible for the vesicular transport of dopamine, noradrenaline, and serotonin.

Neuropeptides

In contrast to the small transmitter molecules, the neuropeptides (e.g., opioid peptides) are synthesized in the rough endoplasmic reticulum of the neuronal perikarya. They are enclosed in vesicles in the Golgi apparatus. The vesicles travel down to the terminals by axonal transport.

Transmitter Release

Neurotransmitters are released from the active zone of the presynaptic axon terminal by the following cascade: arrival of the action potential at the terminal, depolarization, opening of voltage-sensitive Ca^{2+} channels (VGCCs), and exocytosis (Fig. 1) (Südhof 2013). The nerve action potential is mainly carried by Na^{+} entry through voltage-dependent Na^{+} channels. Axon terminal voltage-sensitive Ca^{2+} channels are mainly of the N and P/Q types.

Docking and Priming

During the first step, which is called "docking," complete synaptic vesicles are positioned in the vicinity of the presynaptic plasma membrane (Fig. 1). During the next "priming" step, docked synaptic vesicles are prepared for "fusion" with the presynaptic plasma membrane. Calcium influx through N- and P/Q-type Ca^{2+} channels triggers the vesicle fusion. The calcium channels are clustered in the close vicinity of the Ca^{2+} sensor synaptotagmin in the membrane of the synaptic vesicle. The activation of only a few of calcium channels is sufficient to trigger vesicle fusion.

Exocytosis

Exocytosis consists of the fusion of the vesicle membrane with the plasmalemma, the opening of a pore in the fused membranes, and outward diffusion of the neurotransmitter. Three proteins, the so-called SNARE proteins, are essential in exocytosis. One, synaptobrevin, is a protein of the vesicle membrane; the other two, syntaxin and SNAP-25 (synaptosome-associated protein of 25 kD), are proteins of the plasmalemma (Fig. 1). Upon an increase in cytoplasmic Ca^{2+} from about 0.1 to about 100 μM, the vesicular synaptobrevin grabs hold of the plasmalemmal SNAREs, and the three proteins intertwine into a complex that then tightens like a zipper. This "zippering" of the SNAREs pulls the vesicle and plasma membranes together so that the lipid bilayers merge. Ca^{2+} channels and SNAREs lie closely together. Syntaxin and SNAP-25 in fact interact physically with the α1 subunit of N- and P/Q-type Ca^{2+} channels. The close neighborhood permits the fastness of exocytosis; the time from the arrival of the action potential to pore formation is only 100 μs or less. This fast presynaptic component of synaptic transmission contributes to the overall high speed of synaptic transmission: The time period between the appearance of the presynaptic action potential and the postsynaptic response is frequently less than 1 ms (synaptic delay).

After exocytosis, the synaptic vesicle membrane with its lipids and proteins is recycled, either by immediate re-filling with transmitter or by passing through a vesicle resting pool deeper inside the axon terminal.

Stochastic Release

Action potential-elicited neurotransmitter exocytosis is a probabilistic process (Pulido and Marty 2017). Only a fraction of the action potentials elicits transmitter release: release probability varies greatly among synapse types; probability values of 5–20% are not uncommon. Moreover, even in the case of regular presynaptic action potentials, release events are not regular but occur at variable intervals. The probability of release can be modulated by presynaptic receptors in the axon terminal membrane (Fig. 1). Through these receptors, transmitter substances from neighboring neurones and hormones can increase or reduce the release probability. Axon terminals thus integrate the release command of the action potential and various modulatory chemical messages from the neighborhood to release an appropriate amount of transmitter.

Postsynaptic Receptor Activation

The neurotransmitter released from the presynaptic active zone diffuses through the synaptic cleft to the postsynaptic neuron. The postsynaptic receptors are mostly localized in the cell membrane of the dendrites or dendritic spines, in a region called postsynaptic density (Fig. 1). The presynaptic active zone and the postsynaptic density are closely held together with the help of cell adhesion molecules anchored in the axon terminal and the postsynaptic neuron.

Of the several classes of receptors for endogenous chemical signals (Hofmann 2017), two are used as postsynaptic receptors in synaptic

transmission: ligand-gated ion channels (LGICs) and G-Protein-Coupled Receptors (GPCRs). Due to the large number of transmitters and the existence of several receptor types for almost all, postsynaptic receptor activation is the most diversified step of synaptic transmission. Table 1 shows selected neurotransmitter receptors.

Ligand-Gated Ion Channels (LGIC)

LGICs are hetero- or homo-oligomeric proteins consisting of three, four, or five peptide chains, of which each spans the membrane several times (Alexander et al. 2019a). The three to five subunits surround a pore, which in the absence of transmitter is closed. The nicotinic receptor for acetylcholine, the $GABA_A$-receptor, the glycine receptor, and the 5-HT_3-receptor for serotonin are pentamers. The ionotropic glutamate receptors (iGluRs) are tetramers. The P2X-receptors for ATP are trimers. When transmitter is bound to a ligand-gated ion channel receptor, the open probability of the pore is increased. Ions may then

Synaptic Transmission, Table 1 Selected neurotransmitter receptors

Physiological transmitter	Receptor[a]	Effector mechanisms	Pharmacological agonists	Pharmacological antagonists
Glutamate	AMPA[b] (LGIC)	Na^+ and K^+ conductance ↑	AMPA	
	kainate (LGIC)	Na^+ and K^+ conductance ↑	kainate	
	NMDA[b] (LGIC)	Na^+, K^+, and Ca^{2+} conductance ↑	NMDA	NMDA: ketamine, amantadine
	[c]$mGluR_{1,5}$ (GPCR)	$G\alpha_{q/11}$		
	$mGluR_{2,3}$ -(GPCR)	$G\alpha_{i/o}$		
	$mGluR_{4,6,7,8}$ (GPCR)	$G\alpha_{i/o}$		
GABA	$GABA_A$ (LGIC)	Cl^- conductance ↑	muscimol	bicuculline
	$GABA_{B(1a,1b,2)}$ (GPCR)	$G\alpha_{i/o}$	baclofen	
Glycine	Glycine (LGIC)	Cl^- conductance ↑		strychnine
Acetylcholine	Nicotinic (LGIC)	Na^+, K^+, and Ca^{2+} conductance ↑	nicotine, suxamethonium	tubocurarine, pancuronium
	Muscarinic M_{1-5} (GPCR)	M_1, M_3, M_5: $G\alpha_{q/11}$	muscarine, carbachol	atropine, butylscopolamine, tiotropium, trospium
		M_2, M_4: $G\alpha_{i/o}$		
Dopamine	D_{1-5} (GPCR)	D_1, D_5: $G\alpha_s$	D_{2-3}: bromocriptine	D_{2-3}: haloperidol, amisulpride, risperidone
		D_2, D_3, D_4: $G\alpha_{i/o}$	D_{2-4}: pramipexole	
Noradrenaline	$\alpha_{1A,B,D}$ (GPCR)	$G\alpha_{q/11}$	phenylephrine	prazosin, tamsulosin
	α_{2A-C} (GPCR)	$G\alpha_{i/o}$	clonidine dexmedetomidine	yohimbine, mirtazapine
	β_{1-3} (GPCR)	$G\alpha_s$	isoprenaline	propranolol, metoprolol
			salbutamol	
			mirabegron	
Serotonin	5-HT_{1A-F}	$G\alpha_{i/o}$	5-$HT_{1B,D}$: sumatriptan, ergotamine	
	5-HT_{2A-C} (GPCR)	$G\alpha_{q/11}$		
	5-HT_3 (LGIC)	Na^+ and K^+ conductance ↑		ondansetron
Opioid peptides	μ, δ, κ (GPCR)	$G\alpha_{i/o}$	μ: morphine	naloxone
			μ: fentanyl	

[a]*LGIC* ligand-gated ion channel, *GPCR* G protein-coupled receptor
[b]*AMPA* α-**am**ino-3-hydroxy-5-methyl-4-isoxazole **p**ropionic **a**cid, *NMDA* ***N*-m**ethyl-**D**-**a**spartic acid
[c]*mGluR* metabotropic glutamate receptor

enter or leave the cell, but a selectivity filter in the channel lets only certain ions pass.

LGIC/Glutamate Receptors

The AMPA and kainate receptors for glutamate are cation channels; they are permeable for Na^+ and K^+ (some AMPA receptors also for Ca^{2+}) (Table 1) (Henley and Wilkinson 2016). When they open, the major consequence is a sudden entry of Na^+, and the ensuing depolarization is called excitatory postsynaptic potential (EPSP; Fig. 1). The NMDA receptor is also activated by glutamate, but glycine is needed as a co-activator. The NMDA receptor is blocked by Mg^{2+} at resting membrane potentials, and this blockade is relieved by depolarization. The NMDA receptor ion channel is permeable for Na^+, K^+, and Ca^{2+}. Ca^{2+} entering into the postsynaptic neuron via the NMDA receptor can activate intracellular event cascades involving calmodulin and Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII).

LGIC/$GABA_A$- and Glycine Receptors

The $GABA_A$-receptor and the glycine receptor are Cl^- channels (Table 1). When they open at a resting membrane potential of about −60 mV, the consequence is an entry of Cl^- which elicits hyperpolarization. This hyperpolarization is called inhibitory postsynaptic potential (IPSP).

All these postsynaptic events last only for a few milliseconds; synaptic transmission through LGICs is fast. When the postsynaptic cell membrane is sufficiently depolarized, voltage-dependent Na^+ channels open, and an action potential is generated.

G Protein-Coupled Receptors (GPCRs)

GPCRs are proteins that span the postsynaptic cell membrane seven times (heptahelical receptors) (Alexander et al. 2019b). Small ligands are usually bound within a pocket formed by the seven transmembrane helices. The large neuropeptides bind to the extracellular domains. When the receptors are activated, they interact with the appropriate G proteins that are bound to the inner surface of the cell membrane. The G proteins then pass the information on to various effectors (Fig. 1).

GPCR/$G\alpha_{i/o}$ Coupling

The $mGluR_{2,3,4,6,7,8}$ glutamate receptors; $GABA_B$ receptors; M_2 and M_4 muscarinic receptors; D_2, D_3. and D_4 dopamine receptors; α_2-adrenoceptors; $5\text{-}HT_{1A–F}$ serotonin receptors; and μ, δ, and κ opioid receptors all couple to $G\alpha_{i/o}$ proteins. Via G protein Gβγ subunits, G protein-gated inwardly rectifying potassium (GIRK) channels are activated, and the membrane potential becomes more negative. Activation of $G\alpha_{i/o}$ protein-coupled receptors also lowers the open probability of N- and P/Q-type Ca^{2+} channels. The cytoplasmic level of the second messenger cyclic AMP (cAMP) is lowered as well: less cAMP means less activation of the hyperpolarization-activated cyclic nucleotide-gated (HCN) ion channel family. Neurotransmitters acting on postsynaptic $G\alpha_{i/o}$-coupled receptors usually slow the firing rate of postsynaptic neurons by opening GIRK channels but also by attenuating the cAMP-dependent activation of HCN channels. Presynaptically localized $G\alpha_{i/o}$-coupled receptors inhibit the transmitter release from the axon terminal.

GPCR/$G\alpha_{q/11}$ and $G\alpha_s$ Coupling

The $mGluR_{1,5}$ glutamate receptors; the M_1, M_3, and M_5 muscarinic receptors; and the α_1-adrenoceptors couple to $G\alpha_{q/11}$ proteins and thereby increase the cytoplasmic levels of the messengers inositol trisphosphate, diacylglycerol, and Ca^{2+}. Finally, the D_1 and D_5 dopamine receptors and the β-adrenoceptors couple to $G\alpha_s$ and thereby increase the cytoplasmic level of cyclic AMP.

These cascades of reactions need time in the range of seconds: synaptic transmission through GPCRs is slow. All further postsynaptic changes depend on the type of the postsynaptic cell. For example, activation of β_2-adrenoceptors causes in the heart an increase of the rate and force of contraction; in skeletal muscle glycogenolysis and tremor; in smooth muscle relaxation; in bronchial glands secretion; and in sympathetic nerve terminals, an increase in transmitter release.

Termination of Synaptic Transmission

Once released, neurotransmitters are inactivated by diffusion into the neighboring extracellular space, combined with one of two specific

pathways: either extracellular degradation by enzymes that face the extracellular space or uptake into cells.

Extracellular Degradation of Transmitters

Extracellular degradation removes acetylcholine, the neuropeptides, and ATP. Acetylcholine is rapidly hydrolyzed to choline and acetate by acetylcholinesterase (Rotundo 2017). The enzyme is localized in the presynaptic and postsynaptic cell membrane and in the extracellular matrix and splits about 10,000 molecules of acetylcholine per second. High concentrations of butyrylcholinesterase are found in the brain and many peripheral tissues. Although butyrylcholinesterase is also capable of cleaving acetylcholine, under physiological conditions its contribution to the elimination of acetylcholine released by neurons is minimal.

Elimination of Transmitters by Transporters

Glutamate, GABA, glycine, dopamine, noradrenaline, and serotonin are taken up into adjacent cells by plasmalemmal neurotransmitter transporters (Fig. 1). The transporters for GABA, glycine, dopamine, noradrenaline, and serotonin belong to the SLC6 transporter family (Kristensen et al. 2011). These transporters possess 12 transmembrane domains and use the electrochemical driving force of Na^+ across the neuronal membrane for moving the neurotransmitters against their concentration gradients into the neurons. The GABA transporter GAT1 is responsible for the re-uptake of GABA from the synaptic cleft into the axon terminal. GAT3 and BGT1 transfer GABA into adjacent glial cells. Glycine is transported into glycinergic axon terminals and glial cells by GLYT2 and GLYT1, respectively. The dopamine (DAT), noradrenaline (NAT), and serotonin transporters (SET) are located almost exclusively in the dopaminergic, noradrenergic, and serotoninergic terminals, respectively. In all these cases, cellular uptake means re-uptake into the presynaptic neurone. Re-uptake by the plasmalemmal transporter may be followed by vesicular re-uptake, an economical way of inactivation, reminiscent of the recycling of the storage vesicle membrane.

The glutamate transporters belong to the SLC1 transporter family, possess 12 transmembrane segments, and also use the transmembrane electrochemical gradient of Na^+ to pay for the transport work. Both neuronal (EAAT3, EAAT4, and EAAT5) and glial (EAAT1 and EAA2) transporters can remove glutamate from the synaptic cleft (Fig. 1).

Modulation of Synaptic Transmission

Synaptic plasticity. The strength of synaptic transmission (size of the postsynaptic response to a presynaptic action potential) is often modulated under physiological conditions, and this process is called "synaptic plasticity." The strength can be increased (potentiation) or decreased (depression). The duration of the change in synaptic strength can be short term (under 1 min) or long term (longer than ~30 min, up to life-long). Synaptic plasticity is important for memory and learning.

The mechanisms of synaptic plasticity are manifold. Transmitter release can be rapidly changed by altering the number of docked vesicles in the active zone. The number of postsynaptic receptors within the postsynaptic density can also be quickly changed. Longer time periods are necessary for the synthesis of new synaptic proteins and for morphological changes of the postsynaptic dendrites and dendritic spines. A classical form of long-term potentiation (LTP) is the NMDA receptor-dependent associative LTP of glutamatergic synaptic transmission (Lüscher and Malenka 2012). In this case, the presynaptic axon and the postsynaptic neuron are simultaneously depolarized (associative inputs). Glutamate released from the axon terminal activates postsynaptic AMPA and NMDA receptors. The postsynaptic depolarization enables the NMDA receptor to conduct Ca^{2+}. Ca^{2+} triggers intracellular effectors, and as a result, more AMPA receptors are moved into the postsynaptic density. Thus, the associative stimulus elicits an LTP of the AMPA receptor-mediated glutamatergic synaptic transmission.

Presynaptic Receptors

Transmitter release can be modulated also by activation of presynaptic receptors in the axon terminals. The presynaptic receptors are often autoreceptors (i.e., they respond to the transmitter which was released from the axon terminal) and

mostly $G\alpha_{i/o}$ protein-coupled receptors. Thus, presynaptic autoreceptors mediate negative feedback to limit further transmitter release – this is a presynaptically expressed short-term homeostatic synaptic plasticity. α_2-Adrenoceptors on noradrenergic axon terminals are prototypic presynaptic autoreceptors and were much studied (Starke 2001).

Retrograde Signaling

In the case of retrograde signaling, synaptic information flows retrogradely, i.e., from the postsynaptic neuron to the presynaptic axon terminal. Endocannabinoid-mediated retrograde signaling is probably the most ubiquitous form of retrograde signaling. The endocannabinoid 2-arachidonoylglycerol is released from postsynaptic neurons upon depolarization and subsequent calcium influx or after activation of $G\alpha_{q/11}$ protein-coupled receptors (Araque et al. 2017). After crossing the synaptic cleft, 2-arachidonoylglycerol activates presynaptic CB_1 cannabinoid receptors, which leads to a decrease of transmitter release from the axon terminal. This is an endocannabinoid-mediated short-term synaptic plasticity.

Clinical Pharmacology: Drugs Interfering with Synaptic Transmission

Transmitter Production and Vesicular Storage

Levodopa is administered as the precursor of dopamine to compensate for the loss of dopaminergic neurones in Parkinson's disease (see Chapter "Dopamine System"). After passing the blood-brain barrier, levodopa is converted into dopamine by aromatic l-amino acid decarboxylase. Reserpine blocks VMAT2; thus it depletes noradrenaline from the sympathetic axon terminals, and it was used in the past to treat hypertension. Because reserpine simultaneously empties noradrenergic, dopaminergic, and serotoninergic synaptic vesicles in the brain, many central nervous system side effects occur. A major component of the anticonvulsive effect of valproate is enhancement of GABAergic synaptic transmission in the brain by enhancing GABA synthesis in GABAergic axon terminals.

Transmitter Release

A large number of natural poisons inhibit transmitter release from axon terminals. Tetrodotoxin, produced by the puffer fish, occludes voltage-gated Na^+ channels by binding to the external side of the channel pore: as a consequence, the axon terminal is no longer depolarized. The cone snail *Conus geographus* produces 9-conotoxin GVIA, which blocks N-type Ca^{2+} channels. P-/Q-type Ca^{2+} channels are selectively blocked by the funnel-web spider venom 9-agatoxin IVA.

The most ingenious exocytosis toxins, however, come from the anaerobic bacteria *Clostridium botulinum* and *Clostridium tetani*. The former produces seven botulinum neurotoxins (BoNTs) A–G; the latter produces tetanus neurotoxin (TeNT) (Dong et al. 2019). All eight toxins consist of a heavy (H) chain and a light (L) chain that are associated by an interchain S–S bond. The L-chains enter the cytosol of axon terminals and then specifically split one of the three core proteins of exocytosis: synaptobrevin, syntaxin, or SNAP-25 (SNARE proteins; Fig. 1). BoNT L-chains mainly enter peripheral cholinergic terminals: the inhibition of acetylcholine release is followed by flaccid paralysis, the main symptom of botulism. The TeNT L-chain mainly enters cerebral and spinal cord GABAergic and glycinergic terminals: the inhibition of GABA and glycine release is followed by spastic paralysis, the main symptom of tetanus. BoNT and TeNT are the most potent toxic substances known, able to kill vertebrates at a dose of 0.1 to 1 ng/kg body weight. Due to its ability to inhibit acetylcholine release from cholinergic neurons, BoNT is increasingly used in therapy, for example, for the treatment of muscle spasticity, migraine, axillary hyperhydrosis, and overactive bladder. Undoubtedly, BoNT is most frequently used to alleviate wrinkles on the face – a cosmetic indication (in this setting BoNT is commonly called "Botox").

Mirtazapine is one of the most often-used antidepressant drug: it increases the release of noradrenaline and serotonin from axon terminals by blocking presynaptic α_2 adrenoceptors. The anticonvulsive drug levetiracetam lowers glutamate release by interfering with the docking and fusion

of synaptic vesicles after a primary interaction with the vesicle protein SV2.

Postsynaptic Receptor Activation

The most diversified step of synaptic transmission is also the target of the greatest variety of drugs. Some are included in Table 1. Many drugs and poisons stem from plants, for example, muscimol, bicuculline, strychnine, nicotine, tubocurarine, muscarine, atropine, scopolamine, and morphine.

The therapeutic impact of drugs acting at transmitter receptors is enormous. All volatile (isoflurane) and intravenous (propofol) anaesthetics and also ethanol act primarily on cerebral LGICs, above all $GABA_A$-receptors. All neuromuscular blocking agents are agonists (suxamethonium) or antagonists (pancuronium) at the skeletal muscle nicotinic receptor. There would be no surgery worth mentioning without these drug actions on neurotransmitter receptors. The anti-Parkinson drug pramipexole activates D_2 and D_3 dopamine receptors. Another drug used in Parkinson's disease, amantadine, is an antagonist at the NMDA glutamate receptor. Morphine and related opioid agonists are the most effective analgesics. The benzodiazepines (diazepam, midazolam) are positive allosteric modulators of the $GABA_A$-receptors and possess sedative, anticonvulsive, and anxiolytic actions. Neuroleptics such as haloperidol and olanzapine block D_2 dopamine receptors and are standard drugs to treat schizophrenia.

β_1 adrenoceptor antagonists such as metoprolol are mainly used in cardiology and belong to the most frequently prescribed drugs. The α_1 adrenoceptor antagonist tamsulosin is a first-line drug for the treatment of benign prostatic hyperplasia. Antagonists of muscarinic acetylcholine receptors are used to treat asthma and COPD (ipratropium, tiotropium), overactive bladder (trospium), and biliary and renal colic (butylscopolamine).

Transmitter Inactivation

The cholinesterase inhibitors are the classical drugs that interfere with transmitter inactivation. The prototype was the plant poison physostigmine, and derivatives of physostigmine are used to treat myasthenia gravis. The highly toxic nerve gases such as sarin are also cholinesterase inhibitors. Monoamine oxidases (MAOs) metabolize noradrenaline, dopamine, and serotonin. MAO inhibitors are used in the treatment of depression and Parkinson's disease (tranylcypromine and rasagiline, respectively). The GABA transporter is blocked by tiagabine, an antiepileptic that owes its effect to the ensuing increase of the concentration of GABA in the synaptic cleft. Cocaine is abused because it blocks the dopamine and serotonin transporters and, hence, enhances dopaminergic and serotoninergic transmission in the mesolimbic "reward system." Inhibitors of the serotonin (fluoxetine, citalopram, amitriptyline) and noradrenaline (amitriptyline) transporters are the main antidepressant drugs.

Cross-References

- ▶ Antidepressant Drugs
- ▶ Botulinum Neurotoxins
- ▶ Cholinesterases
- ▶ Dopamine System
- ▶ G-Protein-Coupled Receptors
- ▶ Glycine Receptors
- ▶ Nicotinic Receptors

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Synthetic Lethality

► DNA Damage Response

Syntocinon

► Oxytocin

Systemic Inflammation

► Sepsis

T

T Cell Death-Associated Gene 8 (TDAG8, GPR65)

► Proton-Sensing GPCRs

Tachykinin 1 Receptor

► Neurokinin/Tachykinin Receptors

Tachykinin 2 Receptor

► Neurokinin/Tachykinin Receptors

Tachykinin 3 Receptor

► Neurokinin/Tachykinin Receptors

TGF-β Pathway

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Abbreviations

AON	Antisense oligonucleotide
CAF	Cancer associated fibroblast
SMAD	Sma and MAD related protein
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial to mesenchymal transition
ESC	Embryonic stem cell
IPF	Idiopathic pulmonary fibrosis
MH	Mad homology
MSC	Mesenchymal stem cell
lncRNA	Long non coding RNA
PDGF	Platelet-derived growth factor
SMI	Small molecule inhibitor
TβR	TGF-β receptor
TGF-β	Transforming growth factor-β
VEGF	Vascular endothelial growth factor

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Synonyms

TGF-β signaling pathway; TGF-β/SMAD pathway (Transforming growth factor-beta receptor signaling pathway; TGF-beta receptor signaling pathway; TGF-β receptor/SMAD signaling pathway; TGF-beta signaling pathway; TGFβ pathway); Transforming growth factor-beta pathway

Definition

The transforming growth factor-beta (TGF-β) signaling pathway plays important roles in embryogenesis and tissue homeostasis, by controlling cell proliferation, differentiation and apoptosis, as well as cell migration and immune surveillance. A key step in the TGF-β pathway is the binding of secreted TGF-β ligand to TGF-β receptors at the cell surface that convey the signals via activation of intracellular SMAD transcription factors. The pleiotropic functions of TGF-β are highly dependent on cellular context; in diverse differentiation states and/or microenvironments they can have distinct, and even opposing, functions. Aberrant TGF-β signaling has been associated with multiple human diseases, including fibrosis, immune disorders, and cancer. In cancer, TGF-β can act both as a tumor suppressor in early stages of tumorigenesis, and as a tumor promoter in later stages. Altogether TGF-β is an attractive, but challenging, drug target due to its pleiotropic nature. Nevertheless, several drugs targeting the TGF-β pathway have been developed and are currently being tested in clinical trials.

Basic Characteristics

TGF-β Pathway

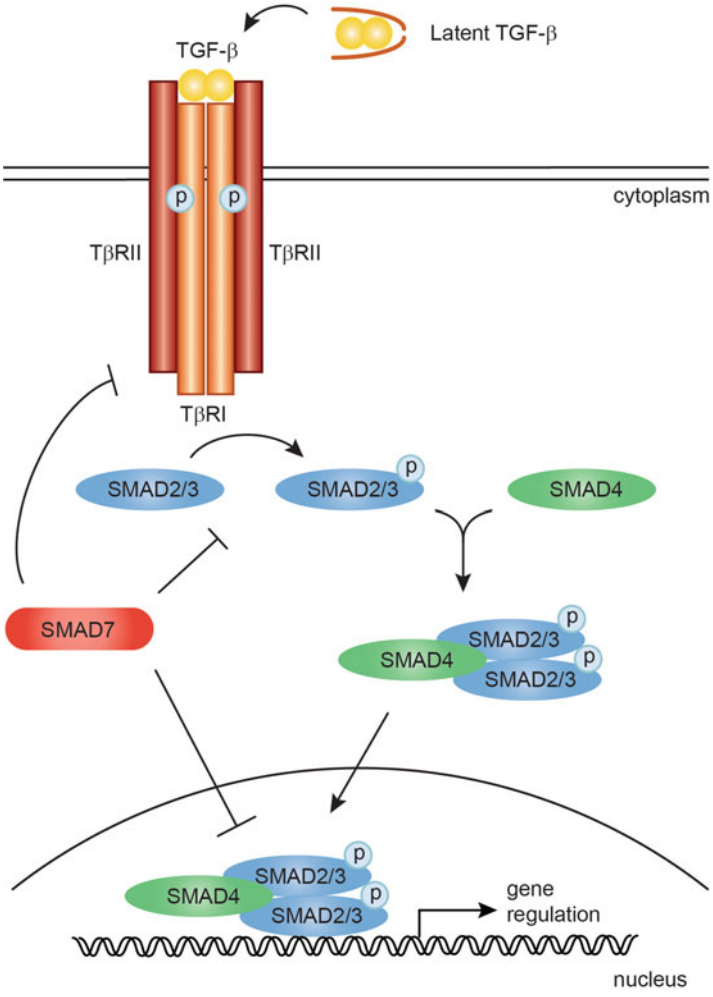
TGF-β1 is the prototypic member of a large family of structurally and functionally related proteins, which includes its close relatives TGF-β2 and TGF-β3 but also activins, nodal, inhibins, Mullerian-inhibiting substance (MIS), growth and differentiation factors (GDF), and bone morphogenetic proteins (BMPs) (Morikawa et al. 2016). Here we do not specify different TGF-β isoforms, and use TGF-β, unless a specific role was shown for a specific isoform.

TGF-β proteins are synthesized as larger precursor proteins consisting of a signal peptide, a pro-domain (which is called latency-associated polypeptide (LAP) for TGF-β) and the mature polypeptide. After the removal of the signal peptide the precursor is proteolytically processed, separating the pro-domain from the carboxy terminal mature polypeptide, which remain however, non-covalently associated (Derynck and Budi 2019). The activation of latent TGF-β occurs via specific proteases that cleave the LAP and/or by mechanical forces generated by cell-surface integrins, resulting in the release of mature, active TGF-β (Derynck and Budi 2019).

TGF-β induces cellular responses by binding to its cognate TGF-β type I and type II receptors (i.e., TβRI and TβRII). The basic structure of type I and II is similar, they both contain a cysteine-rich extracellular domain, a single transmembrane domain and an intracellular region harboring a serine/threonine kinase domain. TGF-β initially engages TβRII, which drives the recruitment of TβRI and the formation of a heteromeric receptor complex. Upon this complex formation, the constitutive active kinase domain of TβRII phosphorylates TβRI in its glycine-serine (GS) juxtamembrane region, leading to its activation. In turn, active TβRI then phosphorylates specific intracellular SMAD effector proteins (Derynck and Budi 2019; Heldin and Moustakas 2016). SMADs can act as transcription factor complexes that relay the extracellular TGF-β signal into the nucleus (Fig. 1).

Canonical TGF-β-SMAD Signaling

The SMAD family can be divided into three groups; (1) receptor-regulated (R-) SMADs (in vertebrates R-SMAD1,-2,-3,-5,-8) that interact with activated type I receptors and are subsequently phosphorylated, (2) the common (Co-) SMADs (in vertebrates SMAD4) that form heteromeric complexes with activated R-SMADs, and (3) inhibitory (I-) SMADs (in vertebrates I-SMAD6/7) which antagonize canonical SMAD signaling. The R- and Co-SMADs contain two conserved *Mad homology* (MH) domains, the



TGF-β Pathway, Fig. 1 Schematic overview of the canonical TGF-β/SMAD pathway. Upon activation, TGF-β induces heteromeric complex formation between TGF-β receptors type II and type I receptors (i.e., TβRII and TβRI). Constitutively active TβRII then (trans)phosphorylates TβRI, which in turn relays the signal to intracellular receptor regulated SMADs (R-SMAD) 2 and 3. The R-SMADs form heteromeric complexes with the common (Co-) SMAD4 and translocate to the nucleus, where they regulate transcription of target genes in collaboration with various cofactors. Inhibitory (I-) SMAD7, inhibits TGF-β signaling through multiple mechanisms, for instance by preventing SMAD2/3 phosphorylation or induction of receptor degradation. The non-SMAD signaling pathways that can be initiated downstream of TβRI are not indicated in the Figure. TGF-β; transforming growth factor-beta, TβR; TGF-β receptor

MH1 domain localizes to the N-terminal side and the M2 domain is located at the C-terminal side of these SMADs, which are connected by a flexible linker region. The MH1 domain of SMADs can bind to DNA, whereas the MH2 domain mediates SMAD heteromeric complex formation and receptor interaction. Heteromeric complex formation of R-SMADs with SMAD4 results in the exposure of nuclear import signals and shielding of nuclear export signals, thus inducing nuclear localization of these complexes. In the nucleus, heteromeric SMAD complexes can act as transcription factors together with other DNA binding transcriptional regulators, such as coactivators, repressors, and chromatin remodelers. This enables diverse transcriptional responses depending on the

combination of DNA binding and regulatory proteins (Derynck and Budi 2019; Massague 2012).

The I-SMADs can inhibit canonical SMAD signaling via multiple mechanisms, including by competing with R-SMADs for binding to activated receptors via their homologous MH2 domain, thereby preventing R-SMAD phosphorylation, or by binding to SMAD4, thereby preventing the interaction between SMAD4 and phosphorylated R-SMADs. Additionally, I-SMADs can recruit E3 ubiquitin ligases SMURF1 and SMURF2 to ubiquitinate activated type I receptors for subsequent proteasomal and lysosomal degradation. Moreover, I-SMADs can attenuate signaling by recruiting phosphatases to the activated TGF-β receptor, thereby mediating receptor inactivation via dephosphorylation of specific residues (Fig. 1) (Derynck and Budi 2019; Massague 2012).

Of note, the TGF-β canonical SMAD pathways can be divided into two branches, one is employed predominantly by TGF-β and activins and signals via R-SMAD2 and -3, the other branch is mainly used by BMPs and signals through R-SMAD1, -5, and -8. Mixed complexes between SMAD2/3 and SMAD1/5 have also been described. I-SMAD6 mainly antagonizes BMP pathways and I-SMAD7 inhibits TGF-β, activins and BMP pathways (Derynck and Budi 2019; Massague 2012). Here we focus on the TGF-β signaling pathway.

Non-SMAD Signaling

In addition to the canonical SMAD pathway, TGF-β family members can trigger so-called non-SMAD pathways to initiate a multitude of intracellular changes. This can either happen directly downstream of TGF-β receptors, or indirectly, for example, via TGF-β/SMAD-induced expression of growth factors, such as epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) that subsequently initiate non-SMAD responses. Among the various non-SMAD signaling pathways that can be activated directly downstream of TGF-β receptors are p38, Jun N-terminal kinase (JNK) and Extracellular Signal-Regulated Kinase (ERK) mitogen-activated protein kinases (MAPK) pathways, phosphoinositide 3 kinase (PI3K)-AKT-mTOR, Janus Kinase/Signal Transducer and Activator of Transcription (JAK-STAT), tumor necrosis factor (TNF)-receptor associated factor 6 (TRAF6) – TGF-β activated kinase 1 (TAK1), nuclear factor kappa B (NF-κB), and the small Rho-like GTPase pathways. Of note, the activation level of these pathways downstream of TGF-β receptors occurs generally to a lower extent as compared to their natural growth factor/tyrosine kinase or cytokine pathways. Furthermore, extensive crosstalk exists between SMAD and non-SMAD pathways (Zhang 2017).

Physiological Roles of TGF-β Signaling

Almost 40 years of TGF-β research has revealed that cellular responses to TGF-β are different and sometimes even opposite, and strongly depend on the cellular context. For example, TGF-β can induce both cell growth and growth inhibition of the same cells depending on the cell culture conditions. TGF-β is a multifunctional cytokine regulating a large range of biological processes, both during embryogenesis and adult tissue homeostasis. Here, we discuss some of the key functions of TGF-β signaling in human physiology, such as regulation of cell proliferation and differentiation and TGF-β-mediated regulation of wound healing and the immune system.

TGF-β Mediates Regulation of Gene Expression

As described above, SMADs can bind directly or indirectly to DNA and thereby regulate gene expression. Several genes, such as *SERPIN1*, encoding plasminogen activator inhibitor protein (PAI-1), inhibitory *SMAD7*, and cyclin dependent kinase inhibitor *p21*, have been established as direct SMAD target genes that are generally and rapidly induced after TGF-β stimulation in many cell types. However, depending on the cofactors SMADs may either activate or repress gene expression. These cofactors, e.g., transcription factors and coregulators, act as DNA binding partners of SMADs for recognition of specific target genes. Importantly, the cofactors themselves are controlled by other (context dependent) signaling pathways. Also, cell-type or lineage-specific transcription factors are responsible for the induction of specific responses in certain cell

types. Additionally, canonical SMAD pathways can be complemented by TGF-β-induced non-SMAD pathways, adding another level of complexity. The combination of signaling crosstalk at multiple levels, together with differences in physiology of different cell types, provides a basis for the context dependency for TGF-β responses (Morikawa et al. 2016). Of note, in addition to protein-encoding genes, TGF-β also regulates expression of noncoding RNAs, such as microRNAs (miRNAs) and long noncoding RNAs (lncRNAs) (Hao et al. 2019).

TGF-β Controls Cell Proliferation and Differentiation

In most cell types, TGF-β has strong growth inhibitory effects, including epithelial, endothelial, and hematopoietic cells. This cytostatic effect happens mainly through two distinct mechanisms; (1) induction of cyclin-dependent kinase inhibitors, such as $p21^{cip1/waf1}$ and (2) elimination of proliferative drivers, such as c-myc. TGF-β ligands have also been suggested to inhibit cell growth indirectly by counteracting the activity of specific mitogens, such as EGF (Morikawa et al. 2016; Siegel and Massague 2003).

In contrast, TGF-β ligands have also been shown to induce proliferation of some cell types, including chondrocytes, osteoblasts, mesenchymal stem cells (MSCs), and fibroblasts under certain conditions. The effects are often context-dependent, as the same cells can undergo growth inhibition or promotion upon TGF-β. The underlying molecular mechanisms of TGF-β-induced growth promotion are largely undefined, and in some contexts, it was suggested to be a secondary effect to the induction of other cytokines, such as PDGF (Morikawa et al. 2016; Siegel and Massague 2003).

During development, TGF-β family members regulate the function of diverse cell types by regulating pluripotency and differentiation of embryonic stem cells (ESCs) and lineage-committed progenitors. BMP ligands, for example, stimulate ESC self-renewal, while TGF-β ligands are implicated in the differentiation of stem and pluripotent cells towards a wide variety of lineages, such as chondrocytes, immune, blood, and neuronal cells. TGF-β is a key regulator of stem-cell state and differentiation from the earliest stages of development to homeostasis of the adult organism (Mullen and Wrana 2017).

TGF-β Induces Epithelial-to-Mesenchymal Transition

Epithelial-to-mesenchymal transition (EMT) is a dynamic and multistep process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties and become mesenchymal cells. EMT is a crucial morphogenetic event in gastrulation, embryonic tissue formation, and regeneration. TGF-β is a key driver of EMT and triggers this process in, for example, heart development and palate fusion. Furthermore, (partial) EMT due to the induction of increased cell motility contributes to tissue regeneration, e.g., wound healing as discussed below. Lastly, pathological versions of EMT triggered by TGF-β contribute to fibrosis and cancer, which is discussed in more detail in the next paragraph. EMT is characterized by down-regulation of E-cadherin and upregulation of N-cadherin and vimentin, these molecular changes are the cause of the observed polarity changes and loss of adherence. EMT is driven by an interactive network of transcriptional repressors, such as SNAIL, SLUG, and many others. TGF-β does not only induce EMT via its canonical signaling route, but often in collaboration with various other (non-SMAD) signaling cascades (e.g., ERK or Wnt signaling) (Hao et al. 2019).

TGF-β Regulates Wound Healing

TGF-β has been shown to control various steps in the wound healing process by regulating multiple cell types. In response to injury, TGF-β expression and activation is rapidly induced and platelets, crucial for wound healing, store large amounts of (latent) TGF-β, which are released at the site of injury. TGF-β is a chemoattractant and, in turn, recruits monocytes and fibroblasts to induce tissue repair. TGF-β also induces production and prevents degradation of various extracellular matrix (ECM) proteins, thus stimulating wound healing. Lastly, at the site of injury epithelial cells undergo TGF-β-induced (partial) EMT and the increased

motility contributes to epithelial wound healing. Cells then reacquire their epithelial phenotype. Combined, TGF-β is a strong contributor to tissue injury repair (Morikawa et al. 2016).

TGF-β Mediates Regulation of the Immune System

TGF-β has a crucial role in immune homeostasis, as illustrated by TGF-β1 knockout mice that suffer from lethal autoimmunity and inflammation and die at 2 or 3 weeks. TGF-β is a powerful immunosuppressant by regulating both cell differentiation and proliferation. It can regulate both innate and adaptive immune cell activities and has diverse effects on different immune cells. TGF-β has potent growth-suppressing activity on most precursor cells of the immune system, and specifically on T and B cells of the adaptive immune system. It is a strong suppressor of cytotoxic T cell proliferation and induces B cell apoptosis. TGF-β also suppresses Th1 and Th2 cell differentiation and stimulates suppressor Treg cells. Overall, TGF-β regulates various aspects of the immune system, mainly exhibiting an immune-suppressive response (Chen and Ten Dijke 2016).

Pathologies Resulting from Deregulated TGF-β Signaling

TGF-β holds crucial roles in various homeostatic processes as described above, and perturbation of this signaling cascade can therefore have catastrophic consequences and induce different diseases. Here, we describe the two most prominent examples of diseases where deregulated TGF-β holds a central role: fibrosis and cancer.

Fibrosis

Fibrosis is a pathological wound healing in which connective tissue replaces healthy parenchymal tissue leading to considerable tissue remodeling and the formation of permanent scar tissue. TGF-β is well-known as a key pro-fibrotic cytokine. Dysregulation and sustained activation of TGF-β signaling mediates both the initiation and progression of fibrosis in various tissues, such as lungs, kidneys, and heart. The pro-fibrotic effects of TGF-β signaling has mainly been attributed to TGF-β/SMAD3 signaling and involves a combination of mechanisms and cell types. For example, in idiopathic pulmonary fibrosis (IPF), TGF-β, that is secreted by alveolar epithelial cells, drives fibrosis by inducing the proliferation, migration, and differentiation of resident mesenchymal cells, resulting in scar tissue formation. Furthermore, TGF-β can promote the activation and differentiation of fibroblasts into myofibroblasts, specialized contractile cells that deposit aberrant extracellular matrix (ECM), which results in altered tissue architecture and thus reduced function. This occurs not only in lung fibrosis, but is especially apparent in cardiac fibrosis. Also, TGF-β-induced EMT plays a crucial role in the induction of fibrosis. For example, in renal fibrosis, TGF-β also mediates to the pathological process by inducing the transformation of tubular epithelial cells into myofibroblasts through EMT. This also occurs in IPF in a similar manner. Of note, endothelial cells have also been identified as a source of fibroblasts in fibrosis. For instance, in heart fibrosis approximately 30% of fibroblasts are estimated to originate from endothelial cells through so-called endothelial-mesenchymal transition (EndoMT). TGF-βs or activators of latent TGF-β, such as integrins, are often highly expressed in fibrotic regions. Altogether, localized excessive TGF-β signaling drives fibrosis via several distinct mechanisms (Hao et al. 2019; Morikawa et al. 2016; Massague 2012).

Cancer

TGF-β has a dual role in cancer and can act as both a tumor suppressor and a tumor promoter. In its tumor suppressive role, TGF-β can cause cell cycle arrest and exert growth inhibitory effects of premalignant cells. Moreover, it can induce apoptosis of these cells. Cancer cells often adapt to avoid these cytostatic effects by inactivating or deletion mutations of genes encoding key players of the TGF-β pathway. For instance, colorectal cancers often harbor *TβRII*, *SMAD2*, or *SMAD4* mutations, thereby rendering the cancer cells insensitive to TGF-β cytostatic effects (Colak and Ten Dijke 2017; Akhurst 2017).

On the other hand, however, elevated TGF-β expression and activity is frequently observed in

many cancers. Additionally, high TGF-β pathway activity has been correlated with tumor progression and poor prognosis, pointing towards a tumor supportive function of TGF-β. Indeed, TGF-β signaling exerts tumor promoting activities through direct effects on the cancer cells themselves and on the tumor microenvironment. First, TGF-β can promote tumorigenesis by inducing EMT by driving tumor cells towards a stem cell-like state, that is, characterized by increased expression of stem cell markers and enhanced tumor initiating capacity. EMT enables increased migration and invasion of cancer cells. These migratory and invasive mesenchymal-like tumor cells extravasate from primary lesions into blood (or lymphatic) vessels and subsequently extravasate at distant secondary sites where they can then form metastases upon undergoing mesenchymal-to-epithelial transition. Moreover, mesenchymal cancer cells are correlated with poor prognosis and associated with chemo- and immunotherapy resistance (Hao et al. 2019).

Angiogenesis, the process of formation of new blood vessels to supply tumors (both primary and metastases) with nutrients and oxygen, is also controlled by TGF-β. TGF-β secreted by tumor cells can bind TβRI on endothelial cells and induce a pro-angiogenic response. Furthermore, TGF-β is a potent inducer of vascular endothelial growth factor (VEGF) expression, thereby promoting angiogenesis as well. In addition, TGF-β can activate so called cancer associated fibroblasts (CAFs) that can contribute to increase cancer cell invasion and immunotherapy resistance. Interesting, failure to respond to immune check point inhibitors was found to be in certain cancer patients caused by overactivated CAFs that excluded cytotoxic T cells from reaching and killing cancer cells. Furthermore, TGF-β has potent direct immunosuppressive effects on both innate and adaptive immune cells, including natural killer cells and T lymphocytes, cells which can normally recognize and clear tumor cells. In addition to inhibiting the proliferation of T cells, it stimulates the differentiation of immune-suppressive regulatory T cells (Tregs) (Chen and Ten Dijke 2016; Colak and Ten Dijke 2017). In summary, TGF-β has a biphasic action in tumorigenesis as in early stages it suppresses tumorigenesis, while at later stages it promotes tumor progression.

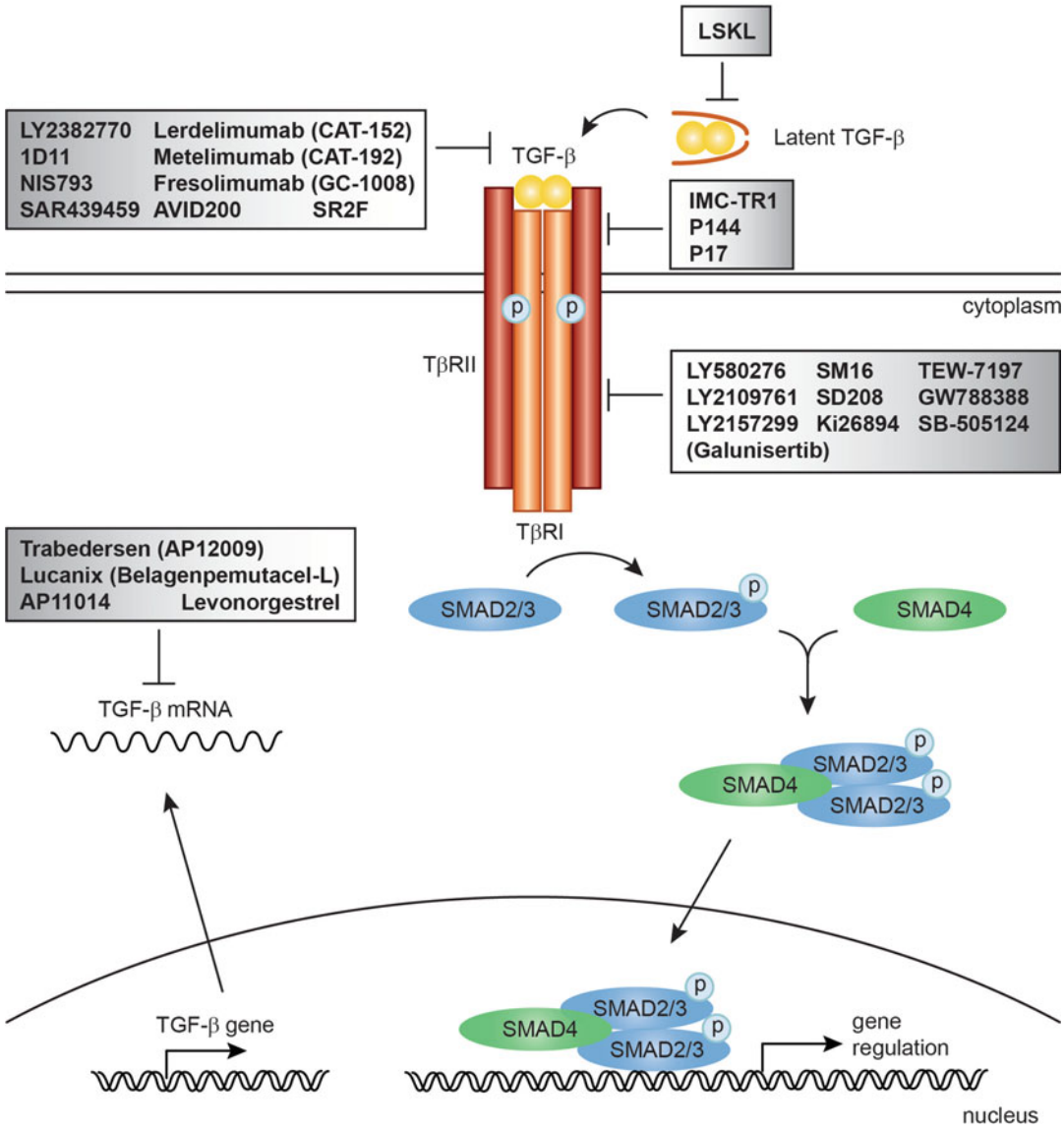
Drugs

Pharmacological Agents Targeting TGF-β Signaling

Pharmacological agents that target different components of the canonical TGF-β signaling pathway have been developed, including antisense oligonucleotides (AONs) that inhibit expression of TGF-β or TGF-β receptor, neutralizing antibodies against TGF-β or TβRII and receptor domain-immunoglobin fusions that interfere with ligand-receptor interactions, and receptor kinase inhibitors also known as small molecule inhibitors (SMIs) (Fig. 2; Table 1) (Akhurst 2017; Akhurst and Hata 2012; Colak and Ten Dijke 2017). The TβRI kinase inhibitors have been tested most extensively in (pre)clinical studies. The translation of anti-TGF-β therapy has been most intensively pursued in the area of oncology, but also for fibrosis and other diseases progress is made, illustrated for instance by the Europe approved drug pirfenidone to treat IPF (Akhurst and Hata 2012).

The number of preclinical examples of TGF-β inhibitors that reduce cancer progression and metastasis continues to increase. Moreover, an expanding number of studies is testing preclinical efficacy of targeting tumors with combinations of TGF-β blockade and cytotoxic drugs or immunotherapy, demonstrating promising combinations for novel treatment opportunities for various cancers (Akhurst 2017).

Because TGF-β inhibitors are not directly cytotoxic, their combined use with chemo-, radio-, or immune therapies gained extensive interest as this may be more efficacious. Several papers have shown the preclinical efficacy of targeting tumors with combinations of TGF-β blockade and cytotoxic drugs, such as doxorubicine or paclitaxel in breast cancer. Combining TGF-β inhibition with radiotherapy is another focus point, and TGF-β pathway inhibition was shown to sensitize glioma



TGF-β Pathway, Fig. 2 Schematic representation of TGF-β pathway blockade. TGF-β signaling can be inhibited in various manners; by antisense oligonucleotides (AONs) that inhibit expression of TGF-β or TGF-β receptor, neutralizing antibodies against TGF-β or TβRII and receptor domain-immunoglobin fusions that interfere with ligand-receptor interactions, and receptor kinase inhibitors also known as small molecule inhibitors (SMIs). TGF-β; transforming growth factor-beta, TβR; TGF-β receptor

cells to radiotherapy and enhance radiotherapy efficiency in breast cancer cells. Of note, radiation can cause fibrosis, which is itself a target for TGF-β therapy, so providing an added benefit of TGF-β inhibition. Also, the combination of TGF-β inhibitors and immunotherapy is suggested to improve the anti-tumor effect of immune cells, as TGF-β has immunosuppressive functions. TGF-β inhibition combined with PD-L1 inhibitor Atezolizumab was suggested to enable T cells to enter the interior of the tumor, illustrating benefits of combination therapies (Akhurst 2017; Colak and Ten Dijke 2017; Hao et al. 2019; Mariathasan et al. 2018).

TGF-β Pathway, Table 1 Summary of pharmacological agents targeting the TGF-β signaling pathway

Drug	Type	Target	Disease (application)	Phase	Clinical trial identifier	Status
Traberdersen (AP12009)	AON	TGF-β2	Glioblastoma and anaplastic astrocytoma	II	NCT00431561	Completed
			Glioblastoma and anaplastic astrocytoma	III	NCT00761280	Terminated
			Melanoma, pancreatic cancer and CRC	I	NCT00844064	Completed
Lucanix (Belagenpumatucel-L)	AON	TGF-β2	NSCLC		NCT00676507	Completed
			NSCLC		NCT01279798	No longer available
			NSCLC		NCT01058785	Completed
AP11014	AON	TGF-β1	Prostate cancer, NSCLC and CRC	Preclinical		
Levonorgestel	Hormone	TGF-β	Ovarian Cancer	II	NCT00445887	Completed
P144 (Disitertide)	Peptide	TβRII-like	Skin fibrosis	II	NCT00781053	Completed
			Skin fibrosis	II	NCT00574613	Completed
P17	Peptide	TGF-β1 receptor binding	Liver and pulmonary fibrosis, metastatic lung cancer, angiogenesis, melanoma, immunosuppression	Preclinical		
LSKL	Peptide	Thrombo-spondin		Preclinical		
Fresolimumab (GC-1008)	Antibody	TGF-β1; TGF-β2	Renal cell carcinoma (RCC?) and melanoma	I	NCT00356460	Completed
			Renal cell carcinoma and melanoma	I	NCT00923169	Completed
			Glioma	II	NCT01472731	Completed
			Mesothelioma	II	NCT01112293	Completed
			Metastatic breast cancer	II	NCT01401062	Completed
			Early-stage NSCLC	I/II	NCT02581787	Recruiting
			Myelofibrosis	I	NCT01291784	Terminated
			Diffuse systemic sclerosis	I	NCT01284322	Completed
			Focal segmental Glomerulosclerosis	I	NCT00464321	Completed
			Idiopathic pulmonary fibrosis (IPF)	I	NCT00125385	Completed
			Osteogenesis Imperfecta	I	NCT03064074	Recruiting

(continued)

TGF-β Pathway, Table 1 (continued)

Drug	Type	Target	Disease (application)	Phase	Clinical trial identifier	Status
			Primary focal segmental Glomerulosclerosis	II	NCT01665391	Completed
LY2382770	Antibody	TGF-β1	Diabetic kidney disorders	I	NCT01113801	Terminated
NIS793	Antibody	TGF-β2	Breast, lung, hepatocellular, colorectal, pancreatic and renal cancers	I	NCT02947165	Recruiting
			Myelofibrosis	I	NCT04283526	Not yet recruiting
SAR439459	Antibody	TGF-β1/2/3	Advanced solid Tumors		NCT03192345	Recruiting
Metelimumab (CAT-192)	Antibody	TGF-β1	Scleroderma and fibrosis	I/II	–	Completed
Lerdelimumab (CAT-152)	Antibody	TGF-β2	Scarring after glaucoma drainage	III	–	Completed
1D11	Antibody	mTGF-β1/2/3	Breast cancer	Preclinical		
IMC-TR1 (LY3022859)	Antibody	TβRII	Advanced solid tumors	I	NCT01646203	Completed
AVID200	Ligand trap	TGF-β1/3	Diffuse scleroderma	I	NCT03831438	Recruiting
			Myelofibrosis	I	NCT03895112	Recruiting
			Advanced solid Tumors	I	NCT03834662	Recruiting
SR2F	Ligand trap	TGF-β1/3	Breast cancer	Preclinical		
LY2157299 (Galunisertib)	SMI	TβRI	Hepatocellular carcinoma (HCC)	I	NCT02240433	Completed
			Prostate Cancer	II	NCT02452008	Recruiting
			Pancreatic Cancer	I	NCT02154646	Completed
			Advanced HCC	I	NCT02906397	Active, not recruiting
			Advanced solid Tumors	I	NCT01722825	Completed
			Rectal adenocarcinoma	II	NCT02688712	Recruiting
			Advanced solid Tumors	I	NCT02304419	Completed
			Advanced solid Tumors, NSCLC, HCC	I/II	NCT02423343	Active, not recruiting
			Metastatic pancreatic Cancer	I	NCT02734160	Completed
			HCC	II	NCT02178358	

						Active, not recruiting
			Myelodysplastic syndromes	II/III	NCT02008318	Completed
			Breast Cancer	I	NCT02672475	Recruiting
			Glioblastoma	II	NCT01582269	Active, not recruiting
			Glioma	I/II	NCT01220271	Completed
			HCC	II	NCT01246986	Active, not recruiting
			Metastatic breast Cancer	II	NCT02538471	Terminated
			Glioma	I	NCT01682187	Active, not recruiting
			Advanced solid Tumors, (metastatic) pancreatic Cancer	I/II	NCT01373164	Completed
			Ovarian Carcinosarcoma	I	NCT03206177	Recruiting
LY3200882	SMI	TβRI	Metastatic CRC	I/II	NCT04031872	Not yet recruiting
			Advanced solid Tumors	I	NCT02937272	Active, not recruiting
TEW-7197 (Vactosertib)	SMI	TβRI	Multiple myeloma	I	NCT03143985	Recruiting
			Metastatic gastric Cancer	I	NCT03698825	Recruiting
			Metastatic pancreatic Cancer	I/II	NCT03666832	Recruiting
			Advanced solid Tumors	I	NCT02160106	Completed
			Myelodysplastic syndromes	I/II	NCT03074006	Active, not recruiting
			Metastatic CRC, gastric Cancer	I/II	NCT03724851	Recruiting
			Metastatic NSCLC	I/II	NCT03732274	Recruiting
			Pancreatic Cancer	I	NCT04258072	Not yet recruiting
			Myeloproliferative neoplasm	II	NCT04103645	Active, not recruiting
			Urothelial Cancer	II	NCT04064190	Not yet recruiting
LY580276	SMI	TβRI	Cancer	Preclinical		
LY2109761	SMI	TβRI/ TβRII	Cancer	Preclinical		

(continued)

TGF-β Pathway, Table 1 (continued)

Drug	Type	Target	Disease (application)	Phase	Clinical trial identifier	Status
SB-505124	SMI	TβRI		Preclinical		
SD208	SMI	TβRI	Cancer	Preclinical		
Ki26894	SMI	TβRI	Breast Cancer	Preclinical		
SM16	SMI	TβRI	Mesothelioma	Preclinical		
GW788388	SMI	TβRI/ TβRII	Fibrosis	Preclinical		

AON antisense oligonucleotides, *CRC* colorectal cancer, *HCC* Hepatocellular Carcinoma, *IPF* Idiopathic Pulmonary Fibrosis, *NSCLC* non small cell lung cancer, *RCC* renal cell carcinoma, *TGF-β* Transforming Growth Factor-beta, *TβR* TGF-β Receptor. Trials targeting ALK1 and integrins are not included in the table

Some of the main concerns in TGF-β drug development are the inadvertent inhibition of the tumor-suppressive arm of TGF-β signaling in cancer and the development of adverse side effects, such as inflammation, autoimmunity, and cardiovascular defects, as discovered in several mouse studies. Clinical safety studies have now provided promising results and considered specific TGF-β blockers, e.g., Galunisertib or LY2157299, a SMI for TβRI kinase, relatively safe in humans, especially compared with cardiotoxicities of other standard chemotherapeutic anti-cancer drugs (Akhurst 2017; Kovacs et al. 2015). Other concerns are appearance of novel unrelated neoplasms, enhanced outgrowth of the primary tumor, and the awakening of dormant cancer cells in response to TGF-β signaling blockade. To date, no clinical report was published describing any of these adverse effects, yet it is important to note that this could be due to the focus on severely ill patients who might have not survived long enough to present these effects. Careful dosing and the use of drug holidays are crucial to avoid on- and off-target side effects of TGF-β therapies. Additionally, as there is considerable phenotypic diversity in the range of responses to decreased TGF-β signaling depending on genetic background, it is key to perform patient stratification and identify which patients will benefit the most from TGF-β therapies and to predict therapy responses. A simple and easy biomarker for patient selection in oncology could be high levels of circulating TGF-β. Phospho-SMAD2 levels or TGF-β target gene expression in ex-vivo cultured blood cells could be noninvasive biomarkers for prediction of patient responses to TGF-β inhibitors (Akhurst 2017; Akhurst and Hata 2012; Colak and Ten Dijke 2017).

The next stage in TGF-β pharmacology will be discovering the most efficacious drug combinations for each disease application and the right dosing regimens. Furthermore, investment to identify novel predictive biomarkers and patient selection criteria for TGF-β therapies is required.

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TGF-β Signaling Pathway

▶ TGF-β Pathway

TGF-β/SMAD Pathway

▶ TGF-β Pathway

Therapy

▶ Rheumatoid Arthritis

Thionamides

▶ Antithyroid Drugs

Thrombin Receptor

▶ Protease-Activated Receptors (PARs)

Thymoleptics

▶ Antidepressant Drugs

Tinzaparin

▶ Heparin and Related Drugs

T-Kinin (Ile-Ser-Bradykinin)

▶ Kinins

Trace Amine-Associated Receptors

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Definition

The term "trace amines" was introduced in 1970 (Boulton 1974) and related to amines, contrary to classical monoamines, having low endogenous concentrations (<100 nM). For a long time, trace amines were considered solely as "false neurotransmitters" modulating function of classical monoamines, such as dopamine and serotonin, via amphetamine-like action. It all changed with the discovery of G protein-coupled trace amine-associated receptors (TAARs) in 2001 (Borowsky et al. 2001; Bunzow et al. 2001).

Traditionally trace amines belong to by-products of amino acid metabolism – β-phenylethylamine (PEA), tyramine (TYR), octopamine (OCT), tryptamine (TRP), and p-synephrine. Later studies revealed much diverse group of endogenous agonists of one or more TAARs. Such compounds include the endogenous thyroid hormone metabolite 3-iodothyronamine (3IT); the catecholamine neurotransmitter metabolites 3-methoxytyramine (3-MT) and normetanephrine, trimethylamine, and isoamylamine; the polyamines putrescine and cadaverine; and possibly agmatine, spermine, and spermidine (Liberles and Buck 2006; Borowsky et al. 2001).

Basic Characteristics

Invertebrates

In invertebrates trace amines play a role of one of major neurotransmitters functioning as invertebrate versions of vertebrate adrenergic systems.

Tyramine and octopamine in invertebrates are not so "trace" as they can be found at high concentration. Invertebrates have also evolutionary distinct receptors – octopamine and tyramine receptors. Both types of receptors have several subtypes and all of them are GPCRs.

Octopamine in invertebrates acts analogous to vertebrate adrenaline system, both peripheral and in the brain: enhances locomotion, mobilizes fat stores, and increases cardiac rate and breathing. Apart from that octopamine regulates immune responses and sensory inputs and has effect on reproductive, feeding, aggressive behavior, learning, and memory. Tyramine action opposes action of octopamine, decreasing locomotion, flying, and foraging. It is also involved in regulation of sensory inputs (Roeder 2005).

Synthesis, Storage, Release, and Degradation

Trace amines are synthesized by decarboxylation of amino acids. β-Phenylethylamine (PEA), p-tyramine (TYR), and tryptamine (TRP) are produced by aromatic L-amino acid decarboxylase (L-AADC)-mediated decarboxylation of phenylalanine, tyrosine, and tryptophan, respectively. OCT and p-synephrine can subsequently be formed by the sequential action of dopamine-b-hydroxylase and phenyl-ethanolamine-N-methyl transferase. Trace amine can also undergo N-methylation forming additional TAAR ligands (Gainetdinov et al. 2018).

Gut microbiota can also be another source of trace amines. Prokaryotes have a large array of decarboxylase enzymes and therefore can synthesize trace amines. With TAARs established to be present throughout the body, it is expected that the role of trace amines and their receptors in mediating host-microbiota interactions will become a growing area of interest.

Ability of prokaryotes to synthesize trace amines brings to us another source – food – that underwent anaerobic fermentation process. Aged cheeses, fermented meats, red wine, soy products, and chocolate are well established as being enriched in one or more of PEA, TYR, and TRP. Another rich source of food-derived trace amines is seafood, from mollusks and crustaceans to fish. In normal conditions consumption of trace amine-rich food does not result in sufficient concentration for receptor activation, but in some cases such possibility should be considered.

Unlike classical neurotransmitters, trace amines appear not to be stored in vesicles. It has been shown that PEA, TYR, and TRP can pass through lipid bilayer. K^+-induced depolarization does not affect trace amine release, and that also strongly suggests that trace amines are not stored in synaptic vesicles and their release is actually a diffusion through membrane. However, some data suggests that there should be regulation of trace amine synaptic levels by transporters. The possible candidate for that is organic cation transporter 2 (OCT2).

The degradation of trace amines mainly occurs by monoamine oxidase. Genetic deficiency in MAO can result in sufficient increase in trace amine levels. There is also well-known "cheese syndrome" in patients treated with MAO inhibitors that could be due to indirect elevation of blood norepinephrine resulting in hypertension, migraine, and even death.

Another way of degradation is via cytochrome P450 pathway.

Involvement in Disorders

Trace amines were for a long time implicated in various human disorders (Gainetdinov et al. 2018). In patients with depression, decreased levels of PEA were found. Other disorders that trace amines are suggested to be involved in are migraine, cluster headache, anxiety disorders, and epilepsy (Berry 2007). There are several studies showing possible involvement of trace amines in schizophrenia. Elevated levels of PEA were shown in urine of schizophrenia patients. Multiple studies have shown an association of schizophrenia with key enzymes involved in the synthesis and degradation of trace amines, particularly aromatic L-amino acid decarboxylase (AADC) and catechol-O-methyl transferase. Also, a decrease in postmortem schizophrenia brain tissue of AADC-positive neurons that have no tyrosine hydroxylase was observed. In Parkinson disease patients after chronic treatment of L-DOPA, the level of TAAR1 agonist 3-MT was elevated that may be

relevant to the development of L-DOPA-induced dyskinesia.

Mechanisms of Action

The mechanisms of trace amine action on brain neurochemistry actually started long before TAARs were discovered. It was shown that application of PEA does not alter basic neuronal activity but influences response on activation of dopamine and norepinephrine, therefore acting as a modulator of activity of other neurotransmitter systems.

PEA was considered to be an "endogenous amphetamine." Indeed, application of high doses of PEA (and TYR also) causes amphetamine-like behavioral stereotypy. Mechanism of that action likely involves amphetamine-like dopamine transporter-mediated elevation of dopamine extracellular levels. Indeed, rapid, short-acting effects of PEA on extracellular dopamine levels were absent, and corresponding locomotor activation and stereotypy were not observed, if the dopamine transporter (DAT) was knocked out. It was observed also that PEA can exert a paradoxical calming effect in hyperactive mice without DAT similar to amphetamine, suggesting similar action on serotonin system to counteract dopamine-mediated hyperactivity. Chronic elevation of PEA also induces downregulation of dopamine D1 receptor, but not D2-like receptors.

TYR has similar effects to PEA: TYR is able to potentiate neuronal responses of dopamine and norepinephrine. No effect of TYR was seen on responses to GABA, glutamate, or serotonin. On the periphery, PEA and TYR were shown to be released from activated platelets being possible mechanism of TAAR-mediated chemotactic responses of leukocytes. TRP action differs from PEA and TYR. TRP is able to promote serotonin inhibitory effects, whereas excitatory responses were either unaltered or ever reversed for inhibitory.

Receptors

Trace amine-associated receptors (TAARs) were initially discovered in 2001 by two separate scientific groups (Borowsky et al. 2001; Bunzow et al. 2001). The new family of receptors was identified for their high affinity to PEA and TYR. At first there was no consistency in nomenclature of receptors. These receptors were identified as TA, TAR, TRAR, or GPR. Not only different nomenclatures were used, but also isoform identification was various resulting in different numbering of same receptors depending on nomenclature system. In 2005, the nomenclature was standardized based on gene sequencing, phylogenetic relationships, and chromosomal organization and these receptors were renamed as TAARs (Lindemann and Hoener 2005).

It is thought that evolutionary TAAR-like proteins first appeared in lamprey, getting conservative TAAR pattern later in evolution, after divergence of jawed vertebrates from jawless fish. Mammal TAARs generally are divided into nine subfamilies (TAAR1–TAAR9). The largest number of TAAR receptor subtypes is found in flying foxes having 26 functional genes. The only known vertebrate species having no TAAR receptors are bottlenose dolphin. Humans have six identified TAAR receptors – TAAR1, TAAR2, TAAR5, TAAR6, TAAR8, and TAAR9. All TAAR receptors, except TAAR2, have one exon. TAAR2 receptors have two exons.

All TAAR are G protein-coupled receptors (GPCRs). Single specific endogenous ligand is not identified for each of the receptor. However, some preferable ligand can be identified. TAAR1–TAAR4 are more potent for primary amines and TAAR5–TAAR9 for tertiary amines. Some (TAAR6 and TAAR8) can even be activated by diamines.

In 2006, TAARs were found in the mammalian olfactory epithelium and showed to be a new class of olfactory receptors sensing innate odors encoded by volatile amines. Moreover, it has been postulated that all TAAR receptors, with exception of TAAR1, are only olfactory and are not present in other cells (Liberles and Buck 2006). Later it has been recognized that not only TAAR1 but other TAARs are also expressed in the body and the brain (Gainetdinov et al. 2018). However, their low expression level and lack of specific ligands make them difficult to study.

TAAR1

TAAR1 is the best characterized receptor among all TAARs. As all TAARs, TAAR1 is a GPCR receptor. It is coupled to Gs proteins, and activation of TAAR1 results in increase of cAMP level (Grandy 2007). There is some evidence that TAAR1 is present both pre- and postsynaptically in neurons. Apart from neurons, expression of TAAR1 was found in astrocytes. TAAR1 is expressed in several brain regions: ventral tegmental area, substantia nigra, dorsal raphe nucleus, amygdala, rhinal cortices, subiculum, prefrontal cortex, nucleus accumbens, hypothalamus, preoptic area, spinal trigeminal nucleus, medullary reticular nucleus, nucleus of the solitary tract, and area postrema (Gainetdinov et al. 2018). Endogenous agonist with high affinity includes PEA, TYR, TRP, OCT, dopamine metabolite 3-MT, and thyroid hormone metabolite 3-iodothyronamine (3IT). Dopamine and serotonin show partial agonism at high but physiologically relevant concentrations (Lindemann and Hoener 2005).

Known to be expressed in regions with dopamine neurons, TAAR1 is considered to be a potent modulator of dopamine system (Lindemann and Hoener 2008). Application of TAAR1 agonists decreases firing frequency of dopamine neurons in the VTA. Consistent with that, deletion of TAAR1 receptors in knockout animals results in increased firing rate of DA neurons in VTA. TAAR1 knockout mice have increased extracellular dopamine level in the nucleus accumbens showed by microdialysis. Agonists and antagonists show corresponding changes in evoked dopamine release that is present only in WT and not in TAAR1-KO mice. However, no difference in dopamine uptake rate was noticed. It seems unlikely that TAAR1-mediated effects occur by regulation of dopamine transporter function, but rather via D2R-mediated mechanism. Indeed, TAAR1 can physically interact with D2 receptor postsynaptic long isoform forming heterodimers. TAAR1 knockout animals have increased expression of D2R, but not D1R, and show supersensitivity of D2 receptors. TAAR1 is also able to transduce the signal via beta-arrestin2 cascade via TAAR1-D2R interaction. TAAR1 normally poorly interacts with beta-arrestin2, whereas TAAR1-D2R heterodimerization significantly increases interaction that results in decrease of GSK3β activation.

TAAR1 receptors are also expressed in serotonergic neurons. TAAR1 agonist inhibits firing rate of serotonin neurons, and lack of TAAR1 in knockout mice results in opposite effect. Overexpression of TAAR1 in mice results in higher level of extracellular serotonin in medial prefrontal cortex. The molecular mechanisms of these effects are not yet understood, but it could be suggested that they are similar to mechanisms involved in dopamine system regulation.

It is also suggested that TAAR1 can regulate glutamatergic transmission. TAAR1 knockout mice have altered glutamate transmission – patch clamp methods revealed deficient NMDA receptor function in prefrontal cortex, together with decrease in the expression of the GluN1 and GluN2B subunits.

Ability to modulate dopamine, serotonin, and glutamate transmission makes TAAR1 a perspective target for the treatment of schizophrenia, mood disorders, narcolepsy, and addiction. TAAR1 variants were reported to be associated with schizophrenia. Preclinical testing indicates that TAAR1 agonists potentially can be used to treat schizophrenia, being able to normalize both dopamine hyper-active and glutamate hypo-responsive circuitry. Schizophrenia is also known to be characterized by development of cognitive symptoms. TAAR1 agonists showed their ability to normalize cognitive deficit. TAAR1 agonists show also some potential antidepressant-like properties together with antipsychotic activity profile. TAAR1 partial agonist has shown to have generalized increase of wakefulness, decreased latency to sleep, and decreased and lighter NREM sleep.

There is growing amount of evidence that TAAR1 agonists can be used in treatment of substance dependence. TAAR1 agonist can prevent the rewarding, pleasurable effects of psychostimulant compounds and decrease craving and drug-seeking behavior. Another application of TAAR1 agonists is related to their abilities

to influence reward system – treatment of feeding behavior disorders. Indeed, it has been shown that TAAR1 agonists can be beneficial in controlling compulsive overeating. TAAR1 agonists decreased food intake in diet-induced obese mice, resulting in weight loss and improved insulin sensitivity. Influence of TAAR1 on feeding behavior is even more evident as TAAR1 is expressed in periphery in the stomach, neuroendocrine cells of the intestine, and beta-cells of the pancreas. Activation of TAAR1 increases glucose-stimulated insulin secretion. As the effect on insulin secretion was observed only at elevated glucose concentrations, reducing the risk of hyperglycemia, TAAR1 agonists have the potential to become novel treatment agents for diabetes. TAAR1 receptors were found also in various populations of leukocytes with shown increase in TAAR1 level after leukocyte activation.

Other Receptors

Other receptors are less studied than TAAR1, and not much information about them is yet available. But what is certain that other TAAR receptors should not be considered only as olfactory as their expression was shown in other areas, including the brain.

For now, TAAR2 expression is shown in lymphocytes and in duodenal mucosal cells of the gastrointestinal system; however it could be suggested that further studies can identify TAAR2 expression also in other areas. Defunctionalizing single nucleotide mutation of TAAR2 is found in 10% of population. It was shown that this mutation is more frequent in patients with schizophrenia; however this data was shown on a small population which is still a matter of discussion, as it was not replicated so far. No information about TAAR2 function is yet known.

TAAR3 and TAAR4 are not expressed in humans and are present only as pseudogenes.

TAAR5 mRNA has also been reported in several mouse brain regions such as the amygdala, arcuate nucleus, and ventromedial hypothalamus, with an overlapping localization of TAAR1 and TAAR5 in the amygdala and ventromedial hypothalamus.

TAAR5 has been also reported to be expressed in various leukocyte populations, the spinal cord, the intestines, and the testes (Gainetdinov et al. 2018).

TAAR6 is predicted to be activated by tertiary amines and/or diamines. TAAR6 was found to be expressed in several brain regions: basal ganglia, amygdala, hippocampus, frontal cortex, and substantia nigra. There is no information yet about TAAR6 function, but there are several studies showing single nucleotide mutations and other genetic variations in patients with schizophrenia and affective disorders.

TAAR6 expression was also found in the spinal cord, but only in rats and not in humans. TAAR6 was also found in several other organs: the intestines, testes, kidney, and leukocyte cells (Gainetdinov et al. 2018).

TAAR7 is a pseudogene in humans but has representation in other mammals: in fact, mice have 15 functional TAAR receptors and one pseudogene, and rats have 17 functional receptors and two pseudogenes (Borowsky et al. 2001).

TAAR8 expression was found in the amygdala, cerebellum, cortex, and spinal cord. Outside nervous system, TAAR8 is found in the islets of Langerhans, intestines, spleen, testes, heart, lungs, kidney, and leukocytes. Like TAAR6, TAAR8 is predicted to be activated by diamines. So far, no information about TAAR8 function is known.

TAAR9 expression was found in the leukocytes, pituitary gland, skeletal muscles, spinal cord, intestines, spleen, and kidney. Loss of function mutation in the TAAR9 gene was shown in up to 20% of human population; however so far, no relevance to any disorder was identified.

TAAR Receptors in Olfaction

TAAR2–TAAR9 receptors are found in the olfactory epithelium, but not in the vomeronasal organ. TAARs serve to detect social or ecological relevant innate cues. TAAR5 has been suggested to be activated by trimethylamine that was proposed to be mouse pheromone. Trimethylamine is present in much higher concentration in male mice urine (compared to female mice urine) and can activate sex-specific behavior (Liberles and Buck 2006).

In rats and humans, smell of trimethylamine has aversive action.

TAARs are also thought to be involved in avoidance reaction for rotting food source (rarely attraction). TAAR6 and TAAR8 are predicted to be responsive for that reaction. Cadaverine and putrescine, substances that are thought to be responsive for "rotten" smell, are suggested to be agonist of these two receptors.

Drugs

Studies showed that a broad number of substances, including psychotropic agents, have high activity agonism at TAAR1. However, ability of such substances to activate TAAR1 differs by species, for example, endogenous hallucinogen N,N-dimethyltryptamine (DMT) and lysergic acid (LSD) show limited activity in human isoform. Also, TAAR1 can be activated by beta-adrenergic agonist ractopamine and dopaminergic agonist apomorphine. A number of TAAR1 full and partial agonists were developed, like RO5263397, RO5166017 (full agonists), and RO5203648 (partial agonist). A mixed 5-HT 1A/TAAR1 agonist was developed by Sunovion Pharmaceuticals and currently being tested clinically in patients of schizophrenia with very promising results (Berry et al. 2017; Gainetdinov et al. 2018).

One putative non-selective TAAR5 agonist was described – 2-(a-naphthoyl)-ethyltrimethylammonium iodide (alpha-NETA) (Belov et al. 2019). The data obtained with alpha-NETA suggests that TAAR5 receptors can be a novel molecular locus for several conditions seen in schizophrenia: sensory gating deficits, cognitive paradigm known to be reflective of schizophrenia-related cognitive deficits. This hypothesis is supported by the fact that injection of alpha-NETA modulated dopamine transmission in the brain.

Conclusion

There are many gaps in our knowledge about trace amine system yet. Only for TAAR1 receptors there is some information on its action in the brain. The discovery of TAARs is not only unraveling new modulatory system in the brain but might bring also a host of new therapeutic target for treatment of various human disorders, from schizophrenia to diabetes.

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Transcription Factors

► Transcriptional Regulation

Transcriptional Regulation

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Synonyms

Chromatin remodeling; Hormonal regulation of transcription; Transcription factors

Definition

Transcriptional regulation encompasses the modulation of the synthesis rate of mRNA due to cell-type-specific factors that may be triggered by external stimuli.

Basic Mechanisms

Background

Frequently prescribed drugs lead to alterations of gene expression in cells, often in a cell-type-specific manner, which are decisive for drug action. For example, cortisol and its synthetic derivatives, a frequently prescribed drug for acute and chronic inflammation, have very specific actions in different types of cells, leading to its potent anti-inflammatory effects and – unfortunately – to its devastating side effects, including atrophies of tissues, fat redistribution, and diabetes.

We will consider the nature of transcriptional regulation in general and will refer to the specific example of the response to cortisol, because much of has been learned about the dynamics of genome-wide transcriptional regulation in response to this hormone.

Gene regulation links a cell-type-specific cellular response to external stimuli, which is predominantly coordinated in the nucleus. Cells must be able to respond to a variety of different conditions, exerted by hormones, cytokines, pathogens, and drugs, all acting on cells out of the extracellular environment. Similarly, cells must also respond to cues from within the cells themselves, for example, to nutrient or oxygen deprivation. To receive an external or internal stimulus, cells must possess sensors (receptors). To allow a cell-type-specific response, they have, in addition, so-called lineage-specific transcriptional regulators that are either unique for a certain cell type or are present in a cell-type-specific combination of factors. Together with the stimuli receiving receptors and their signal transduction, the transcription factors integrate a cell-type-specific gene regulatory response. This leads to the unique gene expression signature that regulates a subset of the approximately 22,000 protein-coding genes present in every cell.

Cortisol-regulated transcription is an excellent example of such cell-type specificity. Cortisol causes in macrophages a transcriptional response that leads to an anti-inflammatory activity of macrophages, dampening inflammation and inducing repair processes. On the other hand, cortisol stimulates adipocytes (fat cells) to proliferate, differentiate, and enhance lipolysis and by this contribute to the metabolic side effects of steroid therapy including fat redistribution and insulin resistance, eventually leading to diabetes. Thus, very different genes are regulated by cortisol in macrophages in comparison to those regulated by cortisol in fat cells.

Gene regulation occurs on several levels, including regulation of transcription, regulation of mRNA splicing and alternative splicing, regulation of mRNA stability by microRNAs and other mechanisms, and protein stability.

Cell-type-specific regulation of transcriptional initiation is the best investigated and understood, and some researchers consider this the main regulatory step of gene regulation.

Transcription Initiation

Transcription of protein-coding genes is performed by RNA-polymerase II (RNApol-II). It synthesizes mRNA from genes. The genes carry a transcriptional start site (TSS) which the RNApol-II must find. Because genes are packed in chromatin, the process of finding the TSS requires specific opening steps that depend on the integration of intracellular signals. These are elicited by activated transcription factors and already expressed cell-type-specific and lineage-specific transcription factors (to be described in paragraphs "Enhancers" and "Epigenetic Regulation" below). The TSSs are located 5′ upstream of the coding region of the DNA. The TSS is part of the, so-called, *promoter* (see below). Often, but not exclusively, this DNA region comprises a TATA box (T/A-rich sequence) preceding the TSS. RNApol-II binds this site with the help of general transcription factors such as TATA binding protein (TBP), along with other co-factors. For binding and subsequent transcription, a large complex of proteins is assembled at the promoter, in a hierarchical manner. TBP-associated factors (TAFs) function in this assembly. TAFII250 (TAF, associated with RNApol-II, 250 kDa molecular weight) catalyzes the formation of other essential elements of the transcriptional initiation machinery: TFIID along with TAFs and TBP bind the TATA box upstream of the TSS. Together, a large assembly of numerous factors together with RNApol-II is formed at the transcriptional start site. It is now called **the initiation complex**. In a manner similar to DNA replication, a DNA helicase is necessary to open the double helix and allow cognate RNA molecules to bind to the DNA. Eventually, RNApol-II is released from the other components of the initiation complex and proceeds to synthesize nascent RNA. To summarize, the precise binding of RNApol-II to the start site requires numerous helper components whose association with RNApol-II must then be disrupted for the enzymatic action to start (Haberle and Stark 2018).

After start of transcription, RNApol-II frequently pauses, stopping the synthesis of RNA and a step that is also subject to regulation. The pausing was discovered by a method which permits to localize RNApol-II on genes in the process of transcription. Using chromatin immunoprecipitation (ChIP) followed by either microarray analysis or next-generation sequencing of the DNA precipitated, the RNApol-II location could be determined. These data revealed that RNApol-II occupancy on a gene does not always correlate with mRNA synthesis. Thus, it became clear that the RNApol-II action can be regulated by recruitment to the gene but also by the starting or pausing during the elongation process. As with initiation, the pausing process is regulated by phosphorylation.

After start of elongation, the TSS is cleared of RNApol-II, important to permit restart by a subsequent new initiation complex. Also paused RNApol-II molecules block transcription, in that prior to release of the pause, subsequent RNApol-II molecules cannot initiate transcription. Arrested RNApol-II thus causes turnoff of transcription. It is yet not clear how to explain the results of recent analyses which identified "bursts" of transcription, the synthesis of large amounts of mRNA, which is then followed by immediate turnoff. These findings add to the complexity of gene regulation. However, the physiological relevance of transcriptional bursts is still not fully understood (Core and Lis 2008).

The initiation of specific gene transcription in response to external stimuli is a complex interplay between transcription factors and specific DNA sequences: the promoters and enhancers.

How is it now ensured that cortisol induces in macrophages and in adipocytes the transcription of different sets of genes leading to cell-type-specific responses? One key feature is cell-type-specific opening of the chromatin accomplished by lineage-specific pioneering transcription

factors which mediate the accessibility of hormone-inducible enhancers and promoters (Zaret and Carroll 2011).

Promoters

The promoter is defined as a DNA sequence that regulates the initiation of transcription, usually located at short distance upstream of the TSS. A general eukaryotic promoter can be broken down into three main parts, the core promoter, the proximal sequences, and distal sequences. The core promoter, which is the site of RNApol-II binding, comprises the TATA box and the TSS. The core promoter consists of many DNA motifs, namely, specific DNA sequences (often called consensus motifs) that interact with specific factors. For example, the TATAWAWR motif binds TBP. Mutagenesis experiments in fruit flies have demonstrated that specific motifs define the assembly of the different components of the transcription initiation complex. Single nucleotide exchanges in these motifs prevent transcription. The proximal promoter 5′ upstream of the core promoter houses regulatory sequences which can be bound by transcription factors which modify the activity of or binding of RNApol-II.

Core promoters have been classified according to nucleosome positions and functional roles. The type of core promoter relevant here for cell-type-specific genes is called "sharp." Sharply defined TATA-dependent promoters are often tightly regulated in response to specific stimuli, also linked to transcriptional bursting, whereby transcriptional activity is turned on quickly, a lot of mRNA is produced, and then turned off immediately after the activation stimulus.

Proximal sequences of the promoter contain regulatory sequences with transcription factor binding sites that are similar to those than enhancers, with the difference that they are still close to the core promoter up to 250 base pairs. Distal sequences contain further regulatory sequences (Haberle and Stark 2018; Lenhard et al. 2012).

Enhancers

Enhancers are DNA sequences required to increase (or decrease) transcriptional rates of their regulated genes. While promoters are found in the vicinity of the TSS acting in *cis* on a given gene, enhancers can be found near or far from, upstream or downstream, or even within the sequence of the gene they regulate. They may even act in *trans*, i.e., affecting gene expression on other chromosomes. The action of an enhancer is mediated by regulatory factors binding to the enhancer DNA sequence. The location close to, or far from the promoter does not affect the stimulatory strength of the enhancer, i.e., a distant enhancer is not necessarily less potent than an enhancer close to the promoter.

Enhancers carry short DNA sequences, sequence motifs to which transcription factors and co-regulators bind. Enhancers may contain multiple such short sequences, which can function independently when cloned and tested in vitro, but when combined bind several specific transcription factors whose action defines a specific gene expression signature for their target gene (Long et al. 2016).

For instance, the enhancer sequence motif for the cortisol-induced transcription factor that elicits so many differential cell-type-specific responses is ACAnnnTGT. To this sequence motif, a dimer of the transcription factor GR ("glucocorticoid receptor") binds if associated and activated by its ligand cortisol. The symmetry of the sequence motifs favors often the interaction with transcription factor dimers. Proteins make contact with DNA through either one of four different DNA amino acid sequences which form specific three-dimensional structures: basic helix-loop-helix, helix-turn-helix, leucine zippers, and zinc (Zn) finger motifs. They all have in common that they make specific contacts to bases in the DNA, e.g., in the major groove of DNA. Often, they are also involved in the dimerization of the transcription factors. In our example of the cortisol-bound receptor, this DNA binding amino acid motif is represented by two zinc fingers. Fingerlike are stabilized by a Zn^{2+} ion that is complexed by cystidine and histidine residues. The amino-terminal Zn finger contacts the major groove of the DNA, whereas the carboxy-terminal Zn Finger stabilizes a dimerization interface that allows dimerization with the second GR molecule (Vandevyver et al. 2014).

One may think that identifying enhancers in the genome via such DNA sequence motifs should be trivial. This is however not the case. The motifs do not tell whether they are within active enhancers. Many enhancer-like sequences found in the genome may not exert any activity, and some are only active in one cell type, but not in another. Complicating the definition of an enhancer motif is variation recognized by a given transcription factor. Certain nucleotide positions can be changed without affecting the interaction.

The chromatin structure determines the functional activity of an enhancer. This is particularly evident in enhancers that respond to inducers such as cortisol. The chromatin must first be opened for the GR. This depends on the repertoire of lineage-specific transcription factors that bind in close proximity to the GR enhancer motif ACAnnnTGT.

In adipocytes members of the CCAAT/enhancer binding proteins (C/EBP) transcription factor family and the decisive lineage transcription factor peroxisome proliferator-activated receptor gamma (PPARγ) perform the chromatin opening. In macrophages, for instance, Pu.1 fulfils this function. These factors are considered as "pioneering factors" because they often precede the binding of the inducible transcription factors such as the GR. The pioneering factors assist the binding of the inducible factors leading to so-called hot spots of transcription factor binding characteristic for certain cell types. The combined binding forces the chromatin to be opened and catalyzes access for the promoter transcription initiation complex and thus enables transcription (Farmer 2006; Greulich et al. 2016).

Chromatin opening results in regions of DNA that are extremely sensitive to experimental digestion with the DNA-cutting enzyme, DNase. They are known as DNase hypersensitivity sites. Open chromatin is assumed to indicate sites of high regulatory and transcriptional activity. However, within these sites of DNA that are easily cut, it is possible to find small regions of protected DNA cut less often by DNase. These are known as "footprints," locations bound by transcription factors and protecting the DNA from being digested. Experimenters can then, by assessing the sequence not digested, predict which transcription factor is likely to be bound, by comparing the protected sequence to the known consensus sequences to which the transcription factors are known to bind (Zaret and Carroll 2011).

Intriguingly the occupancy of enhancers by transcription factors is not static, rather highly dynamic with rapid on and off rates. Approaches using fluorescence lifetime microscopy have shown that transcription factors are resident on DNA only for fractions of a second, before moving on to another site. The functional correlation of residence time of transcription factors with chromatin remodeling and initiation of transcription is still far from being understood (Liu and Tjian 2018).

Chromatin remodeling, i.e., the dynamic opening and closure and thus access of DNA for transcription factors, is regulated by epigenetic mechanisms.

Epigenetic Regulation of Transcription

The chromatin architecture determines when and where a transcription factor and RNApol-II can bind to DNA. In eukaryotes, DNA is tightly wound around so-called nucleosomes which consist of proteins called histones. The nucleosome core is formed by a histone octamer, consisting of the proteins H2A, H2B, H3, and H4 all in duplicate. The DNA is then wrapped around this core, with the DNA-associated histone H1 linking individual nucleosomes. This winding of the DNA makes it relatively inaccessible to transcription factors and polymerases. In addition, the chromatin strings (chains of nucleosomes) are ordered into complex higher structures by winding around themselves. The degree of packaging is regulated by various posttranslational modifications of the histones. These modifications can increase or decrease access for transcription factors and RNApol-II to the DNA. The study of histone and DNA modifications not associated with changes in DNA sequence is known as epigenetics. Unlike the fixed inherited genetic code, histone modifications are under control of regulatory stimuli to cells.

The different chromatin-modifying enzymes can be roughly divided into three formal categories: **readers**, **writers**, and **erasers,** classified by their principal molecular action. Readers recognize

epigenetically modified chromatin, writers add modifications, and erasers remove them. The recruitment to specific chromatin locations is mainly catalyzed by the transactivation functions of enhancer- or promoter-bound transcription factors and is therefore often designated as co-regulators.

Writers, for example, are the histone acetyltransferases (HATs) and histone methyltransferases (HMTs), enzymes which catalyze the addition of an acetyl group or a methyl group, respectively, to lysine residues of histone proteins.

One common co-regulator with HAT activity is the protein EP300. Because EP300 is often involved in inducing transcription, it is also designated as a co-activator. EP300 is recruited to the DNA by many different transcription factors, such as RELA (part of the inflammatory NFκB complex, e.g., in macrophages), PPARγ (a nuclear receptor involved in lipid storage in adipocytes), and the GR, the target of cortisol. These different transcription factors with extremely different physiological roles all recruit EP300 to their binding sites at enhancers or promoters and utilize the HAT activity of EP300 to relax the nucleosomal structure, in order to promote transcriptional activity.

Histone methyltransferases (HMTs), the second example belonging to the category of writers, on the other hand, play almost a role opposite to HATs, at least in general. HMTs catalyze the addition of mono-, di-, or even trimethyl modifications to histone lysine residues, which are involved in silencing of gene expression due to contraction of the chromatin structure (Calo and Wysocka 2013).

Erasers remove modifications. Histone deacetylases (HDACs) remove acetyl groups, while histone demethylases remove methyl groups. Because small-molecule inhibitors inhibit selectively individual HDACs, it has been recognized that each HDAC exerts specific functions. Such studies have revealed that HDACs interact with different preferred partners and regulate different genes.

Histone demethylases (HDMs) are less well characterized than HDACs, but nonetheless, research has identified important roles of these enzymes in physiology. Methyl groups from lysine residues are removed by two major classes of demethylases, flavin adenine dinucleotide (FAD)-dependent amine oxidases, and Fe(II) and α-ketoglutarate-dependent hydroxylases. These enzymes utilize and create elements of the Krebs cycle during their activity and thus link chromatin modification and modulation of transcription to energy demands and metabolic status of the cells (Hyun et al. 2017).

Finally, the *readers*, as their name suggests, have less to do with actively altering the epigenetic modifications, but rather with identifying epigenetic changes. However many readers also have writer or eraser functions. Readers have protein domains with extremely high affinity for specific histone modifications. The plant homeodomain (PhD) and malignant brain tumor (MBT) domains recognize methyl histones, while the bromodomain recognizes acetylated histone. The SWI/SNF (SWItch/Sucrose Non-Fermentable) complex is a bromodomain containing protein complex involved in nucleosome reorganization. The bromodomain is essential for SWI/SNF in its ability to identify locations marked for full opening of the chromatin. SWI/SNF then catalyzes a complete nucleosomal shift in an ATP-consuming manner. This results in a loop of DNA, devoid of nucleosomes and thus completely available for binding of transcription factors and RNApol-II (Calo and Wysocka 2013).

The Histone Code

As introduced above, the degree of chromatin packaging determines the access of regulatory proteins to DNA and transcription. Chromatin density in turn is regulated by histone modifications. Active promoters and enhancers can be now recognized by certain modifications of histones, designated as the histone code. For instance, active TSSs are usually depleted of nucleosomes and thus accessible for DNase hypersensitivity assays. However, the first nucleosomes downstream of the TSS are enriched for acetylated histone H3 lysine 27 (H3K27Ac) and trimethylated lysine 3 (H3Kme3) (Andersson and Sandelin 2020).

Nucleosomes bordering active enhancers carry high H3K27Ac and H3K4me1, which now serves as an epigenetic definition of active enhancers. In

contrast, repression of transcription is associated with different histone modifications, in particular with H3K9 di- and trimethylation and H3K27 di- and trimethylations. Using chromatin immunoprecipitation against these histone modifications, followed by sequencing in combination with the genome-wide determination of DNase hypersensitivity sites (see Box), these codes could be established (Calo and Wysocka 2013).

Cortisol leads to differential patterns of enhancer and promotes activity depending on whether it acts on macrophages or on adipocytes. This is orchestrated by the presence of cell-type-specific repertoires of additional transcription factors which together with the DNA-bound GR recruit co-regulators that lead to the histone modifications and eventually the transcription of macrophage- and adipocyte-specific cortisol-regulated genes that execute the particular physiological responses.

Pharmacological Relevance

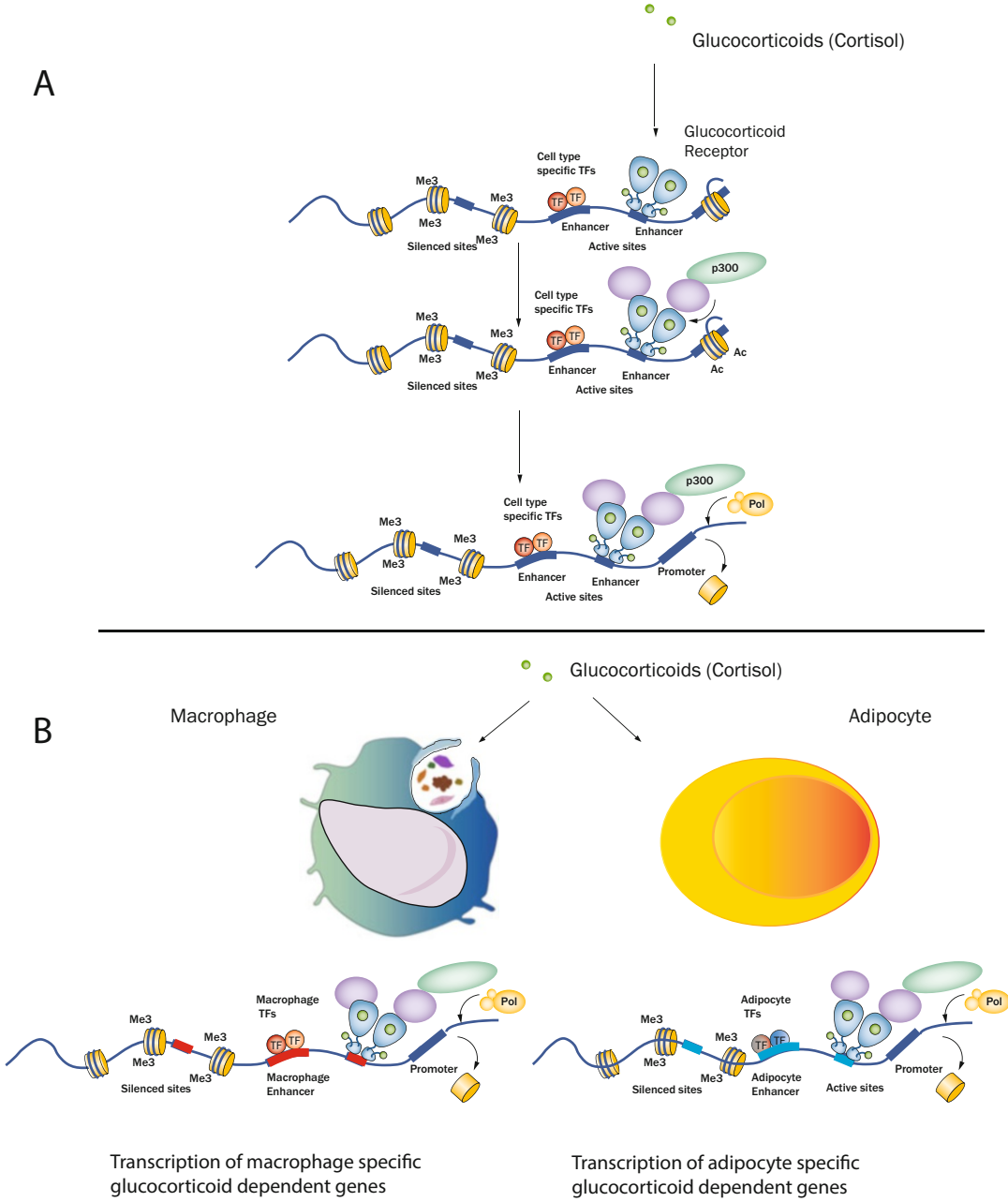
As transcriptional regulation is central to cellular function, its manipulation is therefore of therapeutic value for a number of diseases including chronic inflammation and cancer. The use of ligands, which directly interact with a transcription factor and activate or inactivate the factor or alter histone-modifying enzymes, is an elegant case of transcriptional interference.

Ligands for nuclear receptors have been the first widely applied agents targeting transcription, e.g., estrogens, retinoic acids, and glucocorticoids (e.g., cortisol). More recent pharmaceutical attempts to inhibit the interactions between transcription factors with their coactivators by small molecules have yet been successful in only a few cases. For example, interference with the interaction between the transcription factor p53 and the coactivator CBP hinders the transcriptional activation of p53-dependent target genes, which trigger cell death. As an interesting other approach, the abundance of a transcription factor can be modulated by specifically blocking degradation, again exemplified by small molecules inhibiting the binding of Mdm2 to p53, the first step which targets p53 for degradation. Elevated p53 can be desirable if cancer cells are to be destined for cell death (Grossman 2001; Zhao et al. 2015).

Not all transcription factors can be targeted by small-molecule inhibitors or activators, and thus the concept of oligodeoxynucleotide decoys became of interest. These decoys are short sequences of DNA with specific high-affinity transcription factor binding sites, unique to each transcription factor. These nucleotides are able to enter mammalian cells either through liposome-mediated transfection, viral transduction, or by an active transport by the target cell (e.g., endocytosis). Once inside, the short decoy sequences are bound by their target transcription factor, preventing the target from binding genomic DNA at enhancer or promoter regions, therefore preventing their activity. Early clinical trials of oligodeoxynucleotide decoys have shown safety, but unfortunately not efficacy. For example, a topical application using decoys for the pro-inflammatory transcription factor NFκB was unable to help dermatological conditions over the placebo-treated group (Hecker and Wagner 2017).

Another approach to inhibiting transcription factor function is the use of small molecules, or peptides, to block protein-protein interactions of transcription factors. Many transcription factors have essential dimerization functions, for example, the signal transducer and activator of transcription 3 protein (STAT3). Besides its physiologic life-essential function, STAT3 also acts as an oncogene, promoting cellular growth of cancer cells. It would therefore be a prime target for cancer therapy. STAT3 functions by binding as a homodimer to promoters in the nucleus in order to regulate transcription. Targeting the interface of homodimerization, the Src homology 2 domain (SH2), using peptides which bind SH2, researchers have been able to inhibit the transcriptional activity of STAT3. These types of drugs however have not yet made it past the clinical trial stage (Sgrignani et al. 2018).

The modulation of transcription nicely demonstrates how results of basic science can be translated into medical application. These few examples of drugs in successful use stimulate at



Transcriptional Regulation, Fig. 1 Transcriptional regulation exemplified on cortisol-induced transcription. (A). Glucocorticoids (cortisol) bind to the transcription factor dubbed glucocorticoid receptor, GR. GR binds to DNA in open chromatin that is free from nucleosomes caused by the activity of cell lineage transcription factors. These regions are considered as "active sites." The DNA elements that bind lineage-specific transcription factors or hormone-inducible transcription factors such as the GR are called enhancers. Closed chromatin is covered with nucleosomes that have histone modification that mark them as transcriptional silenced sites, such as trimethylation at H3K9 or H3K27 amino acid residues. Following binding of transcription factors, co-regulatory molecules are recruited with enzymatic activities for chromatin modification. P300, for instance, acts as acetyltransferase. Acetylation on histones such as H3K27Ac by histone acetyltransferases and also methylation by histone methyltransferases leading to trimethylated lysine 3 (H3Kme3) take place. These modifications allow

the same time the intensive study of gaps in our understanding of transcriptional control, hoping to find new specific ways of interference. The extensive research into transcription factors is key to the progression of pharmacological strategies to treat disease. However, due to the vast array of actions that each transcription factor has, by their ability to regulate gene expression on a genome-wide scale, it is essential to understand the mechanisms of transcriptional regulation. The number of fail attempts, which work in vitro, or in a preclinical model, but are unable to show efficacy in human trials, is mounting. Transcription factors, while essential for gene regulation in the cell, are extremely difficult to target therapeutically, especially those without endogenous (and known) ligands.

However, in case that ligands are known and available, such as in the example of cortisol therapy, pharmacologic use has profound effects to attack acute, chronic, and overshooting inflammation. The challenge remains how to develop strategies how to define cell-specific actions and modifications that avoid the odds of side effects.

Given the central role of transcription in gene regulation, modifying this process remains an ultimate goal of pharmacology (Fig. 1).

Box: Assays for Measuring Transcriptional Activity, Transcription Factor Binding, and Chromatin Modifications

GRO-seq

To measure directly regulated transcripts, methods such as global nuclear run-on sequencing (GRO-seq) have been established. While there are many different ways of purifying newly synthesized RNA, they all follow a similar principle – enrichment of labeled RNA molecules by tags, which allow purification before processing and sequencing. GRO-seq uses labeling, by incubating cells with a specific nucleotide (5-bromouridine 5′-triphosphate, BrUTP) which is incorporated into the synthesized RNA and then pulled out specifically using an antibody directed against BrUTP. The importance of these methods is that they show currently regulated transcripts, as opposed to other methods which only identify total, fully processed mRNA molecules, which are not only regulated by transcriptional control and RNApol-II recruitment but also by posttranscriptional processing, decay, or stabilization. GRO-seq can also be used to identify new TSS locations or cryptic promoters in the genome not predicted by genome sequencing.

ChIP-seq

Chromatin immunoprecipitation sequencing (ChIP-seq) is a method used to identify locations of transcription factors, co-regulators, and modified histones that are bound to the genome. These can then be annotated to known promoter or enhancer sites in order to determine which gene they are likely regulating. The tool is even more powerful when combined with other technologies such as GRO-seq or whole-transcriptome mRNA sequencing (RNA-seq) to further identify how transcription factor binding loci can influence transcription. First cells are fixed using formaldehyde to lock the transcription factors in place, bound to the DNA.

(continued)

Transcriptional Regulation, Fig. 1 (continued) to remodel chromatin or even disrupt nucleosomes. Eventually basic transcription factors can occupy the promoter and allow recruitment of mRNA polymerase II to allow initiation of transcription. (B) Glucocorticoids activate the same transcription factor GR in macrophages and adipocytes. Due to different lineage-specific transcription factors however, the hormone-induced genes could differ between macrophages and adipocytes, leading to the cell-type-specific induction of cortisol-regulated genes. (Figure is modified after Baschant et al. 2012)

The DNA is then fragmented, either by kinetic means such as sonication or by enzymatic digestion, and the transcription factors and its covalently associated DNA sequence are fished out using targeting antibodies. The DNA that accompanies the transcription factors is then sequenced in order to identify the specific binding site in the genome. This technique has now become a workhorse for the transcription factor research community, able to identify new binding locations of transcription factors, binding sites that are occupied by multiple proteins, and even finding RNApol-II binding sites to determine transcriptional potential or promoter regions. Furthermore, it is possible to also find sequences marked by histone posttranslational modifications, linking the histone code to transcriptional regulation.

DNase-seq

To determine open, generally active, areas of the genome, DNA can be treated with DNaseI, an enzyme which digests DNA. However, DNaseI can only access chromatin, which is open and thus accessible to the enzyme. This means that areas of DNA bound closely to histones, and therefore closed and not accessible, or bound by transcription factors, are protected from the DNaseI and are sequenced. This allows for an approach called "footprinting" whereby the sequences protected from the DNaseI can be used to predict which transcription factors are binding, by comparing the protected sequence to known specific transcription factor binding sites (Shlyueva et al. 2014.

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Transcriptome Analysis

▶ Gene Expression Analysis and Next-Generation Sequencing

Transforming Growth Factor-Beta Pathway

► TGF-β Pathway

Transient Receptor Potential Channels

► TRP Channels

TRP Channels

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Synonyms

Transient receptor potential channels

Definition

A superfamily of cation channels conserved in mammals, flies, fish, sea squirts, and worms. All TRP proteins bear sequence similarities to the founding member of this superfamily, transient receptor potential (TRP), a light-activated cation channel in the *Drosophila* photoreceptor.

Basic Characteristics

The human genome contains 27 TRP channel-encoding genes and 2 genes encoding the closely related endo-lysosomal two-pore channels. So far, only few examples of TRP genes have been identified in nonland plants and fungi, and no TRP channels have been identified in archaea or bacteria (Hilton et al. 2019). Initially, TRP channels were identified solely on the basis of primary sequence similarity and presumed shared overall transmembrane topology. Based on their amino-acid sequence similarities, the TRP proteins fall into eight subfamilies, seven of which are found in humans: canonical (TRPC), vanilloid (TRPV), melastatin (TRPM), mucolipin (TRPML), ankyrin (TRPA), polycystin (TRPP), and the closely related two-pore channels (TPC) (Table 1). The TRPN (NOMP, NO-Mechano-Potential) has so far only been detected in worm, fly, and zebra fish and is proposed to be a mechano-sensing channel (Flockerzi and Nilius 2007; Nilius and Flockerzi 2014). Almost all TRPs have been shown to form ion channels in the plasma membrane (Vangeel and Voets 2019) or in the membranes of intracellular compartments, the later dubbed "organellar" TRP channels (Zhang et al. 2018). Both plasma membrane and organellar TRP channels essentially contribute to cellular Ca^{2+} signaling, and some like the

TRP Channels, Table 1 TRP Channels

TRPC	TRPV	TRPM	TRPML	TRPA	TRPP	TRPN	TPC
TRPC1	TRPV1	TRPM1	TRPML1	TRPA1	TRPP2	NOMP	TPC1
TRPC2	TRPV2	TRPM2	TRPML2		TRPP3		TPC2
TRPC3	TRPV3	TRPM3	TRPML3		TRPP5		
TRPC4	TRPV4	TRPM4					
TRPC5	TRPV5	TRPM5					
TRPC6	TRPV6	TRPM6					
TRPC7		TRPM7					
		TRPM8					

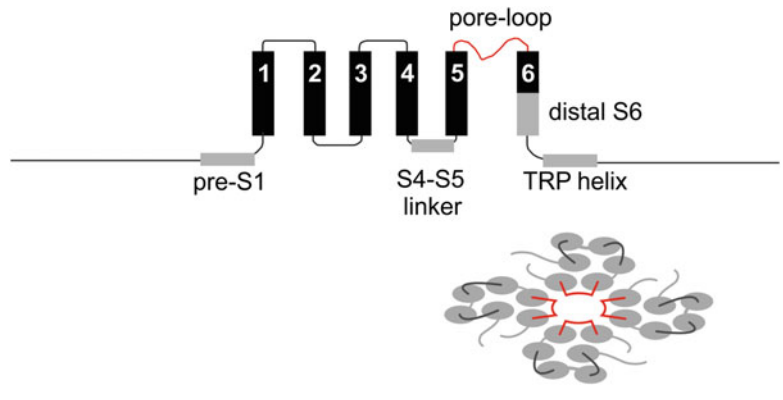
organellar members of the mucolipin subfamily might function as Ca^{2+}, Ni^{2+}, or Zn^{2+} release channels (Nilius and Flockerzi 2014; Vangeel and Voets 2019; Zhang et al. 2018).

Recently, TRP channel structures obtained by electron cryo-microscopy and X-ray crystallography validated many previous assumptions such as the tetrameric architecture in which subunits are arranged in fourfold symmetry around a central ion-permeation path. Up to now, structures of 19 TRP channels have been solved at resolutions between 2.9 and 5 Å (Hilton et al. 2019; Vangeel and Voets 2019; Zhang et al. 2018). These structures provide an unprecedented insight into the molecular architecture of TRP channels. A single TRP protein comprises six transmembrane helices followed by the amphipathic TRP helix which runs parallel to the cytoplasmic plane of the plasma membrane.

Notably, four amino-acid sequence motifs turned out to define a unique signature common to almost all TRP channel proteins (Fig. 1), the pre-S1 helix (motif 1), the S4–S5 linker (motif 2) connecting the S1–S4 helices and the S5-pore-loop-S6 segment, the distal part of helix S6 (motif 3), and the TRP helix (motif 4, also dubbed "TRP box") (Flockerzi and Nilius 2007). According to functional and recent structural data, the motif 3 is part of the S6 helix, and its residues essentially define the lower gate within the central ion-permeation path, whereas the TRP helix (motif 4) interacts with both the pre-S1 helix (motif 1) and the S4–S5 linker (motif 2). The pre-S1 helix and the TRP helix are flanked by large cytoplasmic domains. According to the channel structures, four identical TRP proteins form a tetrameric channel. The central ion-permeation path, which is formed by tetramerized transmembrane helices 5 and 6 and their connecting linker, the pore-loop, comprises an outer vestibule followed by two constriction sites. The outer site formed by the pore-loop defines the selectivity filter, the inner site comprising the distal part of the S6 helix (motif 3), the lower gate, which may close the ion-permeation path. In addition to homo-tetrameric channels, hetero-tetrameric channels composed of more than one type of TRP protein have been identified and characterized *in vitro* and *in vivo*. The hetero-tetrameric channels have different functional properties compared to the homo-tetrameric channels of either protein.

Ion Channel Properties

Most TRP channels do only poorly discriminate between monovalent and divalent cations, especially Na^+ and Ca^{2+}, and, accordingly, are dubbed "non-selective" cation channels. Only TRPV5 and TRPV6 form highly selective Ca^{2+} channels. In these two channels, the permeability to



TRP Channels, Fig. 1 (Top) Cartoon of a single TRP protein/channel subunit depicting pre-S1 helix, transmembrane helices 1 to 6, S4–S5 linker, pore-loop, distal S6, and TRP helix. The four amino-acid sequence motifs, pre-S1, S4–S5 linker, distal S6, and TRP helix, unique to almost all TRPs, are indicated. (Bottom) View from top on a tetrameric TRP channel

Ca^{2+}(P_{Ca}) is more than 100-fold higher than its permeability to Na^{+}(P_{Na}). A single negatively charged aspartic acid residue conserved in the selectivity filter of both channels is responsible for the high Ca^{2+}selectivity (Nilius and Flockerzi 2014; Vangeel and Voets 2019). TRPM4 and TRPM5 are members of a third subgroup, yielding currents carried by Na^{+} but not by Ca^{2+}. Ca^{2+} itself facilitates activation of some TRP channels by directly binding to Ca^{2+} recognition sites formed by the distal cytosolic domains of helices S2 and S3 (TRPM4, TRPM8, TRPC5) or an EF-hand-like structure in the amino-terminal domain of TRPA1. In addition, Ca^{2+} via binding to Ca^{2+} binding proteins like calmodulin modulates channel activity either as an activating (e.g. TRPV3) or inactivating modulator (TRPV5, TRPV6) (Nilius and Flockerzi 2014; Vangeel and Voets 2019).

Modes of Activation

The gating of many TRP channels critically depends on phospholipase C activation by G-protein-coupled receptors or receptor tyrosine kinases. Activation of PLC could be coupled to TRP channel activation via relief of phosphatidylinositol-4,5-bisphosphate (PIP_2)-mediated channel repression (TRPV1, TRPM7) and/or production of inositol-1,4,5-trisphosphate and diacylglycerol, which contribute to TRPC channel activation. Members of the TRPV subfamily are activated by a broad range of stimuli including heat (TRPV1), ligands such as capsaicin (TRPV1) and (endo)cannabinoids (TRPV1, TRPV2, TRPV4), osmolarity and cell volume (TRPV4), hydrolysis of PIP_2, and protons (TRPV1). The Ca^{2+}-selective TRPV5 and TRPV6 channels are also unique in that they represent the only channels within the TRP superfamily which are activated by low intracellular Ca^{2+}. TRPM8 can be activated by a drop in temperature below 26 °C or by agents, such as menthol and icilin, that evoke a cool sensation. Other channels activated by temperature drops are TRPC5, TRPA1, and TRPM3.

Three TRPM proteins, TRPM2, TRPM6, and TRPM7, are distinguished from other TRPs and other known ion channels, in that they consist of enzyme domains linked to the C-termini of the ion channel domains resulting in "chanzymes". TRPM6 and TRPM7 encode TRP proteins linked to functional atypical protein kinases. Currents through TRPM2 channels are activated by ADP-ribose and changes in the redox status within the cell.

Pharmacological Relevance

A clear limitation of studies on some TRP channels is the lack of specific channel agonists and blockers. Organic compounds (e.g., ruthenium red, econazole, miconazole, SKF 96365) and anorganic blockers (e.g., La^{3+}, Gd^{3+}) have been generally found to be of insufficient potency and specificity. The few exceptions include compounds such as capsaicin ($EC_{50} \sim 0.7$ μM) and resiniferatoxin ($EC_{50} \sim 0.04$ μM) as activators and capsazepine ($IC_{50} \sim 0.3$ μM) as blockers of the TRPV1 currents, the endogenous cannabinoid receptor agonist anandamide ($EC_{50} \geq 4.9$ μM) and related endocannabinoids as activators of TRPV1 and TRPV4 currents, and 4α-PDD, a phorbol derivative, as activator of TRPV4 currents ($EC_{50} \sim 0.2$ μM). Several newly discovered compounds proved to be very potent and specific agonists or antagonists for single TRP channels, such as the TRPC4/C5 activating compound englerin A ($EC_{50} \sim 8$ to 12 nM) and inhibiting compound Pico145 ($IC_{50} \sim 0.35$ nM to 1.3 nM).

Biological Relevance and Emerging Roles for Mammalian TRP Channels

While TRP proteins have initially become renowned as cellular and organismal sensors for a wide spectrum of chemical and physical cues, an increasing number of gene deletion approaches in animals and human diseases associated with mutations in TRP genes have made a strong case for a global (patho)physiological role of TRP channels extending well beyond the sensory nervous system. TRP channels control an exceptionally broad spectrum of homeostatic physiological functions such as maintenance of body

TRP Channels, Table 2 Human TRP channelopathies

Channel	Channelopathy	OMIM[a]
TRPA1	Familial episodic pain syndrome 1 (FEPS1)	#615040
TRPC3	Spinocerebellar ataxia 41 (SCA41)	#616410
TRPC6	Hereditary focal segmental glomerulosclerosis 2 (FSGS2)	#603965
TRPV3	Focal nonepidermolytic palmoplantar keratoderma-2 (FNEPPK2)	#616400
	Mutilating palmoplantar keratoderma with periorificial keratotic plaques (Olmsted syndrome, OLMS)	#614594
TRPV4	Brachyolmia type 3 (BCYM3)	#113500
	Spondylometaphyseal dysplasia, Kozlowski type	#184252
	Autosomal dominant metatropic dysplasia	#156530
	Distal hereditary motor neuronopathy type VIII (HMN8)	#600175
	Hereditary motor and sensory neuropathy type IIC (HMSN2C) (alternative title: Charcot-Marie-Tooth neuropathy type 2C)	#606071
	Scapuloperoneal spinal muscular atrophy (SPSMA)	#181405
TRPV6	Transient neonatal hyperparathyroidism (HRPTTN)	#618188
TRPM1	Congenital stationary night blindness-1C (CSNB1C)	# 613216
TRPM4	Progressive familial heart block type IB (PFHB1B)	#604559
TRPM6	Hypomagnesemia with secondary hypocalcemia (HOMG1)	# 602014
TRPM7	Macrothrombocytopenia and atrial fibrillation	605692.0002
	Amyotrophic lateral sclerosis – parkinsonism/dementia complex 1	#105500
TRPML1	Mucolipidosis type IV (ML4)	#252650
TRPP2	Polycystic kidney disease-2 with or without polycystic liver disease (PKD2)	#613095

[a]Online Mendelian Inheritance in Man database (https://www.ncbi.nlm.nih.gov/omim)

temperature, transmitter release from neurons, mineral and trace element homeostasis, energy metabolism, and reproduction. Almost 20 hereditary human diseases in areas as diverse as neurology, cardiology, hematology, pulmonology, nephrology, dermatology, and urology are caused by mutations in 12 TRP genes (Table 2). However, a detailed understanding of the underlying pathophysiology is still missing, thereby obviating targeted therapeutic strategies. It still remains a mystery why the same gene mutations can cause completely different phenotypes and why TRP channel mutations sometimes have a low penetrance and no overt phenotype, while they entail lethal phenotypes in other instances. Accumulating evidence links TRP channels to a wide array of human diseases beyond the hereditary TRP channelopathies, and some TRPs, especially TRPM8 and TRPV6, appear to be upregulated in certain human cancers, for example, of the prostate and breast. Accordingly, TRP proteins are not only most appealing therapeutic targets, but, in addition as "oncochannels", they may represent prognostic markers for cancer diseases.

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True ChE

► Cholinesterases

Tuberculosis

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Synonyms

Consumption; Phthisis; White plague

Definition

Tuberculosis is the most frequent infectious disease in humans worldwide commonly caused by bacteria of the *Mycobacterium tuberculosis* complex, generally affecting the lungs but potentially all parts of the body. Closely related members of the *Mycobacterium tuberculosis* complex cause similar infectious disease in animals such as *M. bovis* and *M. caprae* in ruminants and wild boars. Following the primary infection, a latent phase without any clinical symptoms precedes the reactivation phase, which is characterized by chronic cough, fever, weight loss, and lung tissue destruction. WHO estimations suggest that one fourth of the world population is latently infected with *Mycobacterium tuberculosis*. However, only 5–10% of the latently infected develop active tuberculosis during their lifetime (WHO 2020). *Mycobacterium tuberculosis* is transmitted via infectious aerosols generated by coughing from the respiratory tract of active tuberculosis patients. If untreated, half of all patients succumb to the disease. Prolonged treatment with combinations of different antibiotics has been proven a successful cure. However, recent multi-drug- and extensively drug-resistant isolates display a new threat (WHO 2020).

Basic Mechanisms

The Disease

According to the World Health Organization's estimates, approximately 10 million individuals develop tuberculosis annually. With 1.4 million fatalities in 2018, infections with *Mycobacterium tuberculosis* complex strains are still the most common cause of death from infectious diseases worldwide (WHO 2020). Highest incidences (ca. 300–500 cases per 100,000 population) occur in countries of South Africa, Southeast Asia, and Mongolia (WHO 2020). Patients infected with drug-susceptible strains of *Mycobacterium tuberculosis* can be successfully treated with a four-drug regime of antibiotics. On top of this pandemic, a public health crisis regarding cases of multi-drug- and extensively drug-resistant strains evolved. Nearly half a million patients were infected with *Mycobacterium tuberculosis* strains that are resistant to at least two of the first-line drugs. In countries of Central Asia, Russia, and East Europe, over 25% of new tuberculosis cases are multi-drug- or extensively drug-resistant (WHO 2020). Unrecognized or late diagnosis of multi-drug-resistant tuberculosis often leads to treatment failures. Treatment success rates in those patients are as low as 54%.

The Agent

Firstly discovered by Robert Koch in 1882 as causative agent of tuberculosis, *Mycobacterium tuberculosis* has subsequently been characterized as an aerobic and slow-growing mycobacterium and member of the Actinobacteria family. As facultative intracellular pathogen, it can survive and grow within host macrophages. Researchers estimate that humans acquired tuberculosis during evolution around 6000–9000 years ago since evidence of the disease has been found in human remains of the Neolithic era and in Egyptian mummies (Hershkovitz et al. 2008; Zink et al. 2003). Animal species crossed the species barrier to ruminants upon domestication in Neolithic times. *Mycobacterium tuberculosis* is a Gram-positive bacterium due to a thin peptidoglycan layer between an inner and an outer membrane. The hydrophobic cell wall and outer membrane, consisting of mycolic acids and wax esters, classify mycobacteria as acid-fast. *Mycobacterium tuberculosis* can be visualized using Ziehl-Neelsen's carbol fuchsin staining and remain stained after de-staining by acids and alcohol due to their thick and waxy lipid layers. This

acid-fast stain still represents the most commonly used method for detection of *Mycobacterium tuberculosis* in patient samples such as sputa and monitoring treatment success. Although *Mycobacterium tuberculosis* has an inner and outer membrane with a peptidoglycan cell wall layer in between, it phylogenetically belongs to the Actinobacteria.

The Control

After inhalation of contagious aerosol droplets coughed out by an active tuberculosis patient, *Mycobacterium tuberculosis* enters the upper respiratory tract and encounters pulmonary surfactant and deposits on the lung epithelial barrier, where it can become decorated by surfactant protein A and other humoral factors such as the complement component C3b. Alveolar macrophages, patrolling the alveolar surface, phagocytose opsonized mycobacteria and carry them into deeper lung tissues, thereby initiating the primary infectious focus. Within macrophages, *Mycobacterium tuberculosis* grows, eventually leading to necrotic host cell death and the release of a multiplicity of the initial bacterial inoculum. Concomitantly, additional immune cells become attracted to the site of infection through both danger signals of the dying/dead host cell and chemokine release induced by microbe-associated molecular patterns of *Mycobacterium tuberculosis* in epithelial cells and macrophages. Among the first infiltrating cells are myeloid-derived innate immune cells, such as neutrophilic granulocytes, monocytes, and macrophages, which can serve as host cells for *Mycobacterium tuberculosis*. Failure of these cells to kill the mycobacteria in a process termed early clearance can lead to primary tuberculosis as seen in small children, causing miliary tuberculosis and meningitis with high mortality rates. However, concomitant priming of CD4 and CD8 T cells in the draining lymph nodes causes onset of acquired immunity and production of interferon gamma and tumor necrosis factor alpha. These cytokines activate macrophages to unfold their microbicidal armamentarium rendering them capable to control mycobacterial growth, leading to latent infection in more than 90% of the infected. Histologically, this stage is characterized by the formation of a highly organized multicellular structure called the granuloma. The granuloma consists of a necrotic and lipid-rich core, most probably representing debris of former host cells, in which extracellular mycobacteria are located. The necrotic core is surrounded by multiple layers of immune cell populations (from inside to outside): (1) infected macrophages, foam cells, and giant cells; (2) uninfected macrophages, few neutrophils, and dendritic cells; (3) few natural killer cells and T and B cells (Ramakrishnan 2012). The structure is enclosed by a fibrous cuff consisting of collagen. With the help of antigen-specific T helper 1 cells secreting interferon-γ, host macrophages rather succumb to programmed apoptotic than necrotic cell death. Upon establishment of a hypoxic and nutrient-poor environment within the granuloma, *Mycobacterium tuberculosis* enters a non-replicating, metabolically inactive, dormant state, resulting in immune-controlled latent infection in absence of clinical symptoms (Gengenbacher and Kaufmann 2012). This latency can last for decades and in most cases infection can go unrecognized. However, the granuloma harboring dormant mycobacteria bears the risk of reactivation, primarily through immunocompromising conditions, which can lead to reactivation in 5–10% of all latently infected individuals at least once in their lifetime.

The Exacerbation

The exact immunological, cellular, and molecular mechanisms underlying the event when a granuloma is not able to restrain mycobacterial infection, reactivation, proliferation, and disease exacerbation anymore are still unknown. Genetic as well as environmental factors seem to play important roles in disease progression. Immunocompromising conditions resulting from, e.g., HIV coinfection, immunosuppressive therapies (cancer, autoimmune disease, organ transplantation), type 2 diabetes, age-associated immune senescence, smoking, malnutrition, or iron overload can disrupt maintenance of an intact granuloma structure and cause reactivation of dormant *Mycobacterium tuberculosis*. Mycobacterial proliferation kicks off inflammation, attracting immune cells such as neutrophils, monocytes, and macrophages from the bloodstream to the site of infection. Neutrophils are highly specialized, toxic innate immune cells equipped with

an armamentarium of many different damaging effector molecules. However, after uptake of *Mycobacterium tuberculosis*, neutrophils quickly succumb to a necrotic cell death, which is dependent on one of the toxic mechanisms exhibited by neutrophils, namely, the generation of reactive oxygen species (Corleis et al. 2012). Thus, with the help of several virulence factors, *Mycobacterium tuberculosis* turns the neutrophil's own killing machinery against itself. Necrotic cell death of neutrophils releases other highly tissue-damaging molecules, such as matrix metalloproteinases that degrade the extracellular matrix, reactive oxygen-generating enzymes, lipases, phosphatases, and membrane pore-forming proteins. Release of the cytokine interleukin 8 and neutrophil-derived, danger-associated molecules such as S100 alarmins attract further neutrophils. Neutrophil accumulation causes tissue damage and a massive influx of even more neutrophils, monocytes, and macrophages. Within macrophages that clean up infected and necrotic neutrophils, *Mycobacterium tuberculosis* can grow, before it eventually also induces necrotic cell death in these cells (Dallenga et al. 2017). These patho-dynamics result in establishment of a vicious circle of necrotic host cell death interspersed with periods of mycobacterial replication, lung tissue destruction, and, ultimately, coughing up of infectious particles for disease transmission (Dallenga and Schaible 2016).

Pharmacological Intervention

Classical Treatment

Classical treatment with antibiotics that have been shown to be effective against mycobacteria usually cures drug-susceptible tuberculosis after 2–6 months of treatment. The WHO recommends an intensive treatment phase of 2 months with daily administration of the four antibiotics rifampicin, isoniazid, pyrazinamide, and ethambutol followed by a continuation phase for 4 months with rifampicin and isoniazid (WHO 2020). However, many patients enter treatment only after prolonged periods of disease before being diagnosed. The chronic infectious process can have already led to severe lung tissue destruction and long-term sequelae, such as cavities and impaired lung function. Antibiotic treatment is only targeting the bacterium, which usually also leads to quick reduction of inflammation. Some patients however, although diagnosed as cured and sputum negative for *Mycobacterium tuberculosis*, do not show improvement of lung function and resolution of inflammation. Other patients show no reduction in mycobacterial burden over months of treatment. Those are often mis-diagnosed for susceptible tuberculosis but are infected with multi-drug- or extensively drug-resistant tuberculosis.

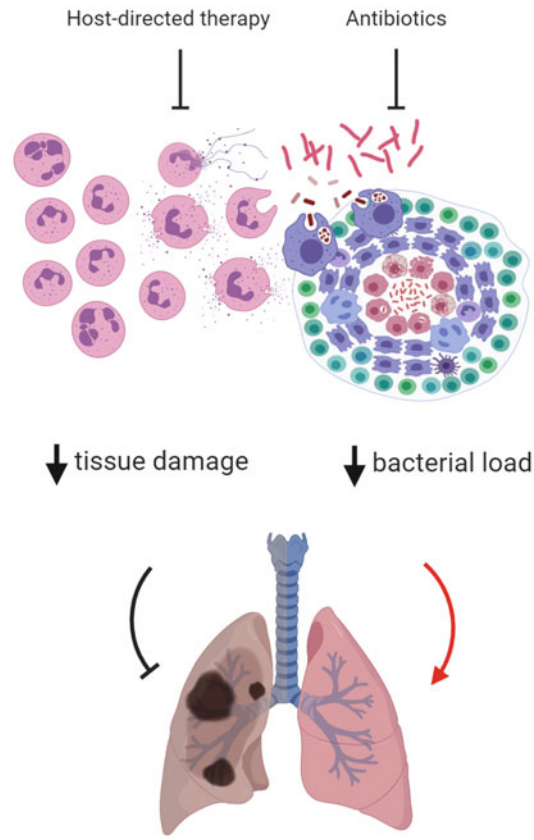
Multi-drug- and Extensively Drug-Resistant Tuberculosis

Successful treatment of multi-drug- or extensively drug-resistant tuberculosis displays a big challenge worldwide. Tuberculosis is considered multi-drug-resistant when the infecting mycobacterial strain is resistant to at least two of the abovementioned first-line drugs, usually to rifampicin and isoniazid. Additional resistances against two second-line drugs, including an injectable one, such as amikacin, define extensively drug-resistant tuberculosis. *Mycobacterium tuberculosis* has been shown to quickly acquire resistances even to newly developed and last resort antibiotics (Hoffmann et al. 2016). Combinations of second- and third-line antibiotics such as moxifloxacin, ethionamide, and rifabutin over a time course of 24 months are used to treat multi-drug- and extensively drug-resistant tuberculosis. However treatment success rates of multi-drug- and extensively drug-resistant tuberculosis are as low as 54% and 30%, respectively (WHO 2020). Moreover, second- and third-line drugs, especially upon long-term usage, display severe side effects such as irreversible hearing loss, burning sensation in the extremities, peripheral neuropathy, renal and liver toxicity, depression, and psychosis (Torun et al. 2005). Suffering from these side effects together with the prolonged period of treatment often causes patients to stop taking their medicines. Reduced patient compliance and withdrawal from the anti-tuberculosis regimen contribute to development of extensively or even totally drug-resistant *Mycobacterium tuberculosis* strains that are considered to be almost untreatable (Parida et al. 2015). The threat of increasing cases of multi-drug- and extensively drug-resistant

tuberculosis cases together with the occurrence of long-term sequelae, lung tissue scaring and cavitation, and impaired lung function requires alternative therapies adjunct to and supporting antibiotic treatment, which only targets the infectious agent.

Host-Directed Therapy

Host-directed therapies that target host immune mechanisms represent such supplemental strategies. Host cell necrosis, exacerbated inflammation, and lung tissue destruction characterize active tuberculosis, causing strong coughing and release of contagious aerosols and even blood. Interestingly, *Mycobacterium tuberculosis* produces a molecule, sulfolipid-1, that actively stimulates the lung-innervating neurons to induce coughing (Ruhl et al. 2020). Among others, this immunopathological condition is thought to be driven by the massive influx of neutrophils to the site of mycobacterial infection, which has been observed in histological examinations of the lungs of active tuberculosis patients (Dallenga et al. 2018). Indeed, increased numbers of infiltrating neutrophils exacerbate tuberculosis but generally also other severe respiratory diseases like influenza virus and SARS-CoV-2 infections (Camp and Jonsson 2017; Kimmey et al. 2015; Laforge et al. 2020). Interfering with neutrophil-mediated pathomechanisms, such as attraction, release of attractants and toxic effectors, and induction of necrotic cell death may represent a promising strategy to ameliorate severe forms of active tuberculosis, mitigate tissue destruction, and reduce long-term sequelae during antibiotic treatment. For instance, when neutrophils were depleted or their attraction to the site of infection was interfered with in experimental mouse models of tuberculosis, bacterial burden, pathology, and death rates were reduced (Vilaplana et al. 2013; Yeremeev et al. 2015). As mentioned above, neutrophils infected with *Mycobacterium tuberculosis* quickly succumb to a necrotic cell death. When this necrotic cell death was prevented by inhibition of one of the enzymes that produces reactive oxygen species, namely, myeloperoxidase, macrophages subsequently engulfing *Mycobacterium tuberculosis*-infected neutrophils were able to control mycobacterial growth (Dallenga et al. 2017). In this *in vitro* study with human cells, release of tissue-damaging molecules was prevented, while simultaneously other immune cells controlled mycobacterial proliferation. Biomarkers, defining disease stage and pathological sequelae, can help to stratify tuberculosis patients for individualized host-directed therapies targeting specific disease-associated mechanisms. Neutrophil-targeting host-directed therapies could be directed by the presence of enhanced neutrophil-associated mRNA signatures or mediators such as interleukin 8, S100, or prostaglandins. In the future, severe tuberculosis patients may benefit from tailored biomarker-educated host-directed therapies in support of antibiotic treatment (Fig. 1).



Tuberculosis, Fig. 1 Adjunct host-directed therapy in tuberculosis. Combined antibiotics and host-directed therapies, e.g., targeting neutrophils, reduce both lung tissue destruction and bacterial burden, resulting in improved disease outcomes and long-term sequelae. (Created with BioRender.com)

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24h-Rhythms

► Circadian Rhythms

Tyrosine Kinases

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Synonyms

Cytoplasmic tyrosine kinases; Hydroxyaryl-protein kinase; Nonreceptor tyrosine kinases; Protein tyrosine kinases; Tyrosylprotein kinase

Definition

Protein tyrosine kinases (PTKs) are enzymes (EC 2.7.1.112) that catalyze the transfer of the γ-phosphate group of ATP to tyrosine residues of protein substrates. The activity of PTKs is controlled in a complex manner by posttranslational modifications and by inter- and intramolecular complex formations.

Basic Characteristics

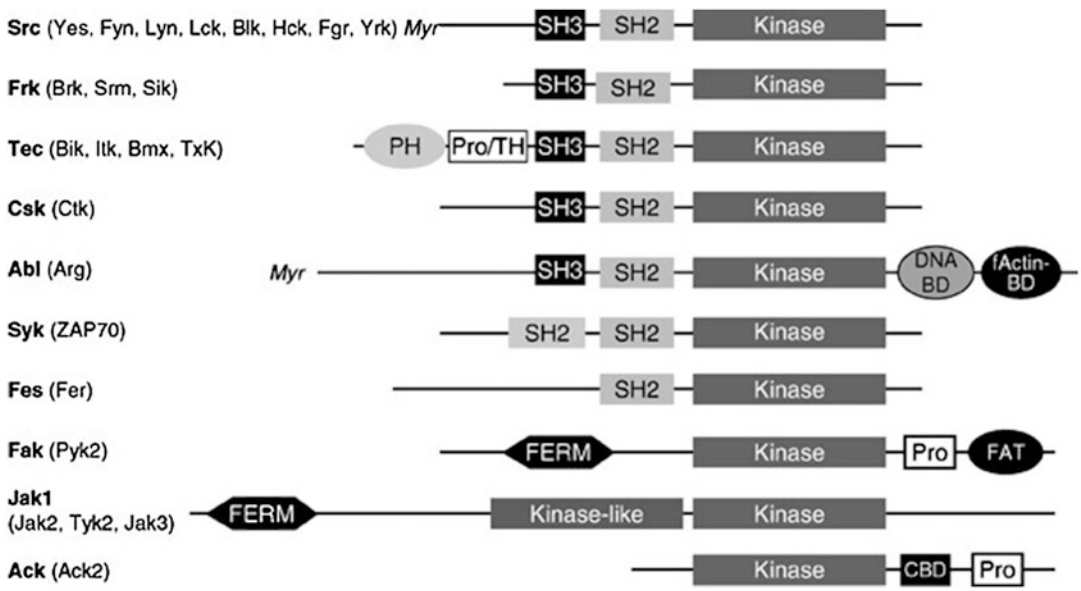
PTKs have been implicated in the regulation of a variety of biological processes such as cell proliferation, migration, differentiation, and survival. They have been demonstrated to play significant roles in the development of many disease states, including immunodeficiency, ► atherosclerosis, psoriasis, osteoporosis, diabetes, inflammatory diseases, and cancer. Currently, PTK inhibitors are clinically used for the treatment of specific hematological cancers and inflammatory diseases, such as rheumatoid arthritis.

PTKs can be subdivided into two large families, receptor tyrosine kinases (RTKs) and nonreceptor tyrosine kinases. The human genome encodes for a total of 90 tyrosine kinases of which 32 are nonreceptor PTKs that can be placed in ten subfamilies (Fig. 1). All nonreceptor PTKs share a common kinase domain and usually contain several additional domains that mediate interactions with protein-binding partners, membrane lipids, or DNA (Table 1). These interactions may affect cellular localization and the activation status of the kinase or attract substrate proteins for phosphorylation reactions.

c-Src

c-Src was the first cellular homologue of a viral oncoprotein (v-Src from the Rous sarcoma virus) that was discovered. It is involved in mitogenic signaling from many types of transmembrane receptors and has been implicated in a variety of cancers. c-Src and the Src-like kinases Fyn and Yes are expressed in most tissues and are at least partially redundant in their function. Hck, Fgr, and Blk are primarily found in hematopoietic cells, whereas Lyn and Lck are also expressed in neuronal cells.

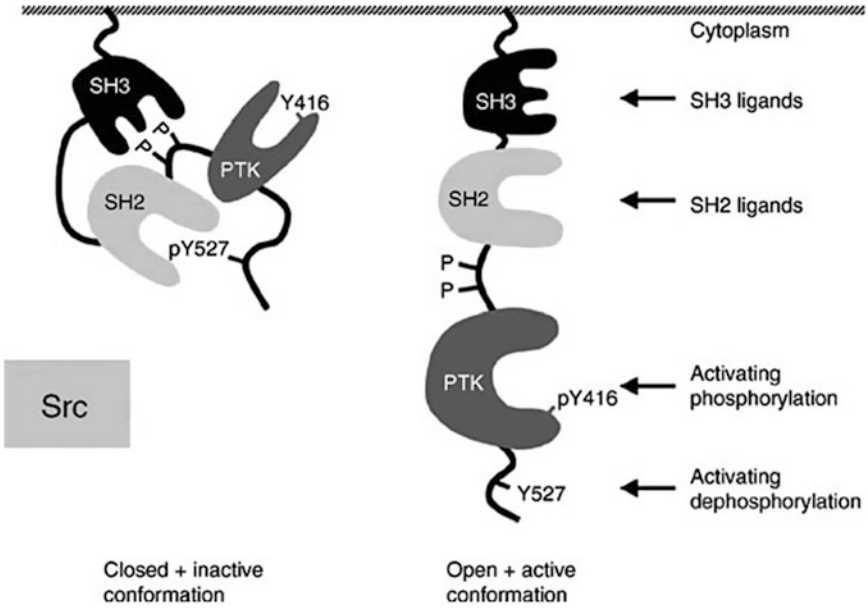
The inactive, closed conformation of Src is maintained by intramolecular interactions of the SH2 and SH3 domains (Fig. 2). The N-terminal SH3 domain binds to a proline-rich sequence in the linker region between the SH2 and the kinase domain. In addition, the SH2 domain binds to a phosphorylated tyrosine residue (Y527 in chicken, Y530 in human) in the C-terminal part of the protein. The kinase executing this phosphorylation is called Csk (C-terminal c-Src kinase) and is member of a distinct PTK family (Fig. 1). Both intracellular interactions together repress Src kinase activity by blocking access to the active site. Src can be activated by



Tyrosine Kinases, Fig. 1 Tyrosine kinases

Tyrosine Kinases, Table 1 Tyrosine kinases

Domain		Function
CBD	Cdc42-binding domain	Binding to the small G protein Cdc42
DNA-BD	DNA-binding domain	Binding to DNA
fActin-BD	f-actin-binding domain	Binding to F-actin
FAT	Focal adhesion targeting domain	Binding to focal adhesion complexes
FERM	4.1/ezrin/radixin/moesin domain	Binding to cytoplasmic regions of transmembrane proteins
Myr	Myristoylation site	Tethering to membranes
PH	Pleckstrin homology domain	Binding to membrane phospholipids, such as phosphoinositides
Pro	Prolin-rich sequences	Binding to SH 3 domains
PTB	Phosphotyrosine binding domain	Binding to phosphorylated tyrosine residues
SH1	Src homology 1 domain, kinase domain	Kinase activity
SH2	Src homology 2 domain	Binding to phosphorylated tyrosine residues
SH3	Src homology 3 domain	Binding to prolin-rich sequences
TH	Tec homology domain	SH3-binding prolin-rich sequences and Zn^{2+}-binding motif



Tyrosine Kinases, Fig. 2 Activation mechanism of Src

dephosphorylation of the inhibitory tyrosine residue and by intermolecular interactions with SH2 and SH3 binding partners. A variety of cytosolic and receptor-type protein tyrosine phosphatases (PTPs), such as PTPα, PTP1B, and SHP-1/2, has been shown to dephosphorylate pY527 and subsequently activate c-Src. Among the SH2 ligands that can activate Src are autophosphorylated RTKs (e.g., epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors) and nonreceptor PTKs (e.g., Fak and Pyk2) as well as tyrosine phosphorylated adaptor proteins (e.g., Shc). For full activation of Src a trans-autophosphorylation of a conserved tyrosine

residue in the activation loop (Y416) has to occur. This model is supported by the elevated Src activity in transformed cells with increased PTP activity; a Src mutant with truncated C-terminus lacking the inhibitory phosphotyrosine residue that has been found in human colorectal cancers and is constitutively activated, and v-Src, which in addition to several point mutations lacks a large part of the C-terminal domain, have transforming potential.

Among the substrates of Src are other nonreceptor PTKs (e.g., Fak, Syk, and Tec kinases), RTKs (e.g., EGF and PDGF receptors), phospholipase Cγ (PLCγ), PI3-kinase, phosphatases (e.g., SHP-2 and PP2A), and adaptor (e.g., Shc and Cbl) as well as focal adhesion proteins (e.g., paxillin, p130 ^{Cas}, and tensin). Src-mediated phosphorylation either modulates enzymatic activity of target proteins or creates docking sites for SH2 or PTB domain-containing proteins promoting the assembly of multimeric protein complexes that function in cellular signaling.

Given its involvement in many receptor-mediated signaling pathways, Src was thought to be an important regulator of cell proliferation, migration, and adhesion. However, the most striking phenotype of c-Src-deficient mice is an osteopetrosis suggesting a role for c-Src in bone remodeling and a compensation by alternative Src family members in other organs. Indeed, combined deletion of c-Src, Yes, and Fyn in mice results in a lethal phenotype.

c-Abl

c-Abl was first identified as the cellular homologue of the transforming gene product of the Abelson murine leukemia virus (v-Abl) and found to encode a nonreceptor PTK. Mammalian c-Abl is expressed ubiquitously and in most cells is primarily localized in the nucleus where it has a role in DNA damage-induced apoptosis.

c-Abl is activated by ionizing radiation in a manner dependent on phosphorylation by ATM (ataxia telangiectasia-mutated), a nuclear protein serine/threonine kinase. c-Abl kinase activity in G0 and G1 phase of the cell cycle is repressed by binding of Rb (retinoblastoma protein) to the activation loop, which is released during S-phase when Rb becomes hyperphosphorylated by cyclin-dependent kinases. Furthermore, c-Abl is negatively controlled by intramolecular SH3 domain interactions and by SH3-binding proteins, such as Pag/MSP23 (human proliferation-associated gene/macrophage 23-kD stress protein). More recently, a role of intramolecular binding of the N-terminal myristoyl residue to a corresponding pocket in the so-called C-lobe of the kinase has been described.

Nuclear substrates of c-Abl include DNA-PK (DNA-dependent protein kinase, an enzyme critical for DNA repair), Rad51 (a homologue of bacterial RecA involved in recombination/repair by catalyzing strand exchange between homologous DNAs), the tyrosine phosphatase SHPTP1, and the p85 subunit of PI3-kinase, negatively regulating their respective activity. In contrast, c-Abl activates JNK (c-Jun N-terminal kinase) and p38 mitogen-activated protein kinases (MAPKs). c-Abl also functions in the cytoplasm, where it is involved in PDGF-induced motility responses and cell adhesion.

In chronic myelogenous leukemia (CML) as well as in a subset of acute lymphoblastic leukemia (ALL), Bcr-Abl, a fusion protein of c-Abl and the breakpoint cluster region (Bcr), is expressed in the cytosol of leukemic cells. This fusion protein forms homo-oligomeric complexes that display elevated kinase activity and is the causative molecular abnormality in CML and certain ALL. The transforming effect of Bcr-Abl is mediated by numerous downstream signaling pathways, including ▶ protein kinase C (PKC), Ras-Raf-ERK MAPK, JAK-STAT, and PI3-kinase pathways.

Fak

Fak (focal adhesion kinase) is expressed in most tissues and is evolutionary conserved across species. It is activated by integrin clustering and by stimulation of several G protein-coupled receptors and RTKs. Fak is associated with focal adhesions and regulates cell spreading and migration. The kinase is essential for embryonic development since the homozygote Fak knockout is embryonic lethal. Pyk2 (proline-rich tyrosine kinase 2), the second member of the Fak kinase family, has a more restricted expression pattern (primarily

neuronal and hematopoietic cells) and does not localize to focal adhesions.

An early step in Fak activation is the autophosphorylation of a tyrosine residue (Y397) proximal to the kinase domain. Phosphorylated Y397 is a high affinity ligand for the SH2 domain of Src, thereby recruiting Src kinases and stimulating their catalytic activity. In a second step, several other tyrosine residues in Fak are phosphorylated, either by Fak itself or by recruited Src. As a consequence, Fak kinase activity is further increased or docking sites for SH2 domain-containing proteins are created, e.g., for Grb2-Sos complexes that link Fak to the Ras-Raf-► MAPK cascade. N-terminal sequences containing the FERM domain anchor Fak to integrins or RTKs, and the C-terminal FAT domain mediates binding to cellular focal adhesions. Among the substrates of the Fak/Scr complex are the adaptor proteins paxillin and p130 ^{Cas} and the focal adhesion-associated protein tensin that bind to C-terminal sequences of Fak and, after their phosphorylation, promote the assembly of signaling complexes at discrete sites within cells.

Fak kinases could be modulators of some aspects of human cancers and may also contribute to the development of vascular diseases involving hyperproliferation and migration of vascular smooth muscle cells.

Jak

Jak1 and 2 were identified, among others, by PCR using degenerate oligonucleotides spanning the conserved kinase domain of Src and therefore initially named Jak, for "Just another kinase." When full-length clones were isolated, it was recognized that they differ markedly from other PTKs by the presence of an additional (pseudo)-kinase domain of unknown function. To denote this unique feature, they were renamed as "Janus kinases" in reference to the ancient two-faced Roman god. Jak1, Jak2, and Tyk2 are ubiquitously expressed, whereas Jak3 is predominantly found in hematopoietic cells. Jak family PTKs mediate signaling downstream of a wide array of receptors, but the most commonly known family associated with Jak activation are cytokine receptors. In response to ligand stimulation, cytokine receptors oligomerize, and subsequently recruited or constitutively bound Jak kinases are activated and phosphorylate the receptors. Some of the receptors' phosphotyrosine residues subsequently bind to SH2 domains of STATs (signal transducers and activators of transcription), which are then phosphorylated by Jaks on a C-terminal tyrosine residue. This leads to STAT oligomerization through a reciprocal interaction between SH2 domains and phosphotyrosines. Dimeric STATs translocate to the nucleus where they initiate transcription of target genes. Alternatively, STATs can be activated by Src kinases that are recruited to Jak-phosphorylated cytokine receptors via their SH2 domain. Jak kinase signaling is negatively regulated by PTPs and by SOCS (suppressors of cytokine signaling) proteins that inhibit Jaks by binding to the activation loop and by targeting the kinases for protein degradation.

STAT 3 and 5 are overexpressed and/or overactivated in several human malignancies, such as breast, non-small cell lung cancer, and head and neck cancer. An aberrant activation of Jak kinases by fusion with the TEL transcription factor and subsequent constitutive dimerization has been observed in T-cell acute lymphocytic leukemia.

Tec

Tec family kinases participate in signal transduction in response to many types of extracellular stimuli that are transmitted by growth factor receptors, cytokine receptors, G protein-coupled receptors, integrins, and, most importantly, B-cell receptors. Tec kinases are involved in the regulation of growth, differentiation, apoptosis, and cell motility. They are primarily found in hematopoietic lineages, but some family members (Btk, Etk/Bmx) also have a broader expression pattern. The defining feature of Tec family kinases is the presence of a PH domain at their N-terminus. The PH domain has a broad binding capacity ranging from lipid products of PI3-kinases, heterotrimeric G protein subunits ($\beta\gamma$ as well as $G\alpha_q$ and $G\alpha_{12}$), and PKC isoforms (βI and δ) to STATs, and other PTKs, like Fak. These interactions may either be involved in Tec activation (phospholipids and G proteins) or recruit potential substrates of Tec kinases (PKC and STATs).

The current understanding of the activation of Tec kinases fits into a two-step model. In the first step an intramolecular interaction between the SH3 domain and a proline-rich region in the TH domain is disrupted by binding of the PH domain to phosphoinositides, G protein subunits, or the FERM domain of Fak. These interactions lead to conformational changes of Tec and translocation to the cytoplasmic membrane where, in a second step, Src kinases phosphorylate a conserved tyrosine residue in the catalytic domain thereby increasing Tec kinase activity. Autophosphorylation of a tyrosine residue in the SH3 domain further prevents the inhibitory intramolecular interaction resulting in a robust Tec kinase activation.

Among the substrates and downstream effectors of Tec kinases are PLCγ2 and PKCβI resulting in a sustained calcium influx and activation of MAPKs. The interaction with the GDP/GTP exchange factor Vav can potentially activate Rac/Cdc42/Rho pathways, which can modulate actin cytoskeleton dynamics and lead to JNK and p38 MAPK activation potentially inducing apoptosis. Paradoxically, Tec kinases may also trigger antiapoptotic signals by stimulating PI3-kinase and Akt and promote proliferation by activating STATs.

Naturally occurring mutations of Bruton's tyrosine kinase (Btk) were identified in human immunodeficiency diseases and X-linked agammaglobulinemia, where a lack of mature circulating B cells and immunoglobulins is observed, supporting a central role for Btk in B-cell biology. In contrast, the knockout of Itk results in a lack of mature T cells and defects in T-cell receptor signaling. Furthermore, Btk and Etk/Bmx are able to complement a weakly oncogenic Src in transformation of hepatocytes and fibroblasts suggesting their participation in anchorage-independent growth and development of cancer.

Syk

The PTKs Syk and ZAP-70 are early intermediates in the transduction of signals from immune receptors, including the B- and T-cell receptors for antigen, activating natural killer-cell receptors, the mast cell and basophil receptor for IgE, and the widely distributed receptors for the Fc portion of IgG. Immune receptors control checkpoints in lymphocyte development and serve to integrate the responses of innate and acquired immunity.

The current model proposes that upon engagement of immune receptors Src-family kinases are recruited that phosphorylate tyrosine residues in specific regions of the receptors, the immunoreceptor tyrosine-based activation motifs (ITAMs). These phosphotyrosines serve as docking sites for the SH2 domains of Syk and ZAP-70 that subsequently autophosphorylate and generate binding sites for SH2 domain containing proteins, like PLCγ, Vav, and the adaptor protein Cbl. Furthermore, Syk and ZAP-70 phosphorylate a number of cytosolic and transmembrane linker proteins, such as SLP-76 (SH2-containing leukocyte protein of 76 kD), LAT (transmembrane linker for activation of T cells), TRIMM (T-cell receptor-interacting molecule), and SIT (SHP2-interacting transmembrane adaptor protein) that function as scaffolds to localize and assemble signaling complexes. Syk has also been implicated in the activation of Btk. In addition to be a major player in immune receptor signaling, Syk has a role in the "inside-out" integrin activation signal that is necessary for fibrinogen binding and subsequent aggregation of platelets during hemostasis.

Drugs

PTKs have been shown to play significant roles in the development of many disease states, including immunodeficiency, atherosclerosis, psoriasis, osteoporosis, diabetes, inflammatory diseases, and cancer. Therefore, development of PTK inhibitors has been in the focus of many pharmaceutical companies. While the majority of these inhibitors are directed against RTKs, the poster child for targeted therapies in oncology is imatinib (*Glivec* ™, *Gleevec* ™) that potently inhibits the activity of the nonreceptor PTK c-Abl. Imatinib is a phenylamino-pyrimidine that competes for binding of ATP to the catalytic domain and stabilizes an inactive conformation of the kinase domain preventing activation of c-Abl. In CML as well as in a subset of ALL Bcr-Abl fusion proteins with elevated kinase activity are the causative molecular abnormalities. In clinical trials

with Bcr-Abl-positive CML patients, once-daily oral doses of imatinib led to impressive overall response rates (ORR) of >90% with a well-manageable adverse event profile resulting in the approval by the US Food and Drug Administration (FDA) in May 2001.

However, as with most anticancer treatments, resistance to imatinib therapy was observed in patients that were either irresponsive from the beginning or relapsed after initial successful treatment. Key mechanisms for this resistance are Bcr-Abl gene amplifications and mutations in the kinase domain that reduce the affinity for imatinib. Second-generation, orally available c-Abl inhibitors, such as nilotinib (*Tasigna* ™), dasatinib (*Sprycel* ™), or bosutinib (*Bosulif* ™), are more potent against Bcr-Abl and active against several imatinib resistance conferring mutations. An exception is the T351I variant that is targeted by third-generation Bcr-Abl inhibitors, like ponatinib (*Iclusig* ™). A typical consequence of broader activity against kinase domain mutants is the less stringent selectivity profile of these inhibitors, and indeed, many of the imatinib followers bind to off-target kinases, such as Src, Btk, PDGF receptor, c-Kit, or VEGF receptor 2, which on one hand may increase their therapeutic potential in CML and other cancer types but on the other hand could also involve safety liabilities. Asciminib that is still in clinical development has a completely different mechanism of action: this compound targets the myristate pocket of Bcr-Abl and is therefore active against all known kinase domain mutations that confer resistance to imatinib.

Meanwhile, imatinib is also approved for the treatment of gastrointestinal stroma tumors (GIST), dermatofibrosarcoma protuberans (DFSP), myelodysplastic/myeloproliferative diseases (MDS/MPD), aggressive systemic mastocytosis (ASM), and hypereosinophilic syndrome/chronic eosinophilic leukemia (HES/CEL). The clinical activity of imatinib in GIST is based on its ability to inhibit the RTK c-Kit, which is frequently mutated in this disease. Other indications, such as DFSP, HES/CEL, and a certain proportion of CML, involve aberrant PDGF receptor signaling that is blocked by imatinib.

Since Btk is a key component of B-cell receptor signaling and has an important role in regulating proliferation and survival of B cells, Btk inhibitors have been explored to treat B-cell malignancies, such as chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), Waldenström macroglobulinemia (WM), marginal zone lymphoma (MZL), and diffuse large B-cell lymphoma (DLBCL). Ibrutinib (*Imbruvica* ™) is an irreversible inhibitor of Btk covalently binding to cysteine 481 and was the first drug against this PTK approved by the FDA in 2013 for the treatment of MCL based on impressive ORRs of 68–72%. Meanwhile, it is also used for the treatment of CLL, WM, and MZL. While ibrutinib is already relatively selective, only inhibiting EGF receptor, Itk, Her2, and Jak3 at 11–32-fold higher concentrations than Btk and the clinical adverse event profile is considered as manageable, more selective inhibitors like acalabrutinib (*Calquence* ™) and zanubrutinib (*Brukinsa* ™) are in development and have received first approvals in MCL based on further improved ORRs of >80%. The promise of higher kinase inhibitor selectivity is a better safety profile requiring less dose reductions and allowing more sustained target inhibition, potentially resulting in improved clinical activity.

General concerns about the irreversible mode of action and first reports about acquired resistance to ibrutinib and other covalent Btk inhibitors through C481S mutations triggered the development of reversible, ATP-competitive Btk inhibitors, like LOXO-305 and ARQ 531, that have shown encouraging signs of clinical activity in patients with refractory B-cell malignancies in phase I studies.

Finally, Btk inhibitors may have potential outside of oncology in diseases like rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and multiple sclerosis. There are several clinical studies ongoing, for instance, exploring the highly selective and presumably safer Btk inhibitors evobrutinib and tolebrutinib in these non-oncology indications.

Jak family PTKs are essential for the signaling cascade downstream of type I and II cytokine receptors, and Jak-dependent cytokines are major contributors to immunopathology. The first Jak inhibitor that gained approval by the FDA in 2011 was ruxolitinib (*Jakavi* ™) for

the treatment of myelofibrosis that is associated with an activating mutation in Jak2. Meanwhile, the Jak2/Flt3/Ret inhibitor fedratinib (*Inrebic* ™) is licensed for the same indication. Despite concerns about the safety profile of kinase inhibitors and the potential implications for the treatment of non-life-threatening diseases, several Jak inhibitors, including the Jak1-selective compound upadacitinib (*Rinvoq* ™) and the pan-Jak inhibitors tofacitinib (*Xeljanz* ™) and baricitinib (*Olumiant* ™), have been approved for the treatment of RA and are clinically explored in other inflammatory diseases.

Experimental drugs against nonreceptor PTKs with therapeutic potential are Tyk2, Syk, and Fak inhibitors. Deucravacitinib is an orally available, selective Tyk2 inhibitor stabilizing the pseudokinase domain of this PTK and has shown preliminary evidence of clinical activity in SLE and psoriasis. Mixed clinical results have been reported about compounds targeting Syk and Fak. While the Syk inhibitor prodrug fostamatinib (*Tavalisse* ™) has shown limited clinical activity in RA, it has been approved for the treatment of chronic immune thrombocytopenia (ITP) by the FDA in 2018. The Fak inhibitor defactinib is still explored for the treatment of solid cancers, and future basic research and clinical studies will show whether these and other inhibitors of nonreceptor PTKs may be as successful drugs as imatinib or ibrutinib for the treatment of cancer or the management of certain chronic immunological diseases.

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Tyrosine-Protein Kinase JAK

▶ Janus Kinase

Tyrosylprotein Kinase

▶ Tyrosine Kinases

U

Ubiquitin/Proteasome

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Synonyms

Posttranslational protein modification; Protein degradation

Definition

Protein modification by the covalent attachment of ubiquitin chains serves as a signal to mark proteins for the degradation by a multicatalytic proteinase complex called the proteasome. Thus, the ubiquitin proteasome system (UPS) controls the stability of proteins in a regulated manner affecting multiple essential cellular processes. In addition, dependent on the mode of linkage, ubiquitin regulates protein-protein interaction, endocytosis, replication, and the formation of signaling complexes in a proteasome-independent fashion. Besides phosphorylation ubiquitination represents the most important posttranslational regulatory mechanism in biology.

Basic Mechanisms

Ubiquitin Conjugation

Ubiquitin is a highly conserved 8.5 kDa polypeptide, which was first described in 1974. The discovery that the ubiquitin proteasome system serves as a general mechanism to target proteins for destruction by the proteasome was awarded with the Nobel Prize for Chemistry in 2004.

Upon cleavage from a precursor, ubiquitin becomes covalently attached to the ε-amino group of lysine (K) residues in specific target proteins by the consecutive activity of a set of E1, E2, and E3 enzymes. First an ubiquitin-activating enzyme (E1) is charged with ubiquitin. This is accomplished by using ATP to form a high-energy bond at the C-terminus of ubiquitin. Subsequently this linkage is attacked by the sulfhydryl group of the active site cystein present in the catalytic domain of the E1 enzyme resulting in a thioester formed between the E1 and ubiquitin. Ubiquitin can now be transferred from the E1 to the catalytic core of a second class of enzymes termed ubiquitin-conjugating enzymes (E2). The E1 is released and can now charge additional ubiquitin residues. Ubiquitin bound to E2 can finally be transferred to target protein substrates, a step which is catalyzed by E3 ligases (Song and Luo 2019). As ubiquitin itself contains several lysine

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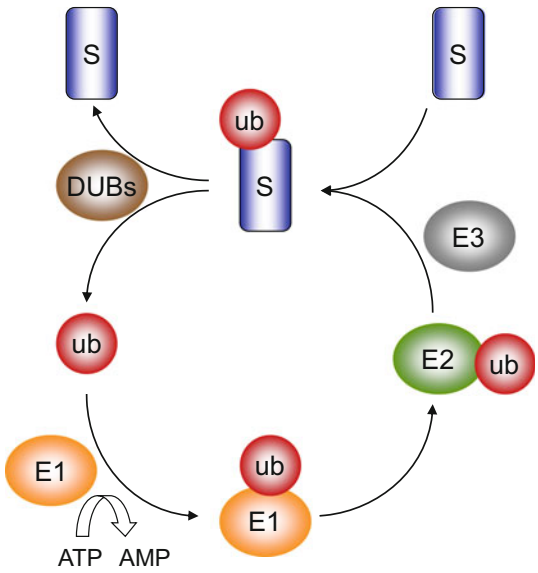
residues, which can serve as targets for the linkage of additional ubiquitin molecules, variable chains of ubiquitin can be attached. Like most posttranslational modification mechanisms, ubiquitin conjugation is a reversible process. Analogous to the activity of phosphatases in protein phosphorylation, deubiquitinating enzymes (DUBs) counteract ubiquitin modification by cleaving the molecule from modified substrates (Clague et al. 2019). The hierarchical order of E1, E2, and E3 activity correlates with an increase in enzymatic specificity which ensures selective ubiquitin modification of distinct target proteins. While only 2 E1 enzymes and around 50 E2 enzymes have been described, more than 700 E3s are encoded in the human genome. The large group of E3 enzymes is mainly responsible to exert specificity in terms of substrate recognition. The existence of more than 90 different DUBs in humans adds further complexity to the system (Fig. 1). In addition to the canonical form of ubiquitination via E1, E2, E3, phosphoribosylated ubiquitin can be linked to serine residues of distinct substrates catalyzed by enzymes of the SidE family. Furthermore, ubiquitin chains can be attached to the N-terminal methionine by the linear ubiquitin chain assembly complex (LUBAC). Complexity is further enhanced as ubiquitin itself can be acetylated on lysine or phosphorylated on serine, threonine, or tyrosine residues. All these variations determine the consequences for the modified protein (Swatek and Komander 2016).



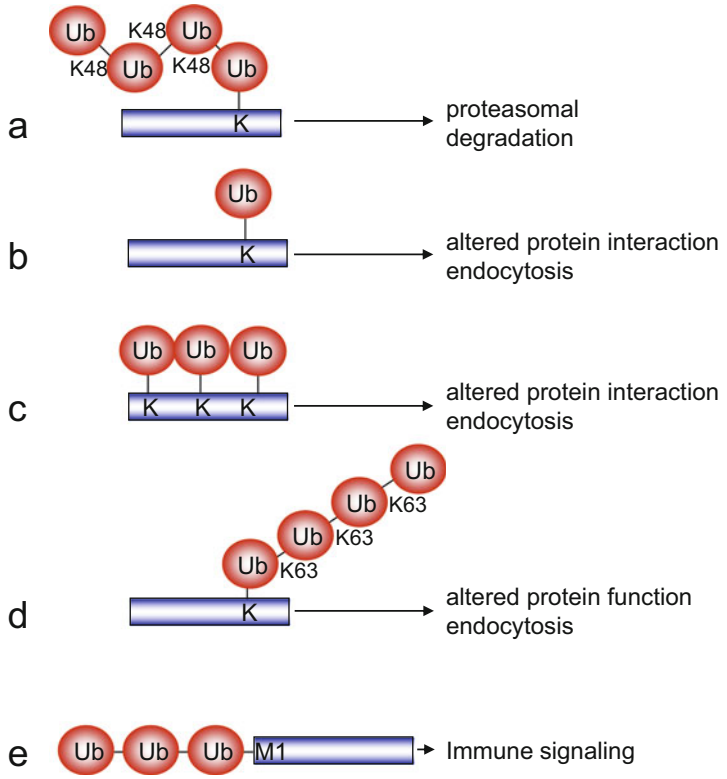
Ubiquitin/Proteasome, Fig. 1 Ubiquitin conjugation cycle. Ubiquitin (Ub) is ligated to target substrates (S) by the consecutive activity of ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3). Deubiquitinating enzymes (DUBs) mediate deconjugation of ubiquitin bound to substrates

Ubiquitin Modification as a Signal for Proteasomal Degradation

Single ubiquitin residues can be connected to one (monoubiquitination) or multiple lysine residues (multi-monoubiquitination) of the target protein. As different lysines within ubiquitin can serve as sites for the addition of further ubiquitin molecules, variable chains of ubiquitin can be connected to the substrate (polyubiquitination). The best-characterized lysines used for ubiquitin chain prolongation are K48 and K63, but also linkages via K6, K11, K27, and K29 and K33 are of relevance. The fate of ubiquitin-labeled proteins depends on the mode of linkage that is formed and has complex consequences (Akutsu et al. 2016). Some classical examples are depicted in Fig. 2.

K48-linked polyubiquitin serves as a signal for proteolytic degradation by the 26S proteasome, a multisubunit complex composed of a core protease termed 20S proteasome and a 19S regulatory particle (PA700), attached at either one or both ends. The 19S regulatory particle can further be dissected into two substructures, a lid and a base. The base harbors six homologous ATPases. The 20S proteasome consists of four heptameric rings (α1, β1, β2, α2) that form a cylindric, barrel-shaped structure harboring three distinct catalytic domains in the β-subunits orientated toward the internal side. These different catalytic subunits were shown to encompass chymotrypsin-like, trypsin-like, and peptidyl-hydrolase-like activity.

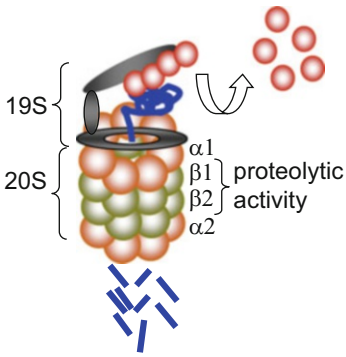
Recognition of ubiquitin-conjugated proteins, detachment of the ubiquitin chains, and unfolding is accomplished by the regulatory 19S subunit. From there the polypeptide is translocated in the



Ubiquitin/Proteasome, Fig. 2 Functional consequences of ubiquitin linkage. Substrates (blue bars) are linked via lysine residues (K) to ubiquitin or ubiquitin chains. (**a**) Attachment of chains connected via lysines in position 48 of ubiquitin (K48) targets substrates for proteasomal degradation. In contrast modification of one (**b**) or multiple (**c**) lysines by a single ubiquitin molecule mediates novel protein interactions or initiates endocytosis. Conjugation of K63-linked polyubiquitin (**d**) alters protein function and can also serve as a signal for endocytosis. (**e**) In linear ubiquitination, ubiquitin chains are linked to the terminal methionine (M1) which plays a major role in immune signaling

lumen of the 20S core proteasome and degraded by the proteolytic activity of the β-subunits (Fig. 3). The pore can be occluded by peptides from the α-subunits, and PA700 functions as a gatekeeper by removing these occlusions. The process of 26S proteasome-catalyzed proteolysis is energy dependent, i.e., depends on ATP hydrolysis.

The modular structure allows adaptation to different physiological demands by the exchange of subunit components in either the regulatory or proteolytic part of the proteasome. IFN-γ, for example, can modify the composition of the proteasome by mediating the exchange of the regulatory complex and replacement of proteolytic subunits. Proteolytic activities in this so-called immunoproteasome are altered in a way that generation of antigenic peptides suitable to be loaded to MHC class I proteins is favored. Thus, the ubiquitin proteasome system exerts an essential function in antigen presentation, necessary for appropriate immune defense against pathogens (Murata et al. 2018).



Ubiquitin/Proteasome, Fig. 3 Structure of the proteasome. Proteasomes form a barrel-like structure and are composed of a 20S core particle and the 19S regulatory subunit located at one or both ends. Two heptameric β-rings (green), which contain the proteolytic domains, are attached to the outer α-rings (orange). Substrates (blue) are recognized by the regulatory particle, unfolded, and upon detachment of ubiquitin chains (red) degraded in the lumen of the 20S core proteasome

The UPS also plays a major role in protein quality control where it is tightly connected and intertwined with autophagy (Pohl and Dikic

2019). In a process known as endoplasmic associated degradation (ERAD), misfolded proteins, which are formed in the endoplasmatic reticulum, are translocated back to the cytoplasm and degraded by the proteasome.

The ubiquitin-mediated degradation of certain proteins is also one of the key mechanisms underlying cellular growth control. Thus, the half-life of critical regulatory proteins of the cell cycle like p53, cyclins, and cyclin-dependent kinases (CDKs) are directly controlled by the ubiquitin system. Furthermore, cell cycle progression depends on a coordinated network of ubiquitin-mediated proteolysis of these factors. Key regulators of cell cycle checkpoints and cyclin degradation are the anaphase-promoting complex (APC) and the Skp1/Cul1/F-box protein complex (SCF) both acting as ubiquitin ligases.

APC is active from mid-M phase (anaphase) to the end of G1 phase and required for disconnecting sister chromatids and exit from M-Phase to G1. The complex mediates the ubiquitination of securin and cyclin B. Degradation of these proteins, which block mitotic progression, promotes anaphase onset and exit from mitosis.

The SCF ubiquitylates proteins from late G1 to early M phase. This complex consists of a core together with different so-called F-box proteins, which are responsible for the substrate recognition. Typically interaction of F-box proteins with their substrate involves sites of phosphorylation, so-called phosphodegrons. Thus, a highly coordinated and specific degradation of different cell cycle regulators in distinct activation stages can be achieved, and it is not surprising that E3 enzymes acting within this context are frequently deregulated in cancer (Senft et al. 2018).

Proteasomal degradation also plays an essential role in the activation of cellular signaling pathways. A prototype for this is the control of NF-κB signaling, which has a pivotal role in inflammatory responses. Upon stimulation the inhibitory IκBα protein is phosphorylated and thereby becomes a target substrate for K48 polyubiquitination. Proteasomal degradation of IκBα releases the transcription factor NF-κB, which subsequently translocates to the nucleus and activates specific target genes.

Non-proteolytic Functions of Ubiquitin Conjugation

In contrast to the proteasome-mediated degradation of proteins linked to K48-polyubiquitin chains, monoubiquitin, multi-monoubiquitin, or K63 linkage of ubiquitin chains is often not a signal for proteasomal degradation, but controls molecular mechanisms like protein activity and interaction, DNA repair, and inflammation. Monoubiquitination and multi-monoubiquitination are crucial events in signal termination. Upon ligand binding surface receptors like receptor tyrosine kinases or G-protein-coupled receptors are hereby marked for endocytosis and degraded via the endosomal/lysosomal pathway. Another example for non-proteolytic functions is the modification of proliferative cell nuclear antigen (PCNA), a DNA processivity factor. While K63-linked polyubiquitin modification results in error-free DNA repair, monoubiquitination of PCNA recruits an error-prone translesion polymerase (Masuda and Masutani 2019).

Ubiquitin modification of substrates can be sensed by proteins, which serve as "ubiquitin receptors." These proteins harbor domains capable of ubiquitin binding and help to translate the signal into the proper physiological response by forming signaling complexes or activating downstream effectors. So far more than 15 different ubiquitin recognition motifs have been identified.

Ubiquitin-Like Proteins

Proteins can also be modified by so-called ubiquitin-like modifiers (UBLs). These proteins have structural similarity to ubiquitin and are linked to substrates in a similar way. Like ubiquitin UBLs are conjugated by the consecutive action of E1, E2, and E3 ligases and sometimes use the same enzymes mediating

ubiquitin linkage. Prominent examples for UBLs are SUMO, NEDD8, FAT10, or interferon-stimulated gene 15 (ISG15), which have been shown to affect multiple cellular functions like protein localization, cell cycle progression, DNA repair, and immune function (Hochstrasser 2009).

Disease Associated with Perturbations of the Ubiquitin System

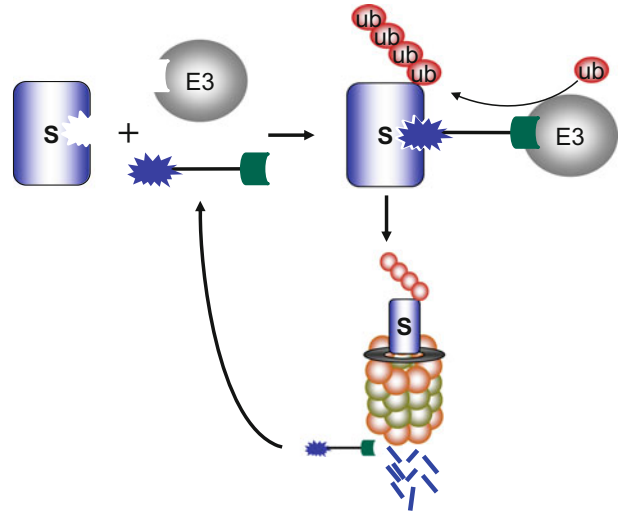
In concordance with the central role of ubiquitin modification in multiple cellular functions, perturbations of this system are associated with a variety of diseases, and only a small selection can be presented here. Defects in the control of cell cycle regulators or genome integrity by components of the ubiquitin system are connected to cancer progression, and many E3 ligases were originally identified as oncogenes (Wang et al. 2017). As the ubiquitin proteasome pathway is a main route for protein clearance, it is not surprising that in proteinopathies (disease caused by aggregate-prone proteins) like sporadic Parkinson or Huntington disease, proteasome activity is reduced. Autosomal recessive loss of function of the E3 ligase parkin is the molecular base for one of the most common forms of familial Parkinson disease. In concert with the kinase PINK1, Parkin plays a major role in the elimination of damaged mitochondria. PINK1, which is normally rapidly degraded, is stabilized upon mitochondrial damage and activates parkin involving parkin and ubiquitin phosphorylation. In its activated form, parkin then assembles ubiquitin chains on numerous proteins on the outer mitochondrial membrane, leading to the recruitment of ubiquitin-binding autophagy receptors and clearance of the damaged mitochondria. Disturbed function of this mitophagy is one etiology of Parkinson disease (Harper et al. 2018).

In cystic fibrosis a point mutation of the cystic fibrosis transmembrane regulator (CFTR) prevents transport of this molecule to the cell surface. Instead this otherwise functional molecule is degraded by the ERAD-ubiquitin proteasome pathway.

Pharmacological Intervention

Pharmacologic Targeting of Components of Ubiquitin and Ubiquitin-Like Modification Systems

Proteasomal inhibition represents a meanwhile established strategy in cancer treatment. Bortezomib (PS-341, Velcade™) was the first proteasome inhibitor to be brought into clinical use for the treatment of refractory and relapsed multiple myeloma, a proliferative disease of plasma cells. Bortezomib inhibits an active site in a proteasome subunit and remarkably shows selective cytotoxicity to certain cancer cells. Later on, second-generation inhibitors of the proteasome were developed which include carfilzomib, ixazomib, oprozomib, delanzomib, and marizomib. In clinical trials these small molecules also showed activity in tumors resistant to bortezomib. Although the precise mechanisms of the effect of proteasome inhibition against cancer are not entirely clear, studies uncovered a general promotion of apoptosis in malignant cells due to destabilization and stabilization of key players in this pathway (Manasanch and Orlowski 2017). The success of proteasome inhibition boosted various approaches to more specifically target distinct components of the UPS to reduce side effects and overcome resistance. An example for such a more specific pharmacological approach is to prevent ubiquitin-mediated degradation of the tumor suppressor p53, which is mediated by the E3 ligase MDM2. Inhibition of MDM2 ligase activity by a family of closely related 7-nitro-5-deazaflavin compounds (HLI98) was shown to stabilize p53. Protein interaction between MDM2 and p53 was effectively prevented by nutlins, a class of *cis*-imidazoline compounds, which exhibited strong antitumor effects in mice. Within the same molecular context, inhibitors were developed for USP7, a DUB that counteracts MDM2 autoubiquitination. Inhibition of USP7 enhances ubiquitination of MDM2 and thus promotes its proteasomal degradation. As a consequence p53 is stabilized and beneficial effects were detected in preclinical tumor models. To



Ubiquitin/Proteasome, Fig. 4 Principle of targeted protein degradation by PROTACS and related molecules. To selectively degrade a particular substrate protein (S), a bispecific adapter molecule (PROTAC) is designed which links the substrate to an E3 ligase-mediating ubiquitin conjugation. This marks the protein for proteasomal degradation, while the adapter is released and can enter a new recruitment cycle

block protein modification by ubiquitin or ubiquitin-like proteins, inhibitors for various E1 activating enzymes were developed. MLN7243 is the first ubiquitin E1 inhibitor to enter a clinical trial for cancer therapy, and also the NEDD8 E1 (NAE1) inhibitor pevonedistat (MLN4924) is currently in clinical trials for the treatment of various types of cancer (Yin et al. 2019). For pharmacologic modulation, the class of ubiquitin- or UBL-deconjugating enzymes (DUBs) represent attractive target structures. As being proteases, DUBs fall under a category of proteins already proven to be druggable, and biochemical assays can relatively easily be developed and adapted to high-throughput format. The large numbers of DUBs with their various functions allow more selective intervention strategies, and small molecules with proven activity were already developed. Many DUB inhibitors addressing severe diseases in oncology, inflammation, and neurodegeneration showed promising effects in preclinical studies or entered clinical trials (Harrigan et al. 2018).

Pharmacologic Exploitation of the Ubiquitin Proteasome System for Targeted Degradation of Proteins

Targeted degradation of proteins is a pharmacologic strategy which gained tremendous interest in recent years and offers great chances for the development of novel therapeutic applications. The approach hijacks the endogenous ubiquitin/proteasome degradation machinery to selectively destroy target proteins associated with pathological conditions. As depicted in Fig. 4, a chemical adapter molecule is constructed which on the one side harbors a specific binding entity for the target protein, while another element in the molecule mediates attachment to an endogenous E3 ligase. Thereby, these so-called proteolysis-targeted chimeras (PROTACs) recruit the target protein to the ubiquitin conjugation machinery, where decoration with ubiquitin chains marks it for proteasomal degradation. In an ideal scenario, the chemical linker leaves the proteasome in its active form and can recruit the next target protein. A single small-molecule ligase modulator can therefore drive the destruction of many molecules of a target protein reminiscent of a catalytic mechanism. In contrast to classical pharmacological approaches where the small molecule needs to block a particular functional domain of a protein, PROTACS offer the conceptual advantage that in principle any protein can be addressed. Even disease-associated proteins previously thought not to be pharmacologically accessible can in

principle be attacked by PROTAC-mediated degradation. These undruggable proteins still constitute the vast majority within the proteome and include transcription factors, adapter proteins, proteins with scaffolding function, aberrant proteins, etc. As the chemical moiety which links the substrate to the ligase only needs to bind the substrate anywhere rather than block a particular enzymatic core, ATP-binding site or whatsoever more options for the compound selection exist.

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Unfolded Protein Response

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Synonyms

Endoplasmic reticulum stress; Misfolded proteins

Definition

The endoplasmic reticulum (ER) is the vital organelle in all eukaryotes, first explored by Porter and his colleagues in 1945 in mice fibroblast (Porter et al. 1945). ER is the complicated tubular meshwork, which controls calcium (Ca^{2+}) homeostasis and serves as a main site for the synthesis of 33–35% of all cellular proteins. Moreover, it is also responsible for folding of transmembrane and secreted proteins that comprise one-third of all the synthesized proteins in the cell (Oakes and Papa 2015). Appropriate folding of proteins is a critical step in preserving its functions, as any abnormality may lead to cellular stress and even causes death, called ER stress (ERS). Various endogenous and exogenous factors interrupt protein folding which causes

accumulation of unfolded proteins in ER lumen, resulting in exaggerating unfolded protein response (UPR).

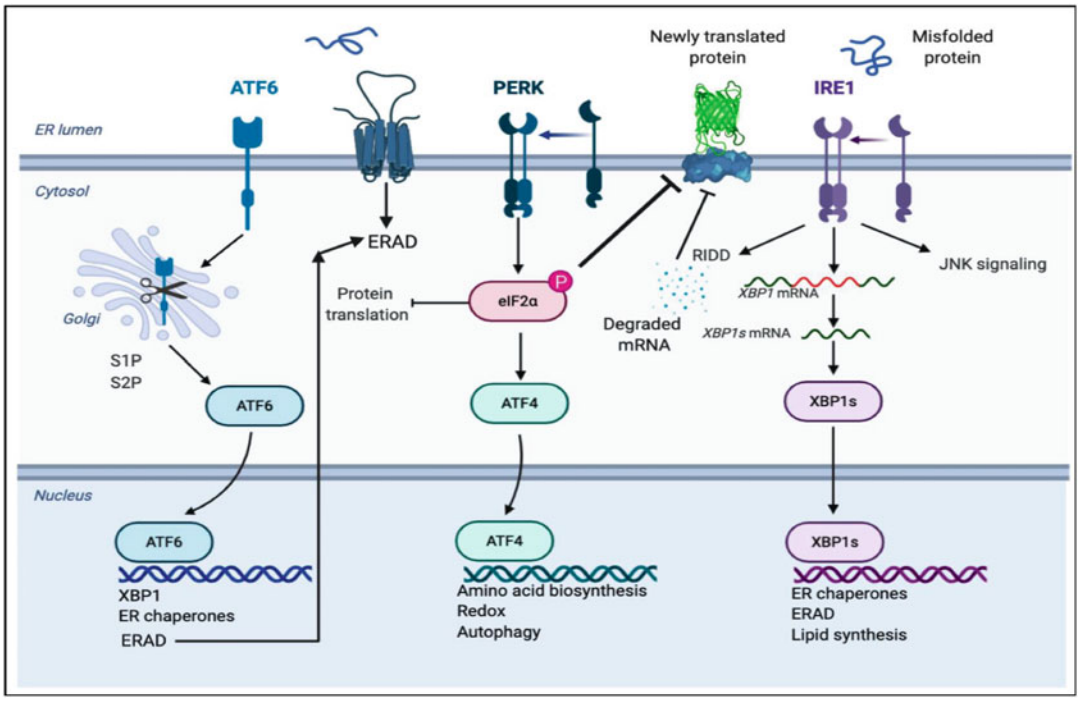
Basic Characteristics of UPR Pathway

The UPR is a highly conserved ER adaptive pathway designed to respond against misfolding of protein in the ER. This pathway consists of three transmembrane stress sensors, namely, inositol-requiring enzyme 1 alpha (IRE1α), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6). During normal physiological conditions, the internal domains of these sensors are inactive via binding with molecular chaperons comprising binding immunoglobulin protein (BiP), also termed as glucose-regulated protein 78 (GRP78) and heat shock protein A5 (HSPA5). Despite outstanding affinity of BiP for unfolded proteins, BiP detaches from ER stress sensors during luminal accumulation of misfolded proteins to activate downstream signaling pathway (Pincus et al. 2010). Mechanistically, accumulated misfolded protein within ER lumen ignites heat shock protein to activate transcription of genes responsible for encoding cytosolic BiP to refold unstable proteins. However, during pathological conditions such as inflammation, neurodegenerative disorder, diabetes mellitus (DM), and cardiovascular disease (CVD), ERS leads to activation of UPR to counterbalance the cellular damage. Furthermore, hydrophobic and basic residues of unfolded proteins directly attach with distinct binding site on IRE1α to induce UPR (Grootjans et al. 2016).

Consequently, upregulated UPR accelerates transcriptional factors and related gene expression to attenuate ERS by repressing synthesis of proteins and activates ER-associated degradation (ERAD) of incomplete folded proteins, thereby elevating synthesis of molecular chaperones to expedite protein folding. The UPR within ER lumen accomplished these compensatory responses via stress sensors by four distinctive approaches. The primary goal of these stress sensors is to preserve proteostasis by rapidly mitigating mRNA translation through PERK-mediated phosphorylation of α-subunit in eukaryotic by translating initiation factor 2 (elF2α), which blocks protein and thus restricts limited amount of protein entry inside the ER. Second, entry of newly synthesized proteins into the ER is reduced by deterioration of ER membrane-bound mRNAs via IRE1-α-dependent decay (RIDD) (Scheuner et al. 2001). Third, elimination of misfolded protein from the ER is mediated by enhancing transcription of ERAD and autophagy like proteins. Lastly, protein folding capacity of ER is increased by the specific genes that mediate its expansion through augmentation of lipid synthesis and biogenesis (Oakes and Papa 2015). Nevertheless, when these compensatory responses are unable to cope with pathological state, the UPR shifts to pro-apoptotic mode as terminal UPR and leads to cellular damage (Grootjans et al. 2016).

Characteristics of IRE1α Signaling

IRE1α is the transmembrane protein consisting of two domains: an endoribonuclease (RNase) domain and a serine/threonine kinase domain. After detaching from BiP, IRE1α undergoes dimerization to induce RNase activity to generate non-conservative splicing of mRNA that encrypts X-box binding protein 1 (XBP1). The resultant translational shift produces a transcriptional inducer, termed as spliced XBP1 (XBP1s), which translocates inside the nucleus to initiate synthesis of GRP78, GRP94, and XBP1, to enhance ER size and facilitate protein folding. Moreover, IRE1α is also responsible for the activation of RIDD which degrades ER membrane-bound mRNAs to limit the quantity of protein which enters inside the ER as shown in Fig. 1. Conversely, during persistent ERS, the compensatory role of IRE1α is impaired, while PERK signaling becomes dominated. Chronic ERS causes activation of cytosolic RNase domain of IRE1α after oligomerization, which leads to non-specific cleavage of apoptosis inhibitory microRNAs (other than RNA of XBP1), thus fans the flame of apoptosis. In summary, during sustained



Unfolded Protein Response, Fig. 1 The accumulation of misfolded proteins within ER lumen stimulates three branches of UPR. After dissociation from BiP, ATF6α translocates to Golgi complex and processed by S1P and S2P to generate p50 cytosolic fragment. P50 fragment serves as transcriptional factor that enhances ER capacity of protein folding. PKR-like ER kinase (PERK) phosphorylates elf2α to halt ribosomal assembly and allow cells to manage temporary ERS. Upon separation from BiP, IRE1α non-covalently splices intron to produce XBP1s mRNA, which encodes proteins to facilitate protein folding capacity of ER

ERS, IRE1α switches to aggravate inflammation and necrosis rather than being adaptive (Amen et al. 2019).

Characteristics of PERK Signaling

The second arm of UPR is PERK which is a local ER transmembrane protein that plays a crucial role during ERS. Moreover, it has been well-established that lipid components in the ER membrane are also capable for the induction of PERK signaling during ERS (Volmer et al. 2013). The primary role of PERK pathway is to suppress synthesis of protein and implicates attenuating eIF2α-mediated translational activities within the ER. During ERS, PERK separates from BiP and undergoes oligomerization to phosphorylate eIF2α, a main substrate for PERK. Phosphorylated eIF2α further blocks eIF2-ternary complex (eIF2-TC) development and thus temporarily mitigates global mRNA translation to facilitate management of transient ERS, although, within ER-stressed cells, translation of majority of mRNA is suppressed by translational activity of some mRNA species which are beneficial during the phase. Importantly, mRNA transcribes activating transcription factor 4 (ATF4) as presented in Fig. 1, which is a critical moderator in UPR and involves transactivation of genes responsible for developing resistance to oxidative stress (OS), metabolism of amino acids, and autophagy (Lei et al. 2017).

Conversely, prolonged translational blockade is not favorable for the survival of cell. Therefore, ATF4 enables protein phosphatase 1 regulatory subunit 15A (PPP1R15A) expression, which encrypts growth arrest and DNA damage-

inducible protein (GADD34). GADD34 directly dephosphorylates elF2α to resume mRNA translation. On the other hand, ATF4 also stimulates transcriptional events leading to the synthesis of C/EBP homologous protein (CHOP), which is implicated in ERS-mediated programed cell death. In order to facilitate cells in the management of ERS, various mechanisms are directed to depress CHOP transcription including PERK-dependent microRNA 211 (miR-211), which suppresses CHOP via methylation of histone. Furthermore, CHOP synthesis is also inhibited by toll-like receptor (TLR) pathway through protein phosphatase 2A (PP2A), which facilitated dephosphorylation of serine residue of elF2Bε subunit (Woo et al. 2012). In fact, strong stimulation of PERK enhances constant level of CHOP; hence, only persistent ERS can switch UPR to pro-apoptotic mode. Based on previous studies, both ATF4 and CHOP during constant ERS functions act as heterodimer to initiate apoptosis by enhancing synthesis of protein, leading to the generation of misfolded protein and cell death (Grootjans et al. 2016).

Characteristics of ATF6 Signaling

ATF6 is a transmembrane transcriptional factor bearing cytosolic N-terminal and ER luminal C-terminal domains that stimulates transcriptional processes, which enable ER to sense ERS. ATF6 signaling pathway comprises of two isoforms, namely, ATF6α and ATF6β. Signal transduction of ATF6 is similar to IRE1α and PERK; however, autophosphorylation and oligomerization are absent. During ERS, N-terminal of ATF6 dissociates from BiP to reallocate itself in Golgi complex (Fig. 1), where it undergoes proteolysis via Golgi resident site 1 protease (S1P) and site 2 protease (S2P) to generate p50 cytosolic fragment that translocates to the nucleus (Amen et al. 2019). The p50 fragment mediates expression of CHOP, GRP78, GRP94, and protein disulfide isomerase (PDI) to promote expansion and protein folding capacity of ER along with stimulation of ERAD pathway (Han et al. 2013).

Therapeutic Approach to Modulate UPR

The lethal connection between deregulated UPR and development of chronic human disorders accelerated researcher to explore small molecules, which modulate UPR signaling during pathological conditions. Previously published findings revealed involvement of UPR signaling pathways in the development of metabolic disorders, cancer, obesity, neurodegenerative diseases, and immune-related disorders (Lebeaupin et al. 2018). Our understanding regarding molecular mechanisms mediating UPR is clearly understood, and the implication of UPR proteins in various disease conditions has been established. Therefore, robust therapeutic approach is needed that enhances the delivery of potential medicaments, mediating through the targeted UPR signaling for the beneficial clinical outcomes.

IRE1α RNase Inhibitors

RNase poses difficult therapeutic targets; direct inhibitors of IRE1α RNase were explored through florescent hairpin RNA, human recombinant protein, and luciferase assays. The chemical structure of all renowned RNase domain inhibitors possesses central aromatic aldehyde with an adjacent hydroxyl group, called hydroxyl-aryl-aldehydes (HAAs). Various HAAs including STF-083010 have been investigated based on their structure activity relationship (SAR), but their in vivo therapeutic potential is still under investigation. Mechanistically, these inhibitors work in a non-competitive fashion by impairing catalytic cleavage, while XBP1 mRNA may modulate and bind to IRE1α (Zhao et al. 2018). However, still further research is needed to explore exact mechanism of impairing catalytic activity.

Allosteric IRE1α Kinase Modulators

Kinases comprise well-defined drug-binding sites. Wang et al. (2012) investigated kinase inhibitors due to their ability to maintain inactive state of IRE1α with subsequent inhibition of RNase,

Unfolded Protein Response, Table 1 Small molecule inhibitors targeting arms of UPR signaling

Class	Disease	Experimental model	Pharmacological action	Reference
IRE1 inhibitors				
STF-083010	Multiple myeloma	MCF-7 xenograft	Inhibition of tumor growth	[12]
KIRA6	Type 1 diabetes	Akita diabetic mouse model	Improved hyperglycemia	[13]
AMG18	Non-obese diabetes	Non-obese diabetic mice	Protect beta cells with enhanced insulin secretion	[14]
PERK inhibitors				
GSK2606414	Prion disease	Tg37 transgenic mice	Neuroprotection with improved behavioral and cognitive function	[15]
GSK2656157	Neonatal diabetes mellitus	Mouse and human islets	Improved hyperglycemia	[15]
elf2α phosphatase blockers				
Salubrinal	Parkinson disease	C57BL/6 mice	Neuroprotection with improved clinical symptoms	[16]
Guanabenz	Amyotrophic lateral sclerosis (ALS)	Transgenic mice	Improved motor neuron survival and performance	[17]
Sephin1	Amyotrophic lateral sclerosis (ALS)	Transgenic mice	Improved motor neuron survival and performance	[17]
ATF4 inhibitors or elf2B activator				
ISRIB	Prostate cancer	Melanoma cell lines	Inhibition of metastatic tumor growth	[18]
2BAct	Vanishing white matter (VWM) disease	Wild-type and R191H mice	Attenuated motor defects and demyelination	[19]
ATF6 modulators				
Compound 147	Cardiac failure	C57B/6J mice	Restore cardiac function and decrease infarct size	[21]
	Dysregulated hepatic proteostasis	C57B/6J mice	Decreased cholesterol content in the liver	[15]
	Renal injury	C57B/6J mice	Preserved renal functions	[21]

using allosteric effect in an in vitro study (Wang et al. 2012). Importantly, they expanded biaryl urea motif after replacing central structure to synthesize KIRA6 (Table 1). Similarly, Amgen and their colleagues explored another kinase inhibitor named AMG18; both the compounds have similar binding affinities that block IRE1α RNase via the same mechanism. Binding of inhibitor mobilizes Cα-helix of IRE1α which causes disruption of contact between dimers and thus diverts the state of equilibrium toward silenced monomeric form of IRE1α (Feldman et al. 2016). Other IRE1α kinase inhibitors may lead to activation of IRE1α such as type I kinase blockers that competitively attach with ATP, and kinase-inhibitor complex undergoes a conformational change similar to active ATP state. Only in vitro findings revealed that these inhibitors stimulate IRE1α signaling, but still there is a dearth in understanding the abovementioned mechanisms via preclinical studies using animal models.

Inhibitors of PERK Signaling

The first reported and extensively investigated PERK inhibitors are GSK2606414 and GSK2656157 as enlisted in Table 1, which are highly potent and well characterized on pharmacokinetic (PK) platforms compatible for in vivo

studies. These inhibitors possess a central structure, indoline, and competitively attached with cytosolic kinase domain of PERK within ATP binding cleft, leading to modification of Cα-helix. This molecular modification accounts for their potent inhibitory effect and selectivity for kinases. Another novel group of PERK inhibitor with structural alterations and distinct binding chemistry was discovered. These agents after binding at the ATP cleavage of PERK cause rearrangement of catalytic moiety and maintain inactive state of PERK. However, AMG44 and AMG52 have similar potential of kinase inhibition but are less potent than GSK2606414 and GSK2656157. Based on pharmacodynamic (PD) study, it attributes to kinase selectivity; AMG52 and AMG44 are favored to explore pharmacology of PERK signaling. In future, long-term investigation with additional selective entities is required (Hetz et al. 2019).

elf2α Phosphatase Blockers

The primary compound, which served as an investigational tool to analyze elf2α phosphatase, is salubrinal (Table 1). Studies based on its mechanism demonstrated prevention of dephosphorylation of elf2α during ERS. More specifically, salubrinal non-selectively blocks protein phosphatase 1 (PP1) holoenzyme phosphatase complexes that regulate P-elf2α as negative feedback during integrated stress response (ISR), facilitated through GADD34 controlling subunits (Colla et al. 2012). In search of selective inhibition of elf2α phosphatases, identification of α-adrenergic agonist guanabenz (Table 1) showed selective blockade of GADD34. This finding motivated further efforts to identify another potent and selective inhibitor named sephin1, lacking α-adrenergic effects as shown in Table 1. However, direct binding of guanabenz and sephin1 with GADD34-PP1 has been disapproved recently. Therefore, extensive molecular study is required to further dissect role of sephin1 and guanabenz to reveal pharmacological basis of their mechanism of action (Crespillo-Casado et al. 2018).

ATF4 Inhibitors or elf2B Activator

Table 1 presented selective ISR or ISRIB inhibitor of ATF4 which was discovered after phenotypic screening by Sidrauski et al. (2013) that effectively depress translational modification of ATF4 reporter during ERS (Sidrauski et al. 2013). Binding of ISRIB to lipophilic cleft at the junction of elf2B β and δ-subunits derives equilibrium dissemination of subunits toward dense population of accumulated decamer complex with highest catalytic activity. The enhanced catalytic potential of elf2B mediated by ISRIB recruits elf2-TC and hampers ATF4 translation. Notably, ISRIB is only limited to elevate catalytic activity of non-sequestered (P-elf2α sequestered elf2B) elf2B, and efficacy of ISRIB during stress is dependent on relative expression of P-elf2α to restricted amount of elf2B. Due to poor physicochemical characteristics, ISRIB is insoluble and poses difficulty in formulation. Moreover, recently it has been reported that ISRIB is effective only during moderate ISR stimulation. Conversely, 2BAct was discovered as new elf2B activator, equipped with excellent pharmacokinetic and physicochemical attributes compatible for oral administration and can be mixed in regular chow diet for long-term in vivo investigations (Wong et al. 2019).

ATF6 Modulators

Small molecule inhibitors and activators targeting ATF6α have been explored using in vitro ERS responsive luciferase reporter assay. Ceapins selectively inhibit ATF6α activation. SAR analysis highlighted pyrazole amide motif enhanced ten-fold increase in the potency of the compound. Presumably, ceapins inhibit translocation of ATF6α from the ER to the Golgi complex by interrupting with coat protein complex II (COPII) facilitated transportation, thereby sequestering ATF6α from S1P and S2P (Gallagher et al. 2016). Similarly, ATF6 activators have been exposed such as 147 (Table 1) and 263 compounds that selectively activate ATF6α signaling pathway. Activated compound 147 modulates ER

local PDI to provoke ATF6α stimulation, while mechanism of 263 remains to be elucidated (Hetz et al. 2019).

Future Prospective

The utilization of small molecules that modulate specific domains of UPR signaling during ERS has confirmed UPR implication in the pathogenesis of several diseases with underlined mechanisms. The understanding of UPR signaling with various in-depth insight paves the way for potential and promising outcomes in the management of multiple human disorders. However, due to broader indications and critical nature of UPR pathways, there are many unanswered questions need to be addressed. Side effects related to these small molecules can be tested in non-human primates and rodents, prior to their investigations at preclinical level. Importantly, comparison between pharmacological inhibition and preclinical investigations using genetic deletion of UPR components is very difficult, as genetic deletion provides permanent inhibition, whereas inhibition via inhibitor occurs in troughs and peaks. One important question needs answer: how the intensity of inhibition is required over the time to achieve beneficial therapeutic outcome with minimum toxic effects? It has been shown that high therapeutic dose with subsequent target inhibition is well tolerated in mice over the months of treatment, while long-term management with on/off treatment cycles needs to be established in future. In addition, future investigations warrant acute vs. chronic elucidation of UPR signal transduction in various disease conditions, to implement unbound strategies and interventions needed in clinical aspects.

Overall, UPR domain plays pivotal role and displayed rapid advancement in drug discovery and development process of small molecules. Modulation of UPR signaling and its core branches provides an opportunity to dissect molecular mechanisms for the expansion of therapeutics in a wider range of ERS-related disorders.

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URAT1

► Physiology and Treatment of Hyperuricemia and Gout

V

Vascular Natriuretic Peptide

► C-Type Natriuretic Peptide and Its Receptors

Vasopressin

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Synonyms

Antidiuretic hormone (ADH); Arginine vasopressin; Argipressin

Definition

Vasopressin is a nonapeptide synthesized in the hypothalamus and secreted from the posterior pituitary gland. It plays a major role in the maintenance of hydromineral balance in the body, exerting antidiuretic action on collecting ducts of the kidney. It is also a very potent vasoconstrictor, acting on arterioles and increasing peripheral vascular resistance. As a neuropeptide, vasopressin regulates affiliative and aggressive behaviors and the response to stress.

Several pathological conditions are directly connected either to malfunction of vasopressin secretion or to the resistance of target tissues to vasopressin action.

The syndrome of inappropriate antidiuretic hormone secretion (SIADH) is characterized by an increased secretion of vasopressin, independent of plasma osmolality, which causes excessive water reabsorption and dilutional hyponatremia. SIADH can be of central origin, when the production of vasopressin in the hypothalamus is affected by an underlying neurological condition or brain injury, but it can also be drug-induced (by selective serotonin-reuptake inhibitors, thiazide diuretics, anticonvulsants) or may even occur as a clinical manifestation of some hormone-producing malignant tumors (e.g., small-cell lung cancer).

Lack of vasopressin due to impaired synthesis, transport, or release leads to the development of central diabetes insipidus, a rare disorder caused by pathological changes in the hypothalamus or/and pituitary gland. In contrast, nephrogenic diabetes insipidus reflects unresponsiveness of the kidneys to water-conserving vasopressin action. Despite their etiological differences, both forms of diabetes insipidus have a similar clinical presentation, comprising polydipsia, polyuria, dehydration, and hypernatremia.

Basic Characteristics

Vasopressin is a cyclic nonapeptide containing an N-terminal cyclic ring core comprising six amino

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acid residues stabilized with a disulfide bridge between residues 1 and 6 and a flexible C-terminal three-residue amidated tail. Structurally, it is very similar to oxytocin, differing from it in only two amino acid residues, in positions 3 and 8.

The vasopressin gene comprises three exons and two introns. In mammals, it is located on the same chromosome as the gene encoding oxytocin, separated by just a few kilobases of sequence and transcribed in the opposite direction to that of oxytocin. It is assumed that the genes for vasopressin and oxytocin descend from the single gene present in Archaemetazoa, the common ancestor of both invertebrates and vertebrates, that was duplicated during evolutionary development.

Vasopressin is synthesized as a pre-prohormone consisting of a signal peptide, the hormone vasopressin itself, the binding protein neurophysin, and the glycosylated peptide copeptin. Due to its stoichiometric generation and stability, copeptin measured in plasma is shown to reliably represent vasopressin release (Christ-Crain 2019). The major site of synthesis is the magnocellular neuroendocrine neurons of the hypothalamic supraoptic and paraventricular nucleus. In the cell bodies of these neurons, the preprohormone is packed into large dense-core vesicles. These vesicles are then transported along the axons to the posterior pituitary gland (de Bree 2000). During axonal transport, the preprohormone is cleaved by proteolytic enzymes, yielding vasopressin that is then stored in vesicles in the posterior pituitary, awaiting osmotic or non-osmotic stimuli that trigger its release (Brown et al. 2013).

Recently, it was shown that some magnocellular neurons axon collaterals project to many sites in the brain, including medial and lateral preoptic area, suprachiasmatic nucleus, lateral habenula, amygdala, locus coeruleus, and arcuate nucleus. These axon collaterals have been implicated in the modulation of different behaviors. As well as containing magnocellular vasopressin neurons, the paraventricular nucleus contains smaller (parvocellular) vasopressin neuroendocrine neurons. Some of these project to the median eminence to regulate ACTH secretion from the anterior pituitary gland; others project centrally and to the spinal cord and are involved in a variety of functions, including cardiovascular regulation and thermoregulation and pain. Vasopressin is also expressed in neurons of the suprachiasmatic nucleus that regulate circadian rhythms and in diverse other populations, including in the olfactory bulbs and retina (Wacker and Ludwig 2019).

Vasopressin secretion from the posterior pituitary increases linearly with plasma osmotic pressure. In short, the magnocellular vasopressin cells are directly osmoreceptive; they express stretch-sensitive ion channels that cause them to be depolarized in response to volume shrinkage that accompanies a rise in extracellular osmotic pressure; they also express specific sodium receptors. In addition, other osmoreceptive neurons in the anterior hypothalamus also sense changes in extracellular osmolality and contribute to stimulation of vasopressin secretion when osmolality rises.

On the other hand, hypotension and hypovolemia are non-osmotic triggers that can increase the secretion of vasopressin. Afferent nerve fibers from cardiopulmonary (volume) baroreceptors located in the atria and pulmonary vasculature synapse within the nucleus of the solitary tract, which interact with neurons of the ventrolateral medulla, including noradrenergic neurons of the A1 cell group. Neurons in both the NTS and in the ventrolateral medulla project directly to the magnocellular neurons of the SON and PVN, and vasopressin neurons in particular receive a strong projection from A1 neurons. Increases in plasma volume normally inhibit the secretion of vasopressin; conversely, under conditions that lead to hypovolemia or a decrease in central venous pressure, atrial pressure and the atrial receptor firing rate decrease, inducing the release of vasopressin from the pituitary gland.

Activation of angiotensin II receptors in the hypothalamus, stress, pain, nausea, and vomiting, as well as some pharmacologically active substances such as nicotine, tricyclic antidepressants, certain antineoplastic drugs, and apomorphine, may also cause the release of vasopressin.

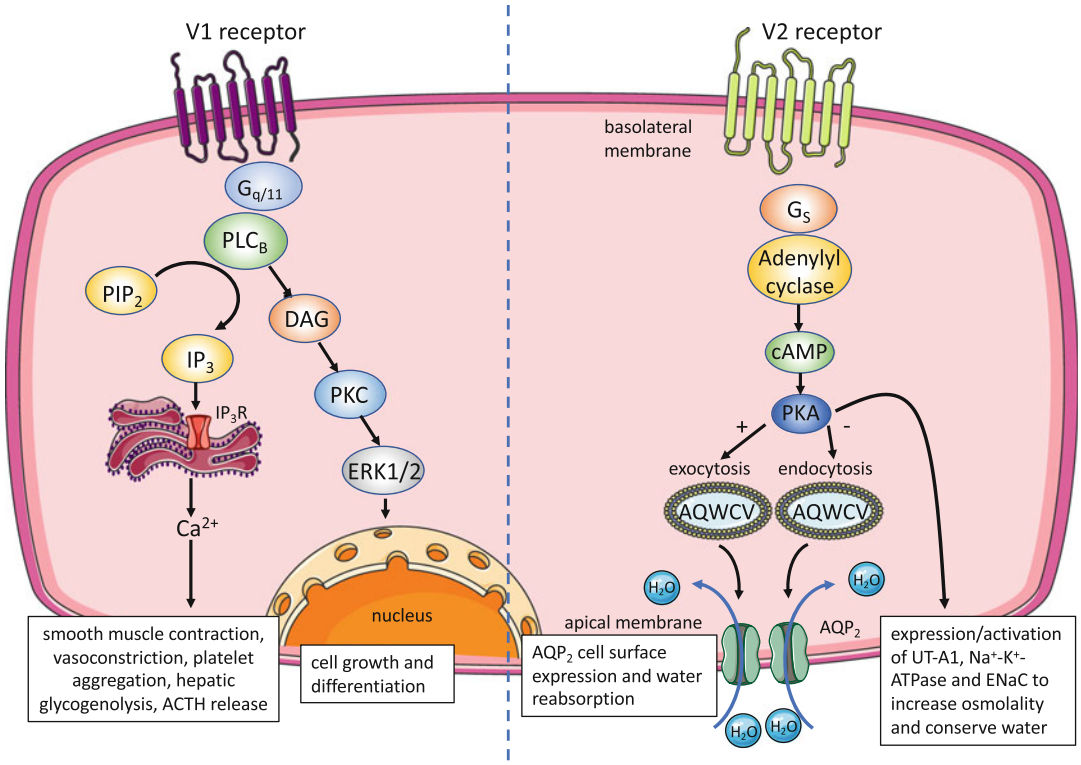
The secretion of vasopressin is inhibited by ethanol, atrial natriuretic peptide, dynorphin, low doses of morphine, dopamine receptor antagonists

(fluphenazine, haloperidol, or promethazine), and anticonvulsive drugs (phenytoin, carbamazepine).

Vasopressin Receptor Activation

Vasopressin elicits its numerous physiological and pharmacological effects through three types of transmembrane, rhodopsin-β adrenergic (type A) G protein-coupled receptors, namely, V1a, V1b, and V2 receptors (Fig. 1). Since the sequence homology between oxytocin and vasopressin receptors is approximately 80% (this high sequence conservation of receptors is especially noticeable in extracellular binding domains), vasopressin may also act as a ligand for oxytocin receptor (Manning et al. 2012).

Vasopressin binding to V1a and V1b receptors activates the Gq-(phospholipase C) PLC- (inositol-3-phosphate) IP_3 pathway (Koshimizu et al. 2012). Once activated, this pathway further leads to mobilization of intracellular Ca^{2+} and activation of protein kinase C (PKC). PKC and Ca^{2+}/calmodulin-activated protein kinases phosphorylate cell-type specific proteins, eventually leading to cellular responses, including vasoconstriction, glycogenolysis, platelet aggregation, ACTH release, and growth responses in smooth muscle cells. Activation of V1 receptors also mobilizes phospholipases A and D (PLA and PLD), which



Vasopressin, Fig. 1 Vasopressin V1 receptor (left side) and V2 receptor (ride side) signalling cascades. Binding to the V1 receptor (V1a and V1b) stimulates membrane-bound phospholipase (PLC_B) via stimulation of a G-coupled protein (G_{q11}), which in turn results in inositol triphosphate (IP_3) formation and mobilization of intracellular Ca^{2+}. A separate phosphorylation cascade occurs via diacylglycerol (DAG). DAG activates protein kinase C (PKC) and ERK1/2 pathway. The binding of vasopressin to the V2 vasopressin receptor stimulates a G_s-coupled protein that activates adenylyl cyclase, in turn causing production of cAMP to activate protein kinase A (PKA). This pathway increases in aquaporin 2 (AQP2) channel formation and apical membrane insertion by increasing the exocytosis of aquaporin water channel-containing vesicles (AQMCV) and inhibiting endocytosis of the vesicles

results in the production of arachidonic acid. Arachidonic acid provides a substrate for eicosanoid synthesis by the cyclooxygenase and lipoxygenase pathways. These locally produced prostaglandins, leukotrienes, and thromboxanes may further activate whole array of signalling pathways.

V1a receptors are present throughout the body. They can be found in vascular and gastrointestinal smooth muscle cells, liver, renal medulla, platelets, myometrium, iris, and various brain regions. V1b receptors are located in the anterior pituitary, kidneys, pancreas, adrenal medulla, and brain.

The V2 receptor activates the Gs protein and adenylate cyclase signalling pathway. Adenylate cyclase converts ATP to cyclic adenosine monophosphate (cAMP), a key signalling molecule in this pathway. In addition, stimulation of V2 receptors also leads to activation of the ERK1/2 MAP kinase pathway, although the exact physiological or pathophysiological importance of V2-regulated MAP kinase pathways needs to be further elucidated.

V2 receptors are most abundant in basolateral membranes of the collecting tubule cells in kidneys. They can also be found on the thick ascending limb of the loop of Henle and in endothelial cells.

Vasopressin Effects

Antidiuretic Action

Acting on V2 receptors, vasopressin increases the permeability to water of the collecting duct cells in the kidney, allowing water reabsorption and excretion of concentrated urine, i.e., antidiuresis. This occurs through the insertion of water channels, aquaporin-2 (AQP2), into the apical membrane of the collecting duct epithelial cells. Consequently, water moves out of the nephron, increasing the amount of water reabsorbed from the forming urine back into the bloodstream. Vasopressin increases transcription of the aquaporin-2 gene, increasing the number of aquaporin-2 molecules in collecting duct cells. Vasopressin also increases the permeability of the inner medullary portion of the collecting duct to urea, allowing increased reabsorption of urea into the medullary interstitium. Finally, vasopressin stimulates sodium and chloride reabsorption in the thick ascending limb of the loop of Henle. This antidiuretic action is crucial for osmoregulation, and defects in vasopressin production, secretion, or action result in diabetes insipidus, characterized by excessive thirst and frequent passing of large volumes of dilute urine.

Osmoreception

Magnocellular vasopressin neurons in the hypothalamus are the "command" neurons that regulate antidiuresis, and the rate of spike discharge increases as extracellular osmolality increases. As a result, vasopressin concentrations in the plasma are proportional to the plasma osmotic pressure above a threshold, known as the "set point," for osmoregulated vasopressin secretion. The set point can vary in different physiological circumstances: for example, in pregnancy the set point is lowered; as a result, more water is retained, allowing for an expansion of plasma volume, and this is accompanied by a lower plasma sodium concentration (Leng et al. 2012).

Cardiovascular Function

Vasopressin is implicated in the maintenance of cardiovascular homeostasis both through its peripheral and central actions. Acting as a vasoactive peptide in periphery, vasopressin controls vascular tone through direct activation of V1a receptors, modulation of ATP-sensitive K^+ channels (KATP), modulation of endothelial nitric oxide system, and potentiation of other vasoactive substances, like noradrenaline or angiotensin II.

Centrally, vasopressin modulates autonomic control of cardiovascular system. Paraventricular parvocellular neurons containing vasopressin project to the intermediolateral column of the spinal cord, regulating renal sympathetic activity, diuresis, and vascular tone. Also, vasopressin modulates baroreceptor reflex sensitivity through area postrema and nucleus of the solitary tract.

Behavioral Effects

Vasopressin does not cross the blood-brain barrier in appreciable amounts, so the central actions of

the hormone reflect the central release, including that from the dendrites of the magnocellular neurons (Ludwig and Leng 2006). Vasopressin has effects on social behavior that seem to complement oxytocin effects; in male prairie voles, vasopressin enhances the expression of aggression toward other males – behavior that seems analogous to territorial behavior designed to protect a mate from access by other males or to defend a nest (Donaldson and Young 2008). The brain vasopressin system also mediates anxiogenic effects (Neumann and Landgraf 2012).

Drugs

Agonists

Vasopressin (synthetic 8-L-arginine vasopressin) is clinically registered for use as a replacement therapy in the treatment of central diabetes insipidus. Due to its short half-life and non-selectivity for V2 receptor, it has been generally replaced by its synthetic analogue desmopressin.

The propensity of vasopressin to cause contraction of smooth muscles in blood vessels and gastrointestinal tract has been used in the management of esophageal variceal bleeding and postoperative ileus and to reduce the possibility of intraoperative bleeding in patients with portal hypertension. Furthermore, vasopressin can be used as a second-line agent during cardiopulmonary resuscitation (CPR) in adults.

Vasopressin increases peripheral vascular resistance and consequently arterial blood pressure, and this can be important for restoring blood pressure in hypovolemic shock and septic shock. Patients with vasodilatory shock are reported to have inappropriately low plasma levels of vasopressin and to be particularly sensitive to the pressor actions of vasopressin. Therefore, vasopressin is indicated for the treatment of hypotension in patients with vasodilatory shock that does not properly respond to therapy with catecholamines and fluids.

The majority of vasopressin side effects are the result of V1a activation, and they include gastrointestinal disturbances due to increased gastrointestinal motility, such as nausea, cramps, and urgency to defecate. Vasopressin can aggravate symptoms of coronary artery disease, cause arrhythmia, and decrease cardiac output. Peripheral vasoconstriction and even gangrene may occur after application of high doses of vasopressin. The most prominent V2 receptor-mediated side effect of vasopressin is water intoxication.

Desmopressin (dDAVP) is a synthetic, long-acting analogue of vasopressin, modified by deamination of cysteine-1 and substitution of L-arginine-8 with D-arginine. Apart from having a longer half-life (~158 min) and duration of action (6–24 h) in comparison to vasopressin, desmopressin is more potent antidiuretic agent, with fewer pressor effects due to higher selectivity for V2 receptor. In fact, desmopressin is 10 times more efficient as an antidiuretic agent than vasopressin, with 1500 times less pronounced ability to induce vasoconstriction. These characteristics make desmopressin the drug of choice in the treatment of central diabetes insipidus (desmopressin is not effective in the treatment of nephrogenic diabetes insipidus and can even be used as a diagnostic method to distinguish between two forms of diabetes insipidus).

In addition, desmopressin is used for primary nocturnal enuresis in both children and adults and enuresis in multiple sclerosis, as well as in idiopathic enuresis. It can also be applied to the management of polyuria and polydipsia occurring after surgical procedures. By activating extrarenal V2 receptors, desmopressin stimulates secretion of procoagulant factor VIII and von Willebrand factor from vascular endothelium into circulation and can therefore be applied to the treatment of mild to moderate hemophilia and von Willebrand disease. The most common side effects of desmopressin include hyponatremia, seizures that may develop as a consequence of hyponatremia, headache, nausea, decreased urinary output, and facial flushing.

Terlipressin is an analogue of vasopressin that shows affinity toward V1a receptors located in the splanchnic smooth muscle cells. Inducing splanchnic vasoconstriction, terlipressin increases renal blood flow, has beneficial effects on hepatorenal syndrome, and also reduces portal pressure and reduces the risk of portal

hypertensive bleeding. Like vasopressin, it is used in the management of variceal bleeding.

Antagonists

Despite various attempts to develop both peptide and non-peptide vasopressin receptor antagonists, to this day only two non-peptide antagonists have been approved for use by regulatory agencies in Europe, Canada, and the USA. These antagonists are called "vaptans" and "aquaretics," the latter due to the tendency of these agents to increase free water excretion in kidneys with no change in sodium or potassium reabsorption.

Conivaptan is a non-selective V1a/V2 receptor antagonist, available only for parenteral (intravenous) administration. It is extensively protein bound, with a half-life between 6 h and 10 h, and is metabolized by the hepatic CYP3A4 system, with minimal excretion by the kidney. It is used in the treatment of hyponatremia in SIADH and in the treatment of hospitalized patients with euvolemic or hypervolemic hyponatremia.

Tolvaptan is a highly selective V2 receptor antagonist with pharmacokinetic properties comparable to those of conivaptan. Like conivaptan, it can be used in patients with SIADH who show signs of hyponatremia and in hypervolemic and euvolemic hyponatremia.

The most dangerous adverse effects of vaptans result from the rapid correction of hyponatremia, with osmotic demyelinating syndrome potentially having fatal consequences. Others include liver damage, polyuria with dehydration, xerostomia, hypotension and dizziness, headaches, gastrointestinal disturbances, and hypokalemia.

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Ventral Tegmental Area (VTA)

▶ Dopamine System

Viral Peptidases

▶ Viral Proteases

Viral Proteases

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Synonyms

Viral peptidases; Viral proteinases

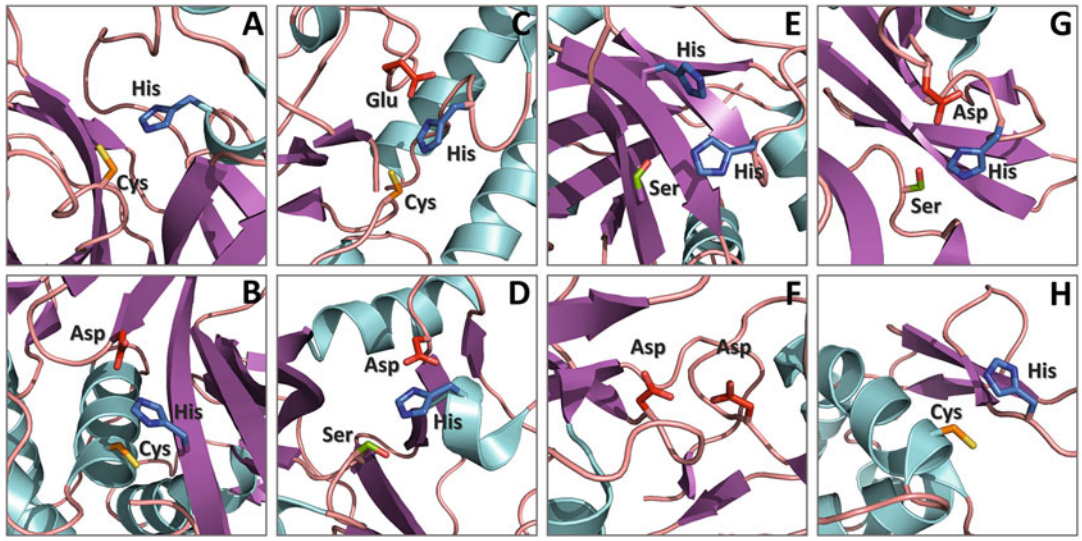
Definition

Viral proteases are proteins encoded by the genome of some viruses that exhibit catalytic activity leading to the hydrolysis of peptide bonds at specific sites within the polypeptide chain. The activity of viral proteases is crucial for a successful completion of the virus replication cycle.

Basic Characteristics

Proteases (peptidases, proteinases) compose a large class of enzymes that catalyze the cleavage of the peptide bond (mainly via the hydrolytic mechanism). Although proteases can be found in all living organisms, they are also encoded by genomes of many viruses. The catalytic activity of proteases is crucial for many biological processes such as protein processing and catabolism, coagulation of blood, immune response, cell signaling, or apoptosis. The enzymatic reaction of proteases is executed by the nucleophilic attack of the catalytic residue (such as serine, cysteine, or threonine) or a water molecule on the carbonyl carbon of the cleaved peptide bond of the substrate. The nucleophile is activated by the neighboring amino acid residue (mainly histidine) or metal ion (e.g., zinc) present in the active center. The structure of the active center as well as specific substrate-oriented binding sites located on the surface of the enzyme allows the hydrolyzed peptide bond to achieve an optimal orientation in close proximity of the enzyme catalytic machinery for the reaction to take place. Considering the catalytic type, proteases can be classified into six groups: serine, threonine, cysteine, aspartic, glutamic, and metalloproteases. The cleavage of the peptide bond can also be catalyzed by the asparagine peptide lyases, i.e., enzymes which break peptide bond utilizing the mechanism of elimination reaction rather than hydrolysis. Additionally, the classification of proteases can be based on other characteristics such as the protein fold (describing topology of the tertiary structure with one of the most common being chymotrypsin-like fold with the characteristic two-β-barrel motif) and the organization of the catalytic center (e.g., catalytic dyad or triad, including not only the number and type of amino acids in the active center but also their order in the amino acid sequence which are the basis of the MEROPS classification system of proteolytic enzymes) (Rawlings et al. 2018) (Fig. 1).

Viruses compose a highly diverse group of infectious agents that invade all types of cellular life forms. The viral replication cycle is possible only intracellularly and is, at least partially, dependent on the host-cell biosynthetic and energetic pathways. Viral genomes are very diverse in terms of their content and organization. The genetic material that has a form of single- or double-stranded RNA or DNA includes at least one open reading frame translated into polyprotein. Proteins encoded by the viral genome are broadly described as structural and/or nonstructural. The first group includes proteins forming capsid and in many cases other proteins involved in the formation of the viral particle such as envelope-building proteins. On the other hand, the nonstructural proteins are important for virus replication, playing regulatory or catalytic functions. The most common enzymes encoded by the viral genomes are polymerases, helicases, and proteases (Mesters et al. 2006). Interestingly, some viruses of the *Filoviridae*, *Paramyxoviridae*, or *Pneumoviridae* families do not show protease activity in their genetic material and are fully dependent on the host proteolytic enzymes (e.g., furin, cathepsins). Still, some viruses code in their genomes two or even more proteolytic activities. The viral proteases constitute a highly diverse group of enzymes that belongs for the most part of cases to cysteine (e.g., severe acute respiratory syndrome coronavirus, SARS-CoV) and serine (e.g., Zika virus, ZIKV) catalytic types. The representatives of other types such as metallo (e.g., variola viruses, VARV), aspartyl (e.g., human immunodeficiency viruses, HIV), glutamyl (e.g., strawberry mottle virus, SMoV) proteases, or asparagine peptide lyases (e.g., poliovirus, PV) are also known (Table 1). Based on the structure organization, the viral proteolytic enzymes are mainly described as chymotrypsin-, papain-, or pepsin-like. Considering the biological characteristics of viral



Viral Proteases, Fig. 1 Structure of the catalytic center of selected viral proteases; the catalytic residues are presented with sticks representation. (**a**) SARS CoV-2 M^{pro} (7jp1.pdb); (**b**) SARS CoV-2 PL (6wx4.pdb); (**c**) HCV NS2 (2hd0.pdb); (**d**) HCV NS3/NS4A (4i31.pdb); (**e**) HSV VP24 (1at3.pdb); (**f**) HIV-1 retropepsin (3gi6.pdb); (**g**) CHIKV CP (5 h23.pdb) (**h**) CHIKV nsP2 (3trk.pdb)

proteases in terms of their substrate specificity, biological activity and function, as well as mechanism of regulation, great differences are observed between viral families. The most common function of viral proteases is the hydrolytic cleavage of viral polyprotein leading to the formation of structural and nonstructural viral proteins and their further processing and maturation. The activity of some viral proteases is also important for proper capsid assembly and maturation that is crucial for the release of new functional virions. The viral proteases can acquire control over cellular processes which allows the virus to shift the biosynthetic machinery of the cell into multiplication of the virus particles. The activity of these enzymes might be regulated by various mechanisms including the presence of a cofactor (e.g., flavivirus protease-cofactor complex, NS2B/NS3), dimerization (HIV protease, HIV PR), membrane association (e.g., coronavirus papain-like protease, PL^{pro}), or self-inhibition (e.g., Chikungunya virus capsid protein, CHIKV CP) (Rawlings et al. 2018).

Drugs

Aspartyl Proteases

HIV-1 Protease

Although HIV-1 was discovered nearly 40 years ago, it is still causing serious health problems worldwide. In 2018 alone, approximately 1.7 million new cases were identified accounting for about 37.9 million people living with HIV around the world. The hydrolase encoded by the HIV-1 is an aspartic protease with a characteristic Asp-Thr-Gly catalytic triad. This enzyme is able to hydrolyze several peptide bonds located in the Gag and Gag-Pro-Pol precursors (Swanstrom and Wills 1997). This activity is required for viral polyprotein processing crucial for the maturation of virus particles. Thus HIV-1 protease has become an attractive molecular target for the anti-HIV-1 agent development.

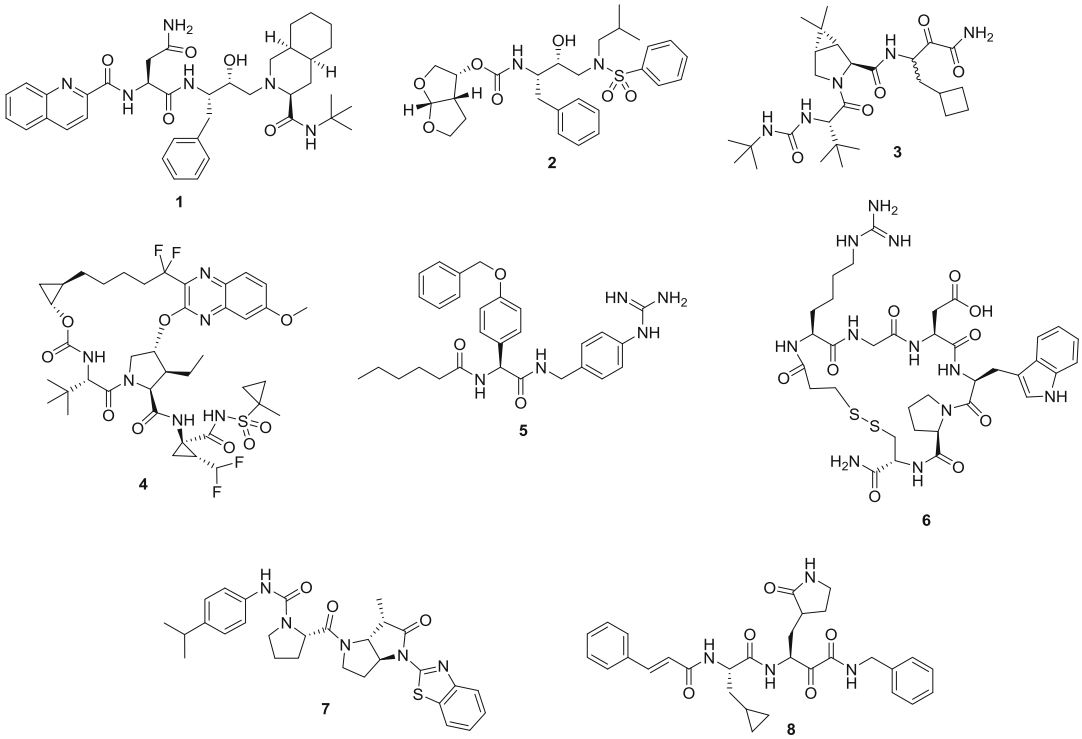
The catalytically active HIV-1 protease functions as a homodimer of which each monomer donates the catalytic residue (Asp25/Asp25′).

Viral Proteases, Table 1 The representatives of the human-infecting viruses expressing proteolytically active proteins (Mesters et al. 2006)

Family	Representative	Protease name	Catalytic type	Catalytic center
Adenoviridae	Human adenovirus C	Adenain (L3)	Cysteine	His-Asp-Cys
Astroviridae	Human astrovirus	Human astrovirus protease ($HAstV^{pro}$)	Serine	His-Asp-Ser
Caliciviridae	Norwalk virus	Calicivirin ($3CL^{pro}$, NS6)	Cysteine	His-Glu-Cys
Coronaviridae	Severe acute respiratory syndrome coronavirus	SARS-CoV papain-like peptidase (SARS-CoV PL^{pro})	Cysteine	Cys-His-Asp
		SARS-CoV picornain 3CLpro (SARS-CoV M^{pro})	Cysteine	His-Cys
Flaviviridae	Hepatitis C virus	HCV peptidase 2 (NS2)	Cysteine	His-Glu-Cys
		Hepacivirin (NS3/NS4A)	Serine	His-Asp-Ser
Herpesviridae	Herpes simplex virus	Assemblin (VP24)	Serine	His-Ser-His
Matonaviridae	Rubella virus	Rubella virus peptidase	Cysteine	Cys-His
Orthomyxoviridae	Influenza A virus	Influenza A PA peptidase	Serine	Ser
Picornaviridae	Poliovirus	Picornavirus capsid VP0-type self-cleaving protein	Asparagine lyase	Asn
		Enterovirus picornain 2A	Cysteine	His-Asp-Cys
		Poliovirus-type picornain 3C ($PV\ 3C^{pro}$)	Cysteine	His-Glu-Cys
Poxviridae	Variola virus	Pox virus metallopeptidase (G1L)	Metallo	Zn
		Vaccinia virus I7L processing peptidase	Cysteine	His-Asp-Cys
Retroviridae	Human immunodeficiency virus 1	HIV-1 retropepsin	Aspartic	Asp-Asp
Togaviridae	Chikungunya virus	Sindbis virus-type nsP2 peptidase	Cysteine	Cys-His
		Togavirin, capsid protease (CP)	Serine	His-Asp-Ser

The substrate binding site is located in a long cleft between both subunits. The HIV-1 protease contains the structural motif of two interlocking loops similar to eukaryotic aspartic proteinases (Lapatto et al. 1989). As for the general substrate specificity of HIV-1 protease, the P1 position is usually occupied by the hydrophobic residues, and the P2 position is commonly occupied by amino acids with branched side chains. The P1′ residue is proline or an amino acid with an aromatic side chain. This structural substrate recognition pattern is quite frequently disturbed. For example, in the p2-NC (p2-nucleocapsid protein) cleavage site, methionine residues are present at P1 and P1' positions.

Saquinavir (**1**, Fig. 2) – the first HIV-1 protease inhibitor – was approved by FDA in 1995. Since then extensive research brought to the drug market several HIV-1 protease inhibitors (PIs) of which nine are currently used in highly active antiretroviral therapy (HAART). In general, the HIV-1 protease inhibitors can be divided into two



Viral Proteases, Fig. 2 Selected inhibitors targeting various viral proteases

groups. The first-generation inhibitors were peptide-based transition state analogs containing hydroxyethylene or hydroxyethylamine function which bind into the enzyme catalytic site. Further studies resulted in a second generation of inhibitors such as lopinavir, atazanavir, tipranavir, and most recently approved HIV-1 protease inhibitor – darunavir. The biggest challenge in the development of HIV-1 protease inhibitors is the design of compounds active also against drug-resistant virus strains. One of the strategies used to fulfil such requirements is to design PIs able to form an extensive hydrogen-bonding interaction network with the backbone atoms located within the HIV-1 protease active site (Ghosh et al. 2016).

Serine Proteases

Hepatitis C Protease

It has been estimated that 71 million people live with chronic hepatitis C virus (HCV) infection worldwide. Although hepatitis C occurs globally, multiple strains or genotypes of various regional distribution have been distinguished. While many patients remain asymptomatic, chronically infected patients have a high chance of developing liver cirrhosis. In 2016 approximately 399,000 people died from hepatitis C-related liver diseases which is a serious consideration around the world. Fortunately, the available antiviral drugs can cure approximately 95% patients with hepatitis C infection.

One of the effective anti-HCV therapies relies on the inhibition of viral serine protease essential for replication. This bifunctional nonstructural protein 3 (NS3/4A) is composed of a functional element (NS3) and its cofactor (NS4A). Interestingly, it has two separate functional activities: serine protease activity and NTPase/RNA helicase activity, both of which located in a single peptide chain and both indispensable for viral polyprotein processing, RNA replication, and new virions formation (Morikawa et al. 2011).

The active site of NS3/4A protease domain is made up by the classical serine protease catalytic triad (His, Asp, and Ser) and represents a chymotrypsin-like fold. This enzyme also contains a zinc binding motif which stabilizes the enzyme structure. The preferably recognized substrate sequence motif is Asp/Glu-X-X-X-X-Cys/Thr↓Ser/Ala-X-X-X (scissile peptide bond is marked by an arrow).

Both the NS3 protein and its cofactor contain α-helical fragments that reassure the membrane association of the complex required for the viral genome replication. Firstly, the *cis* cleavage at the NS3-cofactor junction site is catalyzed and subsequently the polyprotein sequence is processed. The host-cell proteases (e.g., furin, signal peptidase) and other viral proteases can be involved in this process. For example, the NS3/NS4A is not the only protease involved in the HCV replication cycle, and the second proteolytic activity associated with the cysteine protease NS2 responsible for the cleavage between NS2 and NS3 is necessary. The biological role of NS3/NS4A goes beyond the processing of viral polyprotein. Since NS3/4A protease is able to destroy several important cellular proteins such as mitochondrial antiviral signaling protein (MAVS), T-cell protein tyrosine phosphatase (TC-PTP), and TIR domain-containing adaptor inducing IFN-β (TRIF), it blocks the innate immune pathways and modulates the growth factor signaling pathways, thus significantly influencing the persistence and pathogenesis of HCV infection (Morikawa et al. 2011).

In 2003, the NS3/4A protease inhibitor (BILN-2061) was shown to be effective for HCV treatment in a clinical proof-of-concept study. Three years later telaprevir (VX-950) and boceprevir (SCH-503034) were reported to show high potency and effectivity as NS3/4A inhibitors in HCV infections. These two inhibitors form a covalent but reversible bond with the catalytic serine residue located in the NS3 protease active site. In 2011 the FDA approved both drugs for the treatment of HCV infection in combination with PEG-IFN-α and ribavirin. A new generation of non-covalent reversible inhibitors of NS3/4A protease have recently been approved for the HCV treatment including simeprevir, paritaprevir, grazoprevir, glecaprevir, and voxilaprevir (**4**) which are used in combination with other drugs (USA) as well as vaniprevir and asunaprevir (approved in Japan). Although these therapies show a great promise, they are out of reach for many patients due to their high cost. The discovery of NS3/4A protease inhibitor-resistant strains of HCV highlights the need for further studies in this area (Sarrazin et al. 2012; Manns et al. 2017).

Despite the fact that almost all human-infecting viruses carry the information of the protease in their genetic material, until now only HIV-1 and HCV viral proteases have been used as drug targets in clinical practice. We believe that since many research groups have already developed a number of inhibitors active against viral proteases, the breakthrough discoveries in this area are expected in the years to come.

Flavivirus Protease

To the *Flaviviridae* family (*Flavivirus* genus) belong viruses responsible for the development of life-threatening diseases and human mortality such as West Nile virus (WNV), dengue virus (DENV), yellow fever virus (YF), tick-borne encephalitis (TBE), Japanese encephalitis virus (JEV), St Louis encephalitis (SLE), Murray Valley encephalitis (MVE), or Zika virus (ZIKV). These viruses express serine protease – flavivirin (NS2B-3, NS2B/NS3 endopeptidase). The NS3 protease complexed with the NS2B cofactor is responsible for the cleavage of virus polyprotein leading to the generation of the N-terminal nonstructural proteins (NS2B, NS3, NS4A, and NS5) and participates in the processing of the C-terminal regions of the capsid proteins.

NS2B/NS3 is a trypsin-like serine protease with a classic canonical catalytic triad (His, Asp, and Ser). The active NS2B/NS3 protease possesses a chymotrypsin-like fold with two barrels. NS2B cofactor takes part in forming the substrate binding site and directly interacts with the protease substrate: it forms one strand of the N-terminal and two strands of the C-terminal barrels. The NS2B/NS3 flavivirus peptidases recognize basic amino acid residues at the P1 and P2 positions (Lys or Arg, respectively) and small amino acid

residue side chains (Gly, Ala, Ser, Thr) at P1′ (Luo et al. 2015).

The structure of already known potent inhibitors of NS2B/NS3 protease encompasses combination of basic peptides containing a C-terminal electrophilic warhead such as an aldehyde, boronic acid, amide, or phosphonate ester which interact with the catalytic serine residue. Their application as potential drugs is limited by their poor pharmacokinetic properties resulting from their high molecular weight and pronounced polarity of highly basic amino acid side chains. The structure-activity relationships studies focused on the development of DENV and WNV protease inhibitors have not yet delivered any compounds useful for clinical application. Recently Klain et al. developed a new class of dengue and West Nile virus protease inhibitors displaying a submicromolar activity. These compounds are decarboxylated peptides containing a C-terminal 4-guanidinobenzylamino group which is responsible for interacting with S1 pocket of the protease. The most potent inhibitor obtained in these studies (**5**) showed a EC_{50} value of 0.5 μM in cell-based assays with no relevant cytotoxicity (Kühl et al. 2020).

Alphavirus Protease

Alphaviruses similarly to flaviruses are mostly transmitted via bite of an infected mosquito, while replication occurs alternately in a vertebrate host and a hematophagous arthropod. To the alphavirus genus belong among others O'nyongnyong (ONNV), Sindbis (SINV), Semliki Forest (SFV), Venezuelan equine encephalitis (VEEV), Ross River (RRV), and Chikungunya (CHIKV) viruses. The characteristic feature of all alphaviruses is a multifunctional capsid protein (CP) which is crucial for the viral life cycle. Two domains can be distinguished in the structure of capsid protein: unstructured RNA binding N-terminal segment (residues 1–118) and the C-terminal globular protease domain with a typical serine-histidine-aspartic acid catalytic triad (residues 119–267) (Baron 1996). CP protease has a chymotrypsin-like fold, however; viral CP protease has two β-strands instead of the C-terminal α-helix present in chymotrypsin. Such unique structure allows for bending the protein chain in a way that the catalytic center gets access to the substrate cleavage site. After autoproteolytic cleavage, the side chain of tryptophan residue remains bound in a hydrophobic S1 pocket of CP protease, and newly generated C-terminal carboxyl group forms a hydrogen bond network with the catalytic site of the enzyme consequently blocking its trans-proteolytic activity. Hence, alphaviral capsid protein undergoes a single proteolytic event before maturation leading to its assembly into the virus capsid shell forming a nucleocapsid core via encapsidation of viral genomic RNA. The interaction of CP protease with the virus envelope glycoproteins is important for the budding process (Owen and Kuhn 1997).

The site of autoproteolytic cleavage (Glu-Gly-X-Glu-X-Trp↓Ser) is highly conserved among all known alphaviruses. Since it has been shown that the proteolytic cleavage is critical for the life cycle of alphaviruses, inhibitors targeting the CP protease are considered potential anti-alphavirus agents. Recently structure-based drug retargeting studies have identified eptifibatide acetate (**6**), P1,P4-Di (adenosine-5′) tetraphosphate, and paromomycin sulfate as potent CHIKV capsid protease inhibitors. These compounds showed half-maximal effective concentrations (EC_{50}) of 4.01 μM, 10.66 μM, and 22.91 μM, respectively. This report was the first to demonstrate that alphavirus CP protease could be successfully blocked by inhibitors opening a way for the development of novel antiviral drugs (Fatma et al. 2020).

Herpesvirus Protease

Herpesviruses are responsible for several medical disorders including cold sores (herpes simplex virus, HSV-1), genital herpes (HSV-2), chicken pox (varicella-zoster virus, VZV), pneumonitis (human cytomegalovirus, CMV), or Kaposi's sarcoma (Kaposi's sarcoma-associated herpes virus, KSHV). The role of the herpesvirus protease is crucial for a successful capsid assembly and packing the viral DNA molecule. Herpesvirus protease is a part of a complex protein scaffold enveloped by the premature viral capsid. After the protease is self-released, it subsequently degrades the protein scaffold leading to its collapse forming space for

the viral DNA molecule. The herpesvirus protease is essential for the production of fully infectious virions; thus the inhibition of its proteolytic activity could potentially prevent the spread of the virus inside infected cells (Holwerda 1997). The core of the protease is composed of a central barrel formed by seven antiparallel β-strands. The catalytic unit located within the active site is serine and two histidine residues which all together form an uncanonical catalytic triad on the surface of the β-barrel. The substrate recognition pattern is similar for all known herpesvirus proteases: the S1 binding pocket accepts small side chains amino acids such as alanine, the P2 position is usually occupied by a hydrophilic side chain, while small aliphatic amino acids such as valine or leucine are preferred as the P3 residues. Although numerous studies reported the development of many herpesvirus protease inhibitors, none, even highly potent inhibitors which are active in enzymatic tests, significantly suppress the replication of the virus in cell-based assays. One of the most active inhibitors of the herpesvirus protease is *trans*-lactams (**7**) (Skoreński and Sieńczyk 2014).

Cysteine Proteases

Coronavirus Protease

In 2003, China saw an outbreak of an unknown disease which spread worldwide. The observed symptoms of atypical pneumonia, named severe acute respiratory syndrome (SARS), resulted in more than 8,000 infections with approximately 800 deaths in 29 countries (Drosten et al. 2003). Later, it was discovered that the infectious agent responsible for this pandemic was a previously unknown coronavirus, named SARS-CoV. Almost 17 years later in December 2019, a new similar to SARS disease surfaced. Further studies showed that the new disease was triggered by a new coronavirus whose genome displayed a 96% and 79.6% sequence identity to a bat coronavirus and to SARS, respectively. This novel virus was named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and the resulting pneumonia and associated symptoms caused by the coronavirus became to be called disease 2019 (COVID-19) (Zhu et al. 2020; Zhou et al. 2020).

SARS viruses encode two proteases which are tightly involved in the virus replication cycle. One of them is a papain-like protease (NSP3), while the other is a chymotrypsin-like protease (3CL-like protease, main protease, M^{pro}). SARS M^{pro} occurs in three quaternary structures: an inactive monomer, active homodimer, and active octamer (Graziano et al. 2006). The catalytic dyad is formed by cysteine and histidine residues. The high protein sequence similarity between main proteases of SARS-CoV and SARS-CoV-2 was observed, and inhibitors active against SARS-CoV M^{pro} were also found active against SARS-CoV-2 M^{pro}. A majority of M^{pro} inhibitors are peptide-based molecules mimicking part of the substrate with a lactam ring (a rigid isostere of glutamine) at P1 position and C-terminal reactive electrophilic warhead such as α-ketoamide (**8**), boronic acid, α-halocarbonyl, acrylamide, sulfonyl chloride, aldehyde, or isatine (Dömling and Gao 2020; Dai et al. 2020). Recently Wang et al. showed that some of the already known inhibitors such as boceprevir GC-376 and calpain inhibitors II and XII were able to suppress SARS-CoV-2 replication in cell culture studies (EC_{50} ranging from 0.49 to 3.37 μM) (Ma et al. 2020).

In addition to M^{pro}, NSP3 protease of SARS-CoV is considered an attractive drug target since, besides its polyprotein processing function, it participates in the attenuation of the host innate immune responses via stripping ubiquitin and ISG15 (interferon-stimulated gene 15) from the host-cell proteins. A wide range of compounds able to form a covalent bond with the enzyme active site has been described including epoxyketones, α-halo-ketones, alkynes, aldehydes, trifluoromethyl ketones, activated esters, and vinyl sulfones (Báez-Santos et al. 2015).

Although cysteine proteases are a protease type abundant in viruses, they do not compose structurally homogenous group as several different types of the catalytic center organization and composition as well as backbone fold have been described (Table 1). One of the well-known representatives of this group is 3C/3C-like peptidases ($3C^{pro}$/$3CL^{pro}$), which are proteases mainly found in *Coronaviridae*, *Caliciviridae*, and *Picornaviridae* families, but 3C or 3C-like

proteases are also conserved among members of *Pisoniviricetes* and *Stelpaviricetes* classes of viruses. The His-Cys dyad (e.g., SARS-CoV $3CL^{pro}$ or M^{pro}, EC 3.4.22.69) or His-Glu/Asp-Cys triad (e.g., PV $3C^{pro}$, EC 3.4.22.28) can be responsible for the enzymatic activity of 3C-like peptidase, while the sequence cleavage preference can be generally described as Gln-Gly (P1-P1′; Schechter and Berger nomenclature) (Schechter and Berger 1967). The 3C peptidases are catalytically active in the monomeric form; however, $3CL^{pro}$ requires a homodimerization in order to become catalytically active (e.g., SARS-CoV $3CL^{pro}$). After autocatalytic cleavage out, the protease subsequently releases other viral proteins from the polyprotein. Various processes of the virus replication cycle are reported to be dependent on the $3C^{pro}/3CL^{pro}$ activity including stimulation of viral RNA synthesis (via direct interaction with protease or its precursors), inhibition of the host-cell transcription and protein synthesis (by degradation of cellular proteins but also disruption of the nucleus-cytoplasm macromolecules trafficking), immune system suppression, or even initiation of apoptosis (Sun et al. 2016).

Apart from 3C proteinase, the *Picornaviridae* family members encode other cysteine proteases, namely, Leader (Lpro) and A2 (A2pro) but also asparagine peptide lyases (picornavirus capsid VP0-type self-cleaving protein). The biological function of these enzymes is not as broad as $3C^{pro}$ and is limited to few processes such as degradation of initiation factors for translation of host cell or immune response modulation, whereas the asparagine lyases catalyze autolytic cleavage of the capsid protein.

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Viral Proteinases

► Viral Proteases

Viral Vectors

► Gene Therapy Vectors

Vitamin A Metabolites

► Retinoids

Vitamin D

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Synonyms

$1,25(OH)_2D_3$; Calcidiol; Calciol; Calcitriol

Definition

Whether vitamin D is absorbed from the intestine or is derived from the epidermis, it is clear that the liver is the first stop for vitamin D where it is 25-hydroxylated to what is clearly recognized as the major circulating form of vitamin D (Jones et al. 2017) (Fig. 1). The enzyme found largely if not exclusively in the liver is Cyp2r1 that carries out this hydroxylation. However, it is also clear in *Cyp2r1* KO mice some 25-hydroxylation of vitamin D occurs. Although several possibilities have been suggested, the source of the remaining 25 hydroxylation remains undetermined.

At physiologic concentrations of between 10 and 100 ng/mL, $25OHD_3$ does not function directly but must be further hydroxylated on carbon one to $1,25(OH)_2D_3$, the final active form of vitamin D before function. It is $1,25(OH)_2D_3$ that binds to the nuclear receptor programming cells to carry out the well-known functions of vitamin D in the intestine, bone, and other tissues.

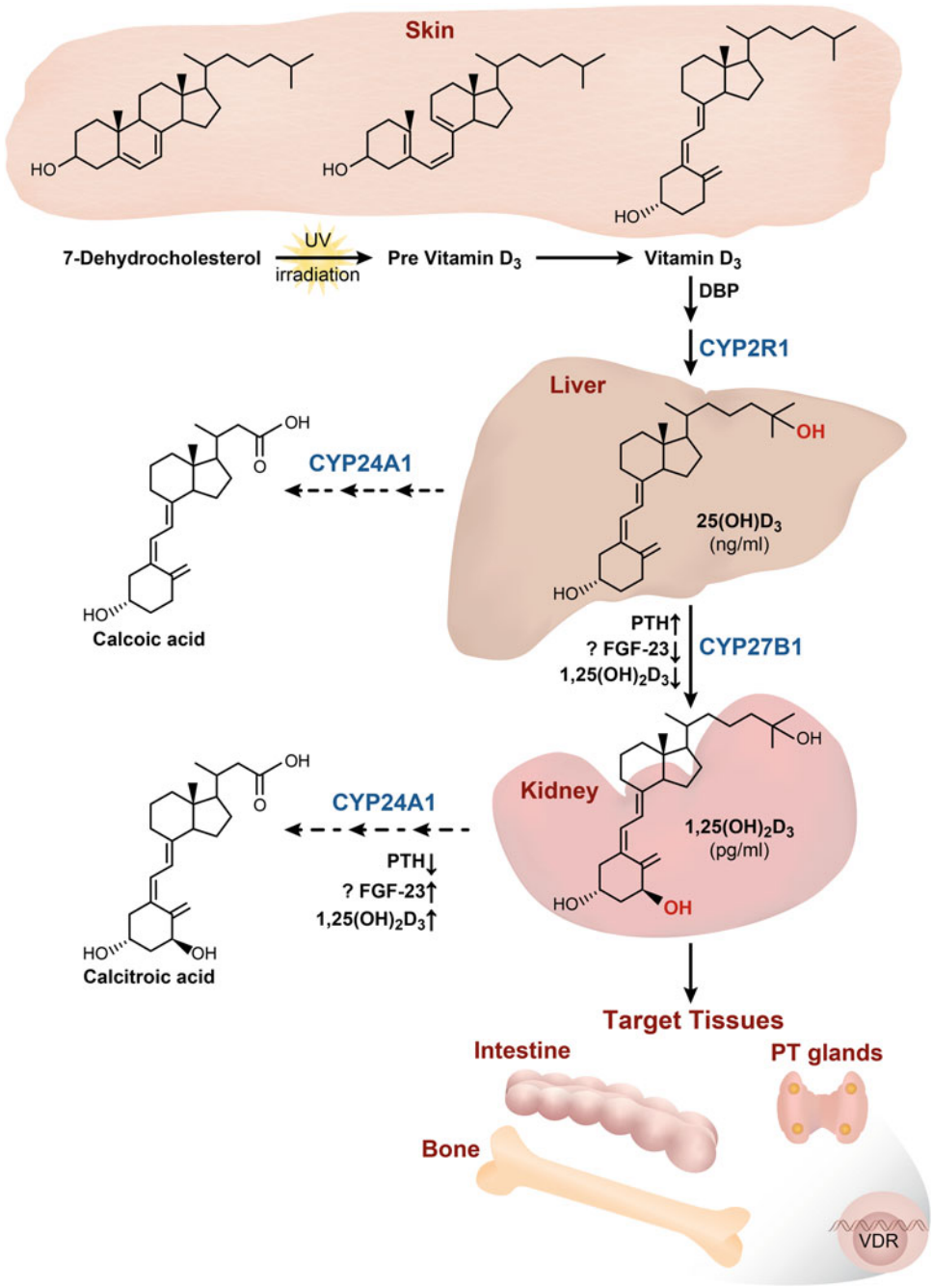
The CYP24A1 enzyme further converts these compounds ultimately to calcitroic acid and calcioic acid (Kaufmann et al. 2019). The CYP24A1 pathway represents the inactivation and elimination pathway of vitamin D metabolism. As described below, the conversion of $25OHD_3$ to $1,25(OH)_2D_3$ or to $24,25(OH)_2D_3$ is tightly regulated by parathyroid hormone and $1,25(OH)_2D_3$ itself and perhaps FGF-23.

Endocrine-derived $1,25(OH)_2D_3$ travels to target tissues. Once inside the target cells, $1,25(OH)_2D_3$ binds the vitamin D receptor. This complex interacts with DNA to alter the expression of a range of genes involved in calcium and phosphorus homeostasis as well as the genes encoding the metabolic enzymes responsible for the production and destruction of $25(OH)D_3$ and $1,25(OH)_2D_3$. More detailed aspects of vitamin D metabolism will be discussed below.

Mechanism of Action

In Vivo Production of Vitamin D

Before the introduction of synthetic vitamin D and/or the fortification of foods by UV irradiation



Vitamin D, Fig. 1 Functional metabolism of vitamin D_3

following the discovery of Steenbock and others, the major source of vitamin D was by sunlight exposure with the exception of some usage of fish liver oils (DeLuca 2014). Sunlight exposure remains the dominant source of vitamin D. However, the exact amount of vitamin D that results from sunlight has been the subject of many papers and disagreement (Holick 2007). So many

factors such as season, distance from the equator, cloud cover, clothing, and skin pigmentation are involved. The angle at which sunlight strikes the skin is of paramount importance. For example, in the winter months of October–April in the northern hemisphere, little or no vitamin D is produced (Webb et al. 1988). A general useful concept is that a 10–20-min exposure of 1/8th body surface to mid-day summer sun will certainly provide an adequate amount of vitamin D (Holick 2007). Dermatologists discourage sun exposure over concern that UV light in excess is carcinogenic. However, a reasonable amount of sun light exposure would provide sufficient vitamin D with a minimum of cancer risk (Webb and Engelsen 2008). Oral intake of vitamin D can certainly be viewed as a great human invention that prevents and treats rickets, osteomalacia, and hypocalcemic tetany, but dermal production represents the true physiologic source of vitamin D.

A new development has been the discovery of how vitamin D produced in the epidermis by UV light is mobilized. Vitamin D binding protein (DBP) is required for this mobilization because *Dbp* -/- mice are unable to utilize vitamin D produced in epidermis by UV light (Duchow et al., 2019). Further, when small amounts of recombinant Dbp are injected in *Dbp* -/- mice, mobilization of vitamin D from skin proceeds. This discovery is supported by a clinical report that a DBP-deficient patient did not respond to UV treatment (Henderson et al. 2019). Thus, DBP now occupies an essential role in the function of vitamin D produced physiologically in the skin. It also focuses on the idea that the synthetic vitamin D in use throughout the world is a superb pharmaceutical solution to disease, but the true physiologic source of vitamin D is the skin produced by the sun's ultraviolet light. Further, vitamin D is not a vitamin since it can be produced in the body.

Metabolic Enzymes and Their Regulation

Enzymes involved in the production of the vitamin D hormone are the 25-hydroxylase (CYP2R1) and 1-hydroxylase (CYP27B1). CYP2R1 is likely the major enzyme that produces $25(OH)D_3$ from vitamin D (Jones et al. 2017). Although other enzymes have been purported to be responsible for 25 hydroxylation, i.e., CYP27A1, CYP2D25, CYP3A4, CYP2C11, and CYP2J1, their involvement is unlikely. Nevertheless, CYP2R1 is probably not the only 25-hydroxylase since mice devoid of Cyp2r1 still produce small amounts of 25OHD as do humans with mutations in the *CYP2R1* gene. So far regulation of CYP2R1 has not been reported. That it is not tightly regulated is suggested by the fact that high blood levels of $25(OH)D_3$ can be produced by providing higher and higher doses of vitamin D_3 (DeLuca et al. 2011). Thus it seems likely that high blood levels of $25(OH)D_3$ are responsible for vitamin D toxicity by acting as an analog of $1,25(OH)_2D_3$ activating genes responsible for calcium absorption and bone mobilization.

CYP27B1 is the sole enzyme responsible for production of $1,25(OH)_2D_3$ from $25(OH)D_3$. When this gene is knocked out or mutated in mice or humans, no $1,25(OH)_2D_3$ is detected. In contrast to the production of $25(OH)D_3$, $1,25(OH)_2D_3$ generation is very tightly regulated in the kidney, and the regulation has been defined at the physiologic level (DeLuca 2014). Meyer et al. (2019) have now mapped the DNA regions important for this regulation. Parathyroid hormone increases the expression of *CYP27B1*, while FGF-23 suppresses renal *CYP27B1*. Not surprisingly, there is a feedback mechanism, so when $1,25(OH)_2D_3$ levels are elevated, the 1-hydroxylase is suppressed. A recent report by Pike and colleagues confirms prior reports indicating the regulators of Cyp27b1 in kidney do not affect expression of Cyp27b1 in non-renal tissues (Meyer et al. 2019). Whether extra-renal production of $1,25(OH)_2D_3$ occurs under physiologic conditions is unclear.

Besides the negative feedback regulation of CYP27B1, control of $25(OH)D_3$ and $1,25(OH)_2D_3$ levels is mediated by another cytochrome P-450, CYP24A1. This enzyme oxidizes the side chains of both molecules, ultimately converting the native hormone to calcitroic acid. Jones and colleagues have also demonstrated $25(OH)D_3$ is converted to an analogous product, calcioic acid (Kaufmann et al. 2019). The discovery of loss-of-function mutations in the *CYP24A1* gene results in hypercalcemia

clearly demonstrates the essential degradation of $1,25(OH)_2D_3$ (Jones et al. 2017). Of great importance is the fact that some of the factors that upregulate CYP27B1 also downregulate CYP24A1 and vice versa, i.e., PTH suppresses and $1,25(OH)_2D_3$ increases CYP24A1 expression.

CYP3A4 is another enzyme that has been reported to contribute to the degradation of the $1,25(OH)_2D_3$ and $25(OH)D_3$. However, individuals with functioning CYP3A4 but with mutations in CYP24A1 still have elevated $1,25(OH)_2D_3$ levels. Only very potent CYP3A4 inducers, such as rifampicin, are able to treat those patients with mutations in CYP24A1 (Hawkes et al. 2017). Of some importance is a recent report of two unrelated human patients that presented with rickets and mutations in the *CYP3A4* gene which resulted in accelerated oxidative removal of $1,25(OH)_2D_3$ while having normal CYP24A1 activity (Roizen et al. 2018).

Conjugation with glucuronides and sulfates in the liver is another proposed catabolic pathway for $25(OH)D_3$ and $1,25(OH)_2D_3$. Conjugates of $25(OH)D_3$ and of $1,25(OH)_2D_3$ have been identified, such as $25OHD_3$-3-*O*-sulfate, $25OHD_3$-3-*O*-glucuronide, and 1,25-glucuronide (Tuckey et al. 2019). It is unclear whether these conjugates play any role except as excretory forms. It has been suggested that they represent enterohepatic circulation, but conclusive evidence supporting this suggestion has not appeared.

Other Metabolites

Lithocholic Acid

Lithocholic acid (LCA) was first identified as a ligand for the nuclear vitamin D receptor nearly 20 years ago. The vitamin D receptor was proposed to act as a bile acid sensor (Makishima et al. 2002). Because of the high concentrations necessary for binding to the VDR, it was proposed to complement the already established role of $1,25(OH)_2D_3$ in the intestine, namely, calcium and phosphate absorption. Subsequent to this finding, the DeLuca laboratory reported that LCA could in fact elevate serum calcium levels in vitamin D-deficient rats (Nehring et al. 2007). However, the concentrations required were nearly 1000X that of $1,25(OH)_2D_3$ calling into question the physiological relevance of this finding.

3-epi-25(OH)D_3 and 3-epi-1,25(OH)$_2D_3$

3-epi-$25(OH)D_3$ and 3-epi-$1,25(OH)_2D_3$ have been reported in human serum. Biologically, these compounds have little activity (Bailey et al. 2013). Whether or not these compounds are generated endogenously or are present as contaminants in the vitamin D preparations remains an important question. Even if 3-epimerization is a bonafide in vivo occurrence, the fact that 3-epimerization markedly reduced biologic activity argues for its lack of importance, except as a possible degradation pathway.

25,26(OH)$_2D_3$ and 1,25,26(OH)$_3D_3$

25,26-Dihydroxy vitamin D_3 was discovered as a metabolite of vitamin D in 1970 (DeLuca et al. 1970). Several laboratories have reported the presence of the 26-hydroxylated forms of $25(OH)D_3$ and $1,25(OH)_2D_3$. In 2001, Endres and DeLuca reported that a contaminant in the ^{3}H-$1,25(OH)_2D_3$ preparation was responsible for the generation of $1,25,26(OH)_3D_3$ in rat (Endres and DeLuca 2001). Thus, it is not clear whether $25,26(OH)_2D_3$ or $1,25,26(OH)_3D_3$ are endogenous metabolites of vitamin D or result from contaminants in the vitamin D preparations. In any case, their biological activities are lower than their respective non-26 hydroxylated counterparts.

24,25(OH)$_2D_3$

$24,25(OH)_2D_3$ is the first product formed when $25(OH)D_3$ is acted on by CYP24A1. This metabolite has recently been proposed to function in the repair of bone fractures, which is a continuation of suggestions made previously (DeLuca 2014). The evidence presented is not convincing, and previous extensive examination of the idea that $24,25(OH)_2D_3$ has bone anabolic activity was quite thoroughly disproved. Overall, it is clear that 24-hydroxylation is the product of CYP24A1 activity and plays a key role in the inactivation of the potent vitamin D metabolites, especially $1,25(OH)_2D_3$ (Masuda et al. 2005).

Clinical Uses

(a) The first clinical area to benefit from the discovery of the vitamin endocrine system is **vitamin D dependent rickets type 1A**. This disorder was discovered by Andreas Prader (Prader et al. 1961) and shown to be an autosomal recessive dysfunction of the *CYP27B1* gene. Before discovery of the vitamin D pathway, clinicians successfully treated this disorder with high doses of vitamin D. This practice generates high blood levels of 25OHD. At serum concentrations of >250 ng/ml, 25OHD acts as an analog of $1\alpha25(OH)_2D$, activating VDR, restoring serum calcium and phosphorus to normal levels, and healing rickets. Current practice is to treat this defect with Ca and 0.5–1.0μg $1,25(OH)_2D_3$ per day (Jones et al. 2017).

(b) **Vitamin D dependent rickets type II** was discovered by Brooks, Balsan, and colleagues and later by extensive recording of several kindreds by Malloy and Feldman (2010). In this disorder high blood levels of $1,25(OH)_2D$ are found with severe rickets. The mutations found are in the vitamin D receptor gene (Malloy and Feldman 2010). In cases where the defect is an early termination codon so no VDR receptor is produced, the only treatment available is the I.V. infusion of calcium (Malloy and Feldman 2010). In other defects where the VDR affinity for $1,25OHD_3$ is altered but still present, high doses of $1,25(OH)_2D$ are more or less effective.

(c) Perhaps the greatest use of the vitamin D metabolites has been in **chronic renal failure patients on dialysis**. In this disorder one of the problems is destruction of the major if not exclusive source (kidney) of $1,25(OH)_2D$ resulting in poor calcium absorption, failure of mineralization, and high levels of parathyroid hormone causing marked bone resorption. Either $1,25(OH)D_3$ or $1OHD_2$ and analogs such as 19-nor-$1,25(OH)_2D_3$ and 22-oxa-$1,25(OH)_2D_3$ have been successfully used to suppress parathyroid hormone production and secretion. $1,25(OH)_2D_3$ with its receptor suppressing the parathyroid gene directly through a VDR binding site on the parathyroid gene. Although oral $1,25(OH)_2D_3$ was initially used for this disorder, on first pass through the intestine, it markedly increases calcium absorption causing unwanted hypercalcemia. This practice was replaced by I.V. $1,25(OH)_2D_3$ which in turn was replaced by less calcemic analogs, i.e., 19-nor-$1,25(OH)D_2$ and $1OHD_2$. Because of cost restriction in the "Bundling" reimbursement for dialysis therapeutics in the USA, "least cost alternative" has resulted in return to low cost $1,25(OH)_2D_3$ for control of secondary hyperparathyroidism without regard to potential hypercalcemia. Nevertheless, 1-hydroxylated vitamin Ds are an important if not essential treatment for renal osteodystrophy.

(d) Another clear application for the 1-hydroxylated vitamin D is **hypoparathyroidism** brought about either by surgery or a genetic circumstance (Gafni and Collins 2019). These patients must be provided with a high calcium intake and a 1-hydroxylated vitamin D to facilitate absorption of calcium. Recall that without parathyroid hormone, the CYP27B1 remains silent in the face of hypocalcemia and little or no $1,25(OH)_2D_3$ is produced. Although parathyroid hormone replacement might be used, its short lifetime, expense, and required intravenous treatment make this approach not practical. Since bone calcium mobilization in vivo by $1,25(OH)_2D_3$ requires PTH, treatment must rely on intestinal calcium absorption.

(e) **Osteoporosis** is another disease area where various vitamin D compounds have been effectively utilized (Cianferotti et al. 2015). It has been especially useful in countries where calcium intake is less than 1 g per day. Although occasional reports that $1,25(OH)_2D_3$ fails to improve either bone density or reduce fractures in post-menopausal women have appeared, these studies are largely flawed by using doses of $1,25(OH)_2D_3$ of 0.25μg/day or less when an estimate of normal production of $1,25(OH)_2D_3$ per day is of the order of 1–2μg/day. In all other studies, impressive increases in bone mass and a reduction of fracture rates

have been achieved with $1,25(OH)_2D_3$, $1\alpha OHD_3$, or ED-71.

(f) **Failed application of 1-hydroxylated vitamin Ds**

One of the most troublesome applications of vitamin D has been in the autoimmune disease multiple sclerosis (MS). This is based largely on the relationship of low sunlight areas with high incidence of the disease (DeLuca and Plum 2017). Animal models of the disease, i.e., experimental autoimmune encephalomyelitis (EAE), have been used to determine if vitamin D plays a role in this disease. The administration of $1,25(OH)_2D_3$ does suppress EAE, but the animals develop hypercalcemia. Clearly hypercalcemia and not vitamin D is responsible for the suppression of EAE. Unfortunately, clinical trials on human MS with high dose vitamin D were initiated resulting in hypercalcemia without benefit to the patient (Hausler and Weber 2019). Quite unexpected is the finding that vitamin D deficiency suppresses EAE as shown in two independent laboratories (DeLuca and Plum 2017). Thus, support for the clinical use of vitamin D in the treatment or prevention of MS is absent. Upon examination of the role of light suppression, a narrow band of ultraviolet light at 313 nm (NBUV) was found effective in suppressing EAE either not altering or slightly increasing serum $25OHD_3$ levels. In a follow-up study, Irving et al. used mice that are unable to synthesize the precursor of vitamin D, i.e., 7-dehydrocholesterol (Irving et al. 2019). These knockout mice responded to the NBUV to the same extent as the wild-type littermates clearly demonstrating that the NBUV suppresses EAE independent of vitamin D. It is not clear what compound mediates the NBUV effect but is under investigation.

(g) **Other possible clinical connections**

A number of investigations have been conducted because of the appearance of the VDR in cells of the immune system (Lang and Aspinall 2017). In particular are the reports that $1,25(OH)_2D_3$ stimulates the synthesis of cathelicidin. Most of the studies carried out are in vitro using nonphysiological concentrations of $1,25(OH)_2D_3$ of at least 10^{-8} M. To our knowledge no report of a well-controlled in vivo effect of vitamin D on suppression of an infectious disease has appeared. An attempt to find an effect of vitamin D on infection of mice by listeria was without success (Baisa et al. 2019). Vitamin D-deficient mice and vitamin D-sufficient mice were equal in their response to listeria infection. A role for vitamin D in immunity has not been found to date despite many suggestions.

The fact that the islet cells of the pancreas are laden with VDR suggests a possible role for vitamin D in type 1 diabetes, an autoimmune disease. Certainly vitamin D-deficient NOD mice develop diabetes more quickly than vitamin D-sufficient NOD mice (Zella et al. 2003). Further vitamin D compounds do suppress diabetes in NOD mice, but unfortunately hypercalcemia is a problem. Thus it is unclear what the role of the vitamin D receptor is in the islet cells and what relationship exists between vitamin D and the development of type 1 diabetes.

Certainly, vitamin D and its functional metabolites play a key if not essential role in calcium and phosphorus metabolism and in prevention of bone disease. It is also an essential element in the control of the parathyroid. So far evidence for a role for vitamin D compounds in immunity or in suppression of autoimmune disorders is lacking. Further the role of the vitamin D receptor in the islet cells of the pancreas and a relationship if any to type 1 diabetes remains unknown.

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Vitamin K

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Synonyms

Phylloquinones (vitamin K1), menaquinones-n (MK-n; vitamin K2), and menadione (vitamin K3, synthetic and intermediate between K1 and MK4). While phylloquinones are synthesized by plants, menaquinones are synthesized in small amounts by human intestinal microbiota using repeated 5-carbon units in the molecule side chain. n stands for the number of 5-carbon units. MK-4 can also be produced by animals (including humans) from phylloquinones and can be found in a number of organs (The Linus Pauling Institute 2014; Biesalski 2016).

Definition

Vitamin K belongs to the group of fat-soluble vitamins. The term "K" refers to its main actions in blood clotting, coagulation, in German named "**K**oagulation."

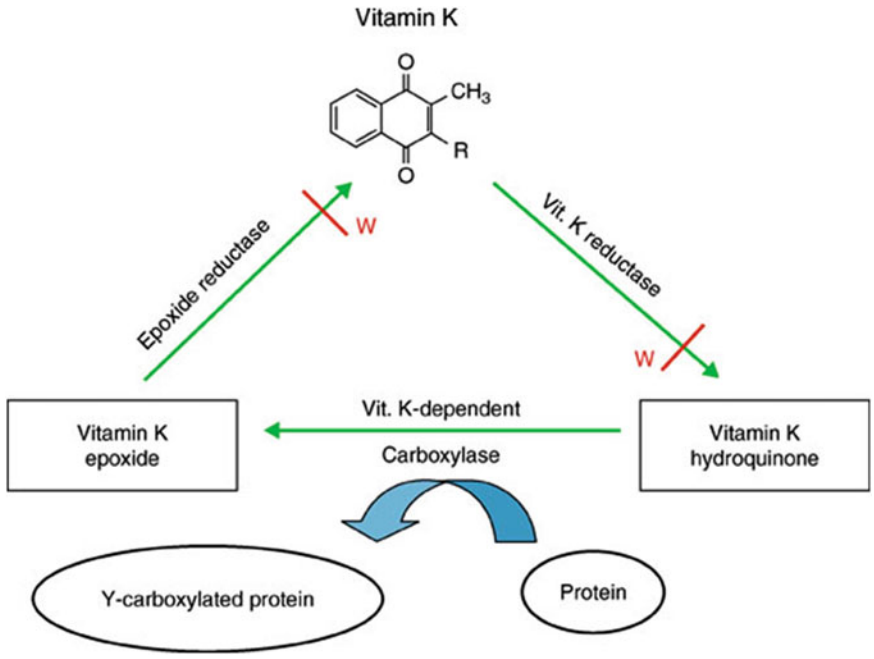
Mechanisms of Action

General Aspects

Although vitamin K is a fat-soluble vitamin, only little stores are found in the body which have to be refilled permanently via dietary input. The role of vitamin K derived from bacteria in the terminal ileum and the colon seems to play a minor role in human nutrition, as the concentration of biliary acids for the resorption of the fat-soluble vitamin K is very low here (Biesalski 2016; Wu et al. 2015). In addition, only diseases of the small intestine lead to a deficit in vitamin K concentration, which cannot be restored by K2 production of colonic bacteria. However, water-soluble vitamin Ks can be resorbed by the colonic mucosa. Maybe because of the little stores for vitamin K, the process of vitamin K-dependent carboxylation of proteins is part of a cycle with several steps during which vitamin K normally is regenerated (see Fig. 1) and thus can be used several times.

Influence on Cell Functions via Gas6

Growth arrest-specific gene 6 (Gas6) is a γ-carboxylated and thus vitamin K-dependent protein found throughout the nervous system and in the heart, lung, stomach, kidneys, and cartilage. It is regarded as a ligand for several families of receptor tyrosine kinases (RTKs). Gas6 plays a role in regulation of cell proliferation, differentiation, migration, and adhesion, release of pro-inflammatory cytokines, platelet aggregation, differentiation of NK cells, adipocyte development, phagocytosis, atherosclerosis, or cancer growth (The Linus Pauling Institute 2014; Wu et al. 2015). Also a role in the developing and the aging nervous system seems realistic (Ferland 2012). Gas6 is hypothesized to act via the Axl receptor with additional effects on adiposity and insulin resistance (Dihingia et al. 2017). However, there are no studies comparing the role of the carboxylated and undercarboxylated form of



Vitamin K, Fig. 1 The vitamin K cycle; w indicates where warfarin or other vitamin K antagonists inhibit the cycle

Gas6 in glucose metabolism and inflammatory pathophysiology.

Coagulation

Binding calcium ions (Ca^{2+}) is a prerequisite for the activation of seven factors in the coagulation cascade that are dependent on vitamin K. The term cascade indicates that the factors involved depend from each other to become activated and to fulfill their special part to stop bleeding. Prothrombin (factor II) and factors VII, IX, and X play central roles in the coagulation cascade; protein Z seems to enhance the thrombin action via promoting its association with phospholipids in cell membranes. The proteins C and S act as anticoagulant proteins such providing an also vitamin K-dependent control and balance of the whole cascade. With both coagulant and anticoagulant factors present in the cascade in the liver, uncontrolled bleeding and/or clotting can well be prevented. On the other hand, liver damage or diseases can disturb this well-controlled cascade, e.g., via reduced clotting factors in the circulating blood resulting in uncontrolled bleeding (hemorrhage) (Olson 1999). One has to be cautious in patients using anticoagulants like phenprocoumon or in elderly persons with vitamin K deficiencies; however, a change in nutrition behavior actually is not recommended.

Bone Mineralization

In bone, several proteins have been described, which are vitamin K-dependent, osteocalcin (bone Gla protein), matrix Gla protein (MGP), Gla-rich protein (GRP; also named Ucma), protein S, and Gas6. Osteocalcin is synthetized by osteoclasts, regulated by the active form of vitamin D, calcitriol. Its capacity to bind calcium needs a vitamin K-dependent γ-carboxylation of three glutamic acid residues. The calcium-binding capacity of osteocalcin indicates its role in synthesis and regulation of bone matrix. Protein S, a coagulant, is also vitamin K dependent in its synthesis by osteoclasts; its role in bone metabolism is still unclear. However, children with reduced protein S levels express enhanced blood clotting and a reduced bone density. MGP has been found in several supportive tissues (bone, cartilage) and in soft tissues including smooth muscle cells in blood vessels. MGP prevents soft tissues (blood vessels) as well as cartilage from calcification (inhibited Ca uptake), while on the other hand, it facilitates bone development and growth (Ca uptake in osteoblasts). Additionally, MGP inhibits bone morphogenic protein2/4 (Wasilewski et al. 2019). Rat studies with warfarin (vitamin K antagonist; see Fig. 1) led to prominent deformations of the skeleton of fetuses as well as newborns including excessive mineralization of growth plates and nasal cartilage and stunted growth. Comparable effects were seen in humans after a therapy with vitamin K antagonists during pregnancy. The so-called warfarin embryopathy includes hypercalcification of the growth plates (chondrodysplasia punctata), a nasal hypoplasia, and disturbances in the growth of facial and hollow bones. The major role of GRP seems to be the Ca regulation in extracellular matrix as an inhibitor of ectopic calcification.

Clinical Use (Including Side Effects)

Vitamin K Deficiency

There are clear-cut differences in deficiencies concerning infants, especially newborns, and adults. Deficiencies of vitamin K are really seldom in healthy adults, because the supply through daily food is (more than) enough, because vitamin K is repeatedly recycled and therefore only a small loss appears, and – although not really accepted – because of the K2 production by intestinal bacteria (see above). On the other hand, those people with severe liver diseases or fat malabsorption or taking vitamin K antagonist anticoagulants have a risk of vitamin K deficiency (cf Schek 2017a). Also, menopausal women are at risk. Symptoms are impaired blood clotting as revealed by routine tests measuring clotting time, unusual bleeding after smaller wounding, nose bleeding, heavy menstrual bleeding, or a great susceptibility to hematoma.

The picture is different concerning infants during the first days of life, especially for those being breastfed (Biesalski 2016). Coagulation-related (vitamin K-dependent) plasma proteins develop

slowly during pregnancy resulting in markedly reduced levels after birth. In addition, human milk is a nutrient low in vitamin K content; the newborns' intestine almost totally lacks menaquinone-synthesizing bacteria, and the vitamin K cycle might not yet be established. Vitamin K supplementation during pregnancy seems not to improve the infant's situation; only VK levels in maternal plasma were enhanced (Shahrook et al. 2018). These deficiencies can lead in healthy newborns to uncontrolled, in worst cases, lethal, intracranial bleedings. Due to that risk, in most countries newborns are supplied with phylloquinones direct postnatally, normally by 1×1 mg injections or by bottle feeding 3×2 mg direct postnatally, between day 3 and 10 and between weeks 4 and 5 (Deutsche Gesellschaft für Neonatologie und Pädiatrische Intensivmedizin 2021).

Osteoporosis

This illness is mainly characterized by an age-related bone loss. The detection of osteocalcin in bone – produced by osteoblasts – was the starting point for a series of studies on the role of vitamin K-dependent proteins in bone development and maintenance and on possible supplementation therapies (see "bone mineralization"). While various studies found a positive correlation between vitamin K status (or intake) and the risk of several fracture types (hip, vertebral, non-vertebral) in several populations, others did not (cf Palermo et al. 2017). Therefore, further prospective studies or clinical trials are needed.

Osteocalcin has been found to be a reliable marker of bone formation, and vitamin K is necessary for the carboxylation of this protein. Therefore, the degree of carboxylation of osteocalcin is a reliable marker for the respective vitamin status (Biesalski 2016). Blood levels of undercarboxylated osteocalcin were found to be higher in postmenopausal women and markedly higher in women over 70. Studies with elderly women resulted in a positive correlation of the blood level of undercarboxylated osteocalcin and the risk of bone fractures. Interestingly, also a correlation of undercarboxylated osteocalcin with the vitamin D nutritional status was described, and a co-supplementation with Ca and vitamin D seems to provide better results in terms of bone mineral density. In addition, K1 may be more effective than MK4 and MK7 (Palermo et al. 2017).

Studies on the effects of an (even long-time) application of vitamin K-antagonistic anticoagulants like warfarin gave no clear-cut results concerning bone density or a changed risk for bone fractures. While negative effects were shown in animal models, in patients with atrial fibrillation or venous thromboembolism, no effect was found in a study with postmenopausal women (cf Palermo et al. 2017).

Supplementation of women >65 years with vitamin MK-7 from Nattō (200μg/day for 3 years) resulted in decreased reduction of bone mineral density (BMD) in the femur, but not in the hip; one over 36 months (180μg/day) had equal results, while 360μg/day for 12 months showed no effects (cf Schek 2017b).

Atherosclerosis

Vitamin K might play a role to counteract the Ca paradox, a loss of BMD on one side together with enhanced arterial calcification in postmenopausal women as well as older men and most prominent in patients with chronic kidney disease on the other side. Vitamin K carboxylates osteocalcin and MGP resulting in more Ca uptake in osteoblasts and reduced uptake in vascular smooth muscle cells, respectively (Wasilewski et al. 2019). However, the role of various fractions of MGP at different vascular sites is further under discussion (Barrett et al. 2018).

Safety and Toxicity

As studies with high supplementation dosages exemplarily show, there is no known toxicity for phylloquinone (K1) or menaquinone (K2) (Biesalski 2016). Therefore no tolerable upper intake level is given by the European Food Safety Authority (EFSA 2021). This is not true for

Vitamin K, Table 1 Vitamin K content of selected nutrients in alphabetic order[a]#

Nutrient	Vitamin K content (µg/100g)		
	MK5–MK9	MK4	Phylloquinone
Apple	–	–	3.0
Banana	–	–	0.3
Bread (wheat)	–	–	1.1
Bread (rye)	–	–	0.7
Broccoli	–	–	156
Brussels sprouts	–	–	817
Butter	–	15	15
Cheese (hard)	71.6	4.7	10.4
Cheese (soft)	52.8	3.7	2.6
Chicken breast	–	9	–
Chocolate	–	1.5	6.6
Cream	–	5.4	5.1
Egg white	–	0.9	–
Egg yolk	0.7	31.4	2.1
Fish (plaice)	2.0	0.2	–
Fish (mackerel)	–	0.4	2.2
Fish (salmon)	–	0.5	0.1
Kale	–	–	817
Margarine	–	–	93.2
Milk (3.5% fat)	–	0.8	–
Buttermilk	2.3	0.2	–
Nattō (fermented soybeans)	1103	–	34.7
Olive oil	–	–	53.7
Porcine steak	1.6	2.1	0.3
Salami	–	9.0	2.3
Sauerkraut	4.4	0.4	25.1
Spinach	–	–	387
Tea. black leaves	–	–	0.3

[a]The adequate intake level (AI) in the USA given by the FNB of the Institute of Medicine in 2001 is 120 µg/day and 90 µg/day for men and women older than 19 years. respectively. In Germany. 70 µg/day for men and 60 µg/day for women <51 years and 80 µg/day and 65 µg/day older than 51 years. 4 µg/day for sucklings <4 months. 10 µg/day 4–12 months. and finally in 3-year steps 50 µg/day for children <15 years are proposed by the German Nutrition Society (DGE) (DGE 2021)

menadione (vitamin K3) that can interfere with glutathione, a natural antioxidant, resulting in oxidative stress and cell membrane damage. Injections of menadione in infants led to jaundice and hemolytic anemia and therefore should not be used for the treatment of vitamin K deficiency.

People using warfarin should try to consume the recommended daily intake of vitamin K very regularly to avoid interferences with their anticoagulant dosage adjusted by the physician. Interestingly, high doses of vitamins A and E seem to antagonize vitamin K, vitamin A interfering with vitamin K absorption and vitamin E inhibiting vitamin K-dependent carboxylase enzymes (Biesalski 2016).

Until 1993 the adequate intake of phylloquinone (there is none given for K2) is set to 1µg/KG BW/day by the EFSA (EFSA 2021).

An idea about the concentration of vitamin K in food sources is shown in Table 1 (Schek 2017a). Interestingly, there are great differences between different sources of such data (e.g., 2, 15) which makes it difficult to find out the real values.

Cross-References

▶ Vitamin D

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Voltage-Dependent Ca^{2+} Channels

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Synonyms

High voltage-activated (HVA) calcium channels; Low voltage-activated (LVA) calcium channels

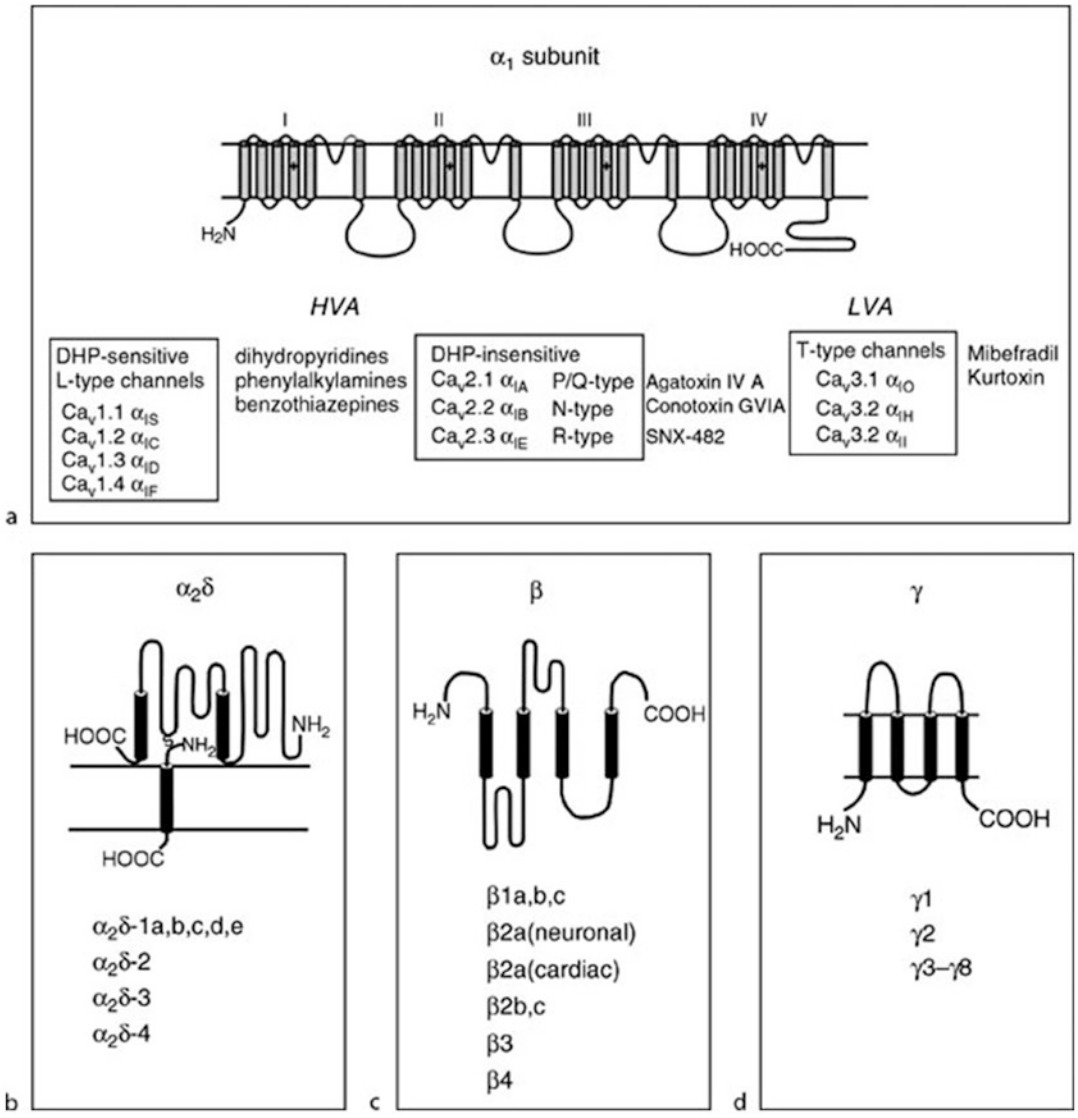
Definition

Voltage-dependent Ca^{2+} channels are a family of multi-subunit complexes of five proteins responding to membrane depolarization with channel opening allowing the influx of calcium into a cell. Voltage-dependent calcium channels are subdivided into three subfamilies: the HVA DHP-sensitive L-type calcium channels, the HVA DHP-insensitive calcium channels and the LVA T-type calcium channels (Hofmann et al. 2014).

Basic Characteristics

Voltage-activated calcium channels regulate the intracellular calcium concentration and contribute thereby to calcium signaling in numerous cell types. These channels are widely distributed in the animal kingdom and are an essential part of many excitable and non-excitable mammalian cell signaling pathways. Electrophysiological studies characterized different calcium currents identified as L-, N-, P-, Q-, R- and T-type current (Catterall et al. 2019; Hofmann et al. 2014). The opening of these channels is primarily triggered by depolarization of the membrane potential, but is also modulated by a wide variety of hormones, protein kinases, protein phosphatases, toxins and drugs. Site-directed mutagenesis has identified sites on these channels, which interact specifically with other proteins, inhibitors and ions.

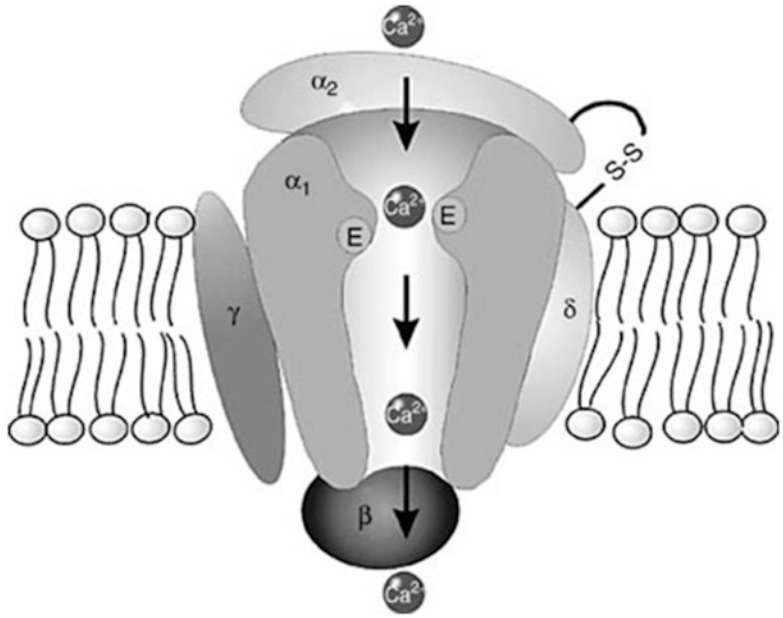
HVA calcium channels are biochemically heterooligomeric complexes of five proteins encoded by four gene families (Fig. 1): The α_1 subunits of ~190–250 kDa contain the voltage-sensor, the selectivity filter, the ion-conducting pore, the binding sites for most calcium channel blockers, and the interaction sites for heterotrimeric GTP-binding protein subunits and other proteins. Seven α_1 genes have been identified (Fig. 1). Four genes ($Ca_v1.x$) encode L-type, dihydropyridine-sensitive channels. Three genes ($Ca_v2.x$) encode the dihydropyridine-insensitive neuronal N-, P-, Q-, R-current. These α_1 subunits are associated with four auxiliary proteins (Fig. 2). The $\alpha_2\delta$ subunits, disulfide-linked dimers, are transcribed from four different genes and are clipped posttranslational into the extracellular located α_2- and the transmembrane δ-protein. The intracellular located β subunit and the



Voltage-Dependent Ca^{2+} Channels, Fig. 1 Structure, identity and blockers of calcium channel subunits

transmembrane γ subunit are encoded by four and up to eight distinct genes, respectively. With the exception of the skeletal muscle calcium channel, a heterooligomer containing the $Ca_v1.1$ (α_{1S}), β1, $\alpha_2\delta$-1 and γ1 subunit, the exact composition of individual channels is not known. The identity of these complexes is further complicated by the existence of several splice variants for most genes that confer significant effects on the electrophysiological and/or pharmacological properties of the channels.

The exact subunit composition of the Low Voltage-activated (LVA) Ca^{2+} channels is unknown (Lacinova et al. 2000; Perez-Reyes 2003). Three α_1 subunits, $Ca_v3.x$, have been identified which induce large T-type current after expression in *Xenopus* oocytes and in HEK cells in the absence of additional subunits. The T-type



Voltage-Dependent Ca^{2+} Channels, Fig. 2 Subunit composition of a HVA calcium channel. The selectivity filter of the channel is created by four glutamates (E)

current can be affected by the $\alpha_2\delta$ and the γ6 subunit suggesting a maximal subunit composition of $\alpha_1/\alpha_2\delta./\gamma$.

Properties of the α_1 Subunits: Each α_1 subunit contains four repeats that are composed of six transmembrane helices and a pore region between helix S5 and S6. The selectivity filter of the channels is located in the pore region and calcium selectivity of the HVA channels is created by four glutamates (E) in each pore, whereas the LVA channels have glutamates in the pore of repeat I and II and aspartates (D) in that of repeat III and IV. Conformational changes of the protein allow the channel to occupy one of three states: the closed, open or inactivated state. The change in membrane potential is sensed by the S4 helices of each repeat which contain a number of positive charged amino acids. Movement of these helices induces opening of the channel pore. HVA channels are activated approximately at a membrane potential of −30 mV with a maximal activation around 0 mV, whereas the LVA channels activate at potentials around −60 mV and have a maximal inward current around −10 mV. The LVA channels activate and inactivate faster than the HVA channels. Inactivation of all channels is voltage-dependent and accelerated if the membrane is depolarized for prolonged time. Voltage-dependent inactivation may be mediated by sequences present in repeat I and II. Some of the HVA channels, especially the $Ca_v1.2$ channel, show calcium-dependent inactivation. Calcium flowing through the channel binds on the internal side to calmodulin, a calcium binding protein tethered to the carboxy terminal tail of the α_1 protein by an isoleucine/glutamine (IQ)-motive. The calcium–calmodulin complex induces a conformational change in the α_1 protein that leads to inactivation of the channel. The $Ca_v1.4$ and the LVA channels do not have this type of inactivation.

The intracellular loop between repeat II and III couples and signals to intracellular effectors. The II–III loop of the $Ca_v1.1$ protein couples directly with the ryanodine receptor 1 located at the sarcoplasmatic reticulum in the skeletal muscle triad. In contrast, the cardiac $Ca_v1.2$ channel is in close proximity but not in direct contact with the cardiac ryanodine receptor 2. This cardiac ryanodine receptor channel is activated by the calcium flowing through the open $Ca_v1.2$ channel. The II–III loops of the neuronal presynaptic

localized Ca $_v$2.1 and Ca $_v$2.2 channels interact directly with the SNARE complex of the neurotransmitter containing vesicles and facilitates together with the inflowing calcium fusion of the vesicle and plasma membrane resulting in opening of the vesicle. Similar interactions occur probably between the Ca $_v$1.2 II–III loop and insulin containing vesicles of the murine pancreatic islets.

Neurotransmitter receptors inhibit the neuronal Ca $_v$2.x channels by activation of G-proteins. The β/γ subunit of the heterotrimeric GTP-binding proteins binds to a QxxER sequence that is located at the I–II loop. Binding of the β/γ subunits confers the "reluctant phenotype" to these channels. This phenotype includes a reduced channel activation and current at normal depolarized membrane potentials. The I–II loop interacts also with the calcium channel β subunit with high affinity allowing transport of the α $_1$ protein from the Golgi to the membrane surface and a shift in the voltage-dependence of channel opening and closing.

Recent results show that the positive inotropy mediated by the cardiac beta-receptor includes cAMP-dependent phosphorylation of the β-2 subunit. This modification weakens the interaction between the β-2 subunit and the small GTPase rad. Rad inhibits the Cav1.2 current, if bound to the β2-subunit.

► Channel blockers: Distinct blockers have been identified for the various HVA calcium channels which are listed in Fig. 1. Dihydropyridines, phenylalkylamines and benzothiazepines block all Ca $_v$1.x L-type calcium channels. These compounds are used mainly to lower the blood pressure in hypertensive patients. In therapeutic concentrations, they block mainly the smooth muscle Ca $_v$1.2 channel. The dihydropyridines bind with high affinity to the inactivated state of the L-type channels. Binding requires amino acids on the IIIS5, IIIS6 and IVS6 helices. Mutation of Thr1061 in IIIS5 to tyrosine abolished completely the high affinity interaction of dihydropyridines with the Ca $_v$1.2 channel protein. The difference in the sensitivity between the cardiac and the smooth muscle L-type calcium channel is in part caused by the use of alternative exons (No. 8) coding for the IS6 helix (Welling et al. 1997). The phenylalkylamine and benzothiazepine binding sites contain amino acids present in the IIIS6 and IVS6 helices and the glutamates in the pore region of repeat III and IV. The latter two groups include compounds that block neuronal Ca $_v$2.x channels at similar concentrations as the Ca $_v$1.x channels.

The LVA α $_1$ subunits are blocked by moderate to low (10μM) concentrations of nickel and bind the channel blocker mibefradil and kurotoxin. Both compounds are not specific LVA channel blockers because they block also Ca $_v$1.x and Ca $_v$2.x channels at about tenfold higher concentration. Interestingly, the endogenous cannabinoid anandamide binds to LVA channels and stabilizes the inactivated state. This effect decreases T-type calcium current and neuronal firing activities.

The α $_2$δ subunit 1 and 2 bind gabapentin and pregabalin with high affinity. This interaction may be causally related to its antiepileptic and neuropathic pain alleviating property.

Localization of channels: The Ca $_v$1.1 channel is present in skeletal muscle at the triad. The Ca $_v$1.2 channel is widely distributed and represents the major L-type calcium channel in most tissues. In contrast, the Ca $_v$1.4 channel has been detected only in the retina, so far. The Ca $_v$1.3 (α $_{1D}$) channel is mainly found in neuroendocrine cells and the inner ear. The Ca $_v$2.1, Ca $_v$2.2 and Ca $_v$2.3 are mainly localized presynaptic. The Ca $_v$2.1 and Ca $_v$2.2 channels interact with the vesicular release machinery and regulate the release of neurotransmitters.

The LVA channels are expressed in a wide variety of tissues. In the cardiac sinus node and the thalamus, activation of LVA channels seems to be necessary to generate action potentials upon depolarizing the membrane.

Mutation and Deletion

Deletion of the Ca $_v$1.1 and Ca $_v$1.2 gene is not compatible with viable mouse pups (Moosmang et al. 2007; Muth et al. 2001). The Ca $_v$1.2 channel is absolutely required for the contraction of the developing mouse heart after embryonal day

14. Mutation of the human $Ca_v1.1$ gene is associated with hypokalemic periodic paralysis. Deletion of the $Ca_v1.3$ gene leads to viable pups that are deaf and have cardiac arrhythmia at rest. Mutation of the human $Ca_v1.4$ gene is associated with X-linked congenital stationary night blindness.

Mutation in the neuronal $Ca_v2.1$ channel is associated with familial hemiplegic migraine and episodic ataxia in humans. Deletion of the $Ca_v2.1$, $Ca_v2.2$ and $Ca_v2.3$ gene is compatible with life accompanied by a variety of central and peripheral defects.

Deletion of the $Ca_v3.1$ channel in thalamocortical relay neurons prevents absence epilepsy. Block of the neuronal LVA channels alleviates certain forms of epilepsy. Deletion of $Ca_v3.2$ leads to coronary artery constriction and focal myocardial fibrosis.

Deletion of the ß1 subunit is lethal, whereas deletion or mutation of the $\alpha_2\delta$-2, β3, β4, γ1 and γ2 genes is associated either with no or various neuronal phenotypes.

More recent analysis of tissue specific gene deletions showed that the $Ca_v1.2$ channel is involved in a wide variety of function including hippocampal learning, insulin secretion, intestine and bladder motility. Further analysis will be required to unravel the functional significance of voltage-dependent calcium channels for specific cellular functions.

Drugs

Numerous dihydropyridine calcium channel blockers have been introduced to treat hypertension and stable angina pectoris. Nifedipine, nitrendipine, nisoldipine, nilvadipine, nicardipine, amlodipine, felodipine, isradipine block preferentially the vascular, smooth muscle $Ca_v1.2$ calcium channel at therapeutic doses. Nimodipine that has a short half-life has been used to alleviate cerebral vasospasms after subarachnoidal bleeding. The phenylalkylamines verapamil and gallopamil and the benzothiazepine diltiazem have been used as antihypertensive drugs and to treat supraventricular tachyarrhythmia. Mibefradil – a compound thought to inhibit preferentially T-type channels – that blocks at low concentrations vascular $Ca_v1.2$ L-type channels in the mouse, has been used for a short period as an antihypertensive drug. It has been removed from the market due to intolerable interactions with other drugs.

Gabapentin and pregabalin are prescribed in certain epileptic diseases such as absence epilepsy and in neuropathic pain. Their therapeutic target for pain suppression is the $\alpha_2\delta$-1 subunit.

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Voltage-Gated K^+ Channels

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Synonyms

Kv-channels

Definition

Voltage-gated potassium (Kv) channels are membrane-inserted protein complexes, which form potassium-selective pores that are gated by changes in the potential across the membrane. The potassium current flow through the open channel follows by the electrochemical gradient as defined by the Nernst equation. In general, Kv channels are localized in the plasma membrane.

Basic Characteristics

Functional Characteristics

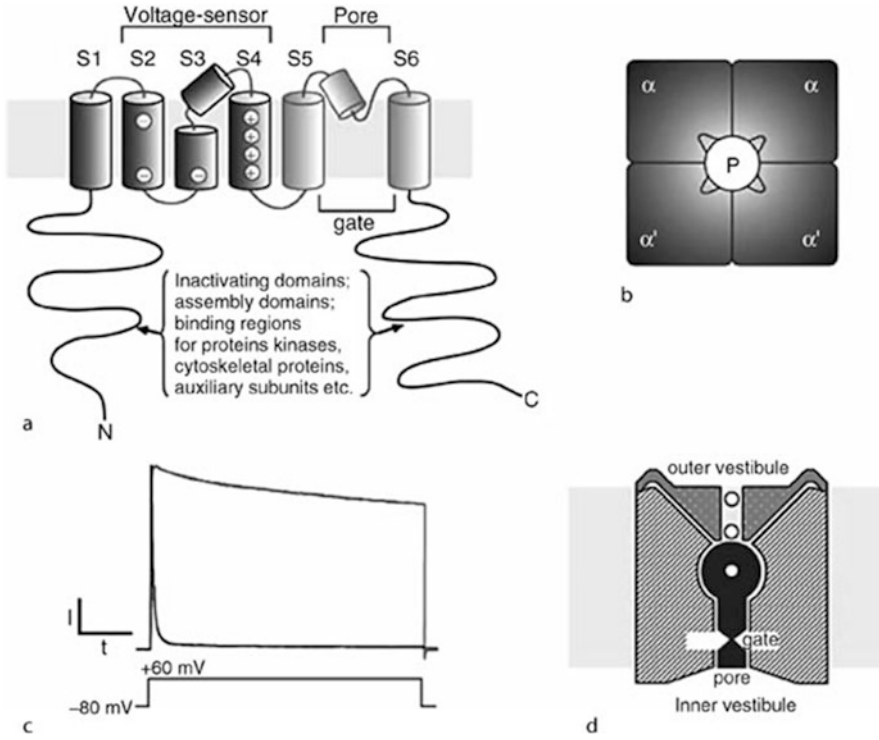
Kv channels may be important determinants of cellular activities correlated with changes in membrane potential. Examples range from neural signal transduction, action potential wave forms, action potential propagation, action potential frequency, pacemaking, and secretion to the regulation of cell volume and cell proliferation. In addition to changes in voltage, Kv-channel activities may be regulated by various physical and/or chemical stimuli. They include Na^+, Ca^{2+}, Mg^{2+}, ATP, O_2, pH, pressure, redox potential, phosphorylation/dephosphorylation, G protein binding, calmodulin binding, KChiP binding, interaction with cytoskeletal proteins, and more (Hille 2001). Once Kv channels have been activated, they catalyze a rapid passage of K^+ through the open pore along the electrochemical gradient as defined by the Nernst equation. Activated Kv-channels often tend to inactivate (Jan and Jan 1997). The kinetics of inactivation may occur in time ranges of ms to tens of seconds. The inactivation mechanism of Shaker-channels, which inactivate rapidly, has been thoroughly investigated. One mechanism utilizes an amino-terminal inactivating domain. This domain is able to bind to the open pore of Shaker-channels. Thereby, the pore becomes both occluded and locked in an open state. Upon repolarization, inactivated Kv-channels recover from inactivation. Inactivated Kv-channels are refractory to activation. In most circumstances, intracellular K^+ concentrations are higher than the extracellular ones, and the membrane potential is positive to the Nernst potential. Therefore, the direction of K^+ current flow through Kv-channels is mostly outward. But there are important exceptions, where either the membrane potential at which the Kv-channel opens is negative to the Nernst potential or the extracellular K^+ concentration is not very different from the intracellular one. For example, inactivated Kv-channels like HERG-channels (see above) may recover from inactivation at very negative membrane potentials. During recovery they pass through an open state permitting an inward flow of K^+ current. Depending on the particular conditions, hyperpolarizing Kv-channel activity may attenuate cellular excitability (e.g., the firing of action potentials) or they may balance depolarizing activities (e.g., clamp the membrane potential to a certain value to allow a steady inward flow of calcium ions). Frequently, repolarizing Kv-channel activity shifts the membrane potential into a hyperpolarizing direction.

Kv-channels are closed in the resting state. Upon depolarization of the cellular membrane potential, closed Kv-channels undergo a series of voltage-dependent activating steps until they reach an activated state from which they can open and close in a voltage-independent manner. HERG-channels represent a particularly interesting example of Kv-channel inactivation. HERG-channels have faster inactivation than activation kinetics, and they very rapidly recover from inactivation at negative membrane potentials. This behavior may result in a situation where most of the current carried by HERG-channels occurs during their recovery from inactivation at negative

potentials, that is, it represents an inward rather than outward current.

Structural Characteristics of Kv-Channels

Kv-channels are frequently heteromultimeric protein assemblies of pore-forming membrane-integrated α-subunits and of auxiliary subunits (Jan and Jan 1997; Pongs and Schwarz 2010). The first Kv-channel subunits were cloned from *Drosophila*. This work initiated the subsequent identification and cloning of many more Kv-channel genes constituting a superfamily of related proteins. The design of the proteins is structurally and functionally highly conserved (Jiang et al. 2003; Wang and MacKinnon 2017). Kv-channel α-subunits have cytoplasmic amino- and carboxy-termini, which



Voltage-Gated K^+ Channels, Fig. 1 Basic features of voltage-gated potassium channels. (**a**) Schematic drawing of the membrane topology of Kvα-subunits. Transmembrane segments are numbered S1–S6. The linker regions between segments S3/S4 and S5/S6 contain small α-helical regions marked as cylinders. Negative charges (−) in segments S2 and S3 and positive charges (+) in segment S4 contribute to the voltage-sensor of Kv-channels. Pore-forming residues are located within the S5/S6 linker region and segment S6. The gate that opens and closes Kv-channels is not exactly known. Amino acid residues of the S4/S5 linker region, segment S5, and segment S6 are directly and/or indirectly involved in the gating machinery. Brackets give examples for additional functions and properties associated with sequences and domains of the cytoplasmic amino- and/or carboxy-termini. (**b**) Assembly of four Kvα-subunits is needed to form functional Kv-channels with a central pore P. α and α′ indicate that assembly of homo- and heteromultimers is possible. (**c**) Typical examples of potassium outward currents (I) mediated by Kv-channels upon jumping from a holding potential of −80 mV to a test potential of +60 mV. Black trace: rapidly inactivating outward current; gray trace: noninactivating delayed-rectifier type current. Time-scale (t) is in ms. Upon repolarization from a depolarizing test potential to a hyperpolarizing holding potential an inward current or tail current can be observed that reflects the closure of open channels. (**d**) Schematic diagram of pore structure. A hypothetical sagittal section through the pore is shown. The pore has an outer vestibule, a selectivity filter (dotted gray) in the upper third of the membrane, an aqueous cavity, a gate (black), and an inner water-filled vestibule. Potassium ions are drawn approximately to scale as white circles

frame a membrane-spanning core domain. The core domain consists of six hydrophobic membrane-spanning segments S1 to S6. Segments S5 and S6 are linked by the so-called P-loop. This P-loop enters and exits the plasma membrane from the extracellular face. Figure 1a shows a cartoon of the most likely membrane topology of Kv-channel α-subunits. Four subunits are necessary to form a functional channel. Homo- as well as heterotetrameric assembly of Kv-channels is possible (Fig. 1b). Kv-channels can be expressed in vitro in heterologous expression systems (Fig. 1c). The relative ease to in vitro mutate Kv-channel cDNAs and to express Kv-channel cDNAs heterologously in the *Xenopus* oocyte or tissue culture expression systems has produced a detailed understanding of many basic features concerning Kv-channel activity. The results showed that the voltage-sensing apparatus of Kv-channels is mainly formed by amino acids residing in segments S2 to S4. Most notably is the occurrence of a repeat sequence $(R/KXX)n_{=3-5}$ in segment S4, lining up several positive charges in the membrane electric field. The charges apparently move in the electric field when the Kv-channels become activated, giving rise to a gating current across the membrane. Gating currents are observed during the voltage-dependent activation of Kv-channels.

Amino acid residues residing in the S5–P–S6 region are engaged in forming the pore, most notably a highly conserved P-loop sequence TVGY/FGD/N, which has been dubbed the K-channel signature sequence. This sequence forms part of the selectivity filter of the Kv-channel pore. Crystal structures of several Kv channels provide detailed high-resolution images of the pore of a K-channel with its surrounding transmembrane helices (Jiang et al. 2003; Wang and MacKinnon 2017; Whicher and MacKinnon 2019; Sun and MacKinnon 2020). K-channel pore structures are apparently highly conserved (Lu et al. 2001; Gonzáles et al. 2012). Hallmarks of the pore structure are an outer vestibule with a relatively flat surface. Beneath, in the upper-third of the membrane, lies a narrow selectivity filter. This is followed by a central aqueous cavity narrowing into the internal mouth of the pore (Fig. 1d). Kv-channels, during voltage-dependent activation and opening, undergo marked conformational changes. In the resting state, a "gate" closes the pore. Upon activation, the gate opens. Amino acid residues in the carboxy-terminal half of the S6 region apparently play a pivotal role for the structure and function of the Kv-channel gate. Other Kv channel domains, for example, the S4-S5 linker region, which links gate and voltage sensor, participate in channel gating via allosteric mechanisms. Furthermore, intracellular domains may also participate in the regulation of gating. Thus, gating involves considerable structural rearrangements typically seen in allosteric proteins (Wang and MacKinnon 2017; Whicher and MacKinnon 2019; Sun and MacKinnon 2020; Long et al. 2005; Barros et al. 2019). In addition, cytoplasmic domains play important roles in Kv-channel function (Wang and MacKinnon 2017). Many of these functions are related to subunits assembly, channel trafficking to and from the plasma membrane, and interactions with cytoskeletal components (Fig. 1a). A tetramerization (T) domain for subunit assembly has been well defined in Shaker-channels, where it is localized in the amino-terminus. Other Kv-channels (e.g., eag, HERG, KvLQT1) have comparable domains within the cytoplasmic carboxy-terminus. ER retention and retrieval signals have been found in the cytoplasmic amino- or carboxy-termini. Thus, channelopathies may be associated with defects in ion channel function as well as with defects in ion channel folding, trafficking, and membrane localization/retention (Curran and Mohler 2015). Also, cytoplasmic Kv-channel domains may contain recognition

Voltage-Gated K^+ Channels, Table 1 Auxiliary subunits and their reported interactions with Kvα-subunits

Auxiliary subunit	Kvα-subunits
Kvβ1, Kvβ2, Kvβ3	Kv1-family, Shaker
MIRP2	Kv3.4
KChIPs (KChIP1,2,3,4)	Kv4.1, Kv4.2, Kv4.3
KCNE1–KCNE4	KCNQ1-KCNQ5
	HERG, Kv3.4, Kv4.3
sloβ1–sloβ4	BK(slo)α-subunits

sequences for a variety of serin/threonine and/or tyrosine protein kinases, which contributes to additional structural and functional diversity (Vacher and Trimmer 2011). Finally, motifs have been characterized that interact with a variety of cytoskeletal components. For example, the conserved carboxy-terminal amino-acid motif TDV is recognized by MAGUK(PSD)-proteins of the post- and presynaptic densities and KvLQT1 contains a functionally important C-terminal domain for calmodulin binding (Yi et al. 2001; Sun and MacKinnon 2017).

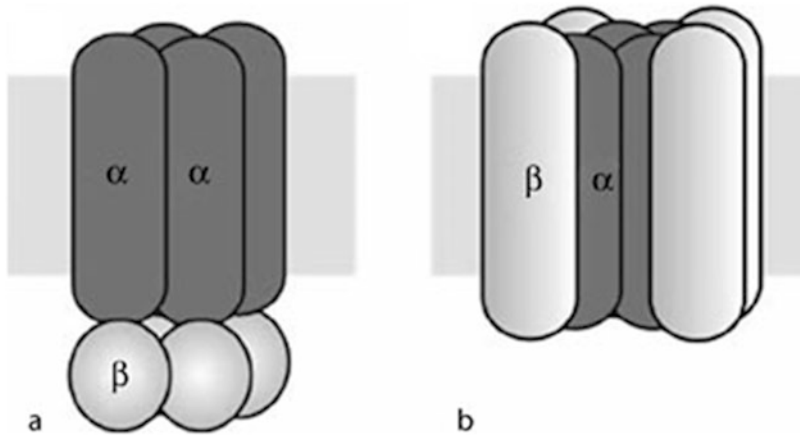
Auxiliary Subunits

Kvα-subunits may coassemble with auxiliary subunits (see Table 1) (Pongs and Schwarz 2010). Auxiliary subunits (e.g., Kvβ-subunits) may bind to cytoplasmic regions of the Kvα-subunits extending the reach of the membrane-integrated core channel into the cytoplasm (Fig. 2a). A particularly interesting group is the KChIP-family. KChIP stands for K-channel interacting protein. The proteins are small Ca^{2+}-binding proteins being related to the superfamily of neuronal calcium sensors. They are tightly associated with somato-dendritic rapidly inactivating Kv-channels (Kv4.1, Kv4.2, Kv4.3). Alternatively, the auxiliary subunits may be membrane integrated proteins like Kvα-subunits (Fig. 2b). Examples are members of the KCNE-family of auxiliary subunits that may assemble with a great variety of Kv-channels including KCNQ-channels, HERG-channels, and Shaker-related Kv-channels.

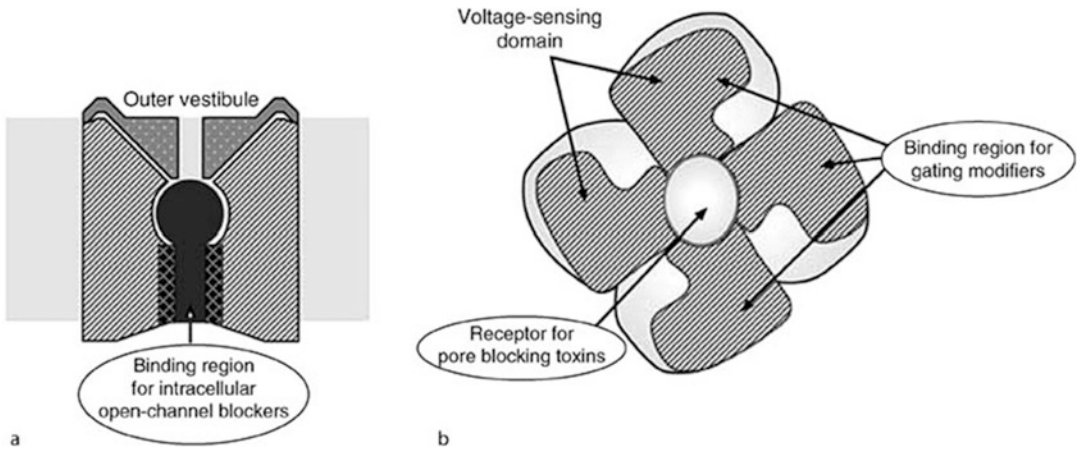
Functional activities of auxiliary subunits may include chaperone activities during assembly, for example, Kvβ-subunits have been shown to exert a chaperone function for the assembly of Shaker α-subunits or the recognition of ER-retention signals as discussed for KChIPs. Notably, auxiliary subunits may modulate the biophysical and pharmacological properties of Kv-channels. Certain Kvβ (Kvβ1.1 and Kvβ3.1) confer a rapid-inactivation behavior to otherwise noninactivating Kv-channels. Association of KCNE1 with KvLQT1 leads to a significant slowing of Kv-channel activation, a depolarizing shift in the voltage dependence of activation and a change in drug sensitivity (e.g., for mefenamide). On the other hand, association of KCNE3 with KvLQT1 leads to voltage-insensitive channels.

Drugs

In the animal kingdom, a great number of toxins from snakes to scorpions, insects, spiders, and sea anemones that block various Kv-channels with



Voltage-Gated K^+ Channels, Fig. 2 Schematic drawing of voltage-gated potassium channels as heteromultimeric assemblies of pore-forming Kvα-subunits and auxiliary subunits. (**a**) Assembly of Shaker-type Kv-channels with cytoplasmic subunits, for example, Kvβ-subunits and KChIPs. (**b**) Assembly of Kv-channels with membrane-integrated auxiliary subunits, for example, MinK and MirPs. Membrane is shaded gray



Voltage-Gated K^+ Channels, Fig. 3 Schematic drawing of Kv-channel binding sites for toxins and drugs. (**a**) Side view of a cut-open Kv-channel. Intracellular open-channel blockers may bind to the inner vestibule of Kv-channels (hatched) formed by segment S6. (**b**) View of a Kv-channel from the top. Pore-blocking toxins bind to receptor sites formed by the Kv-channel pore and the outer vestibule. Gating modifiers have binding sites outside of the pore (striped) and interfere with the voltage-sensing machinery

nano- to picomolar affinities have been identified. Two kinds of block may be discerned in general. The toxin may bind to the outer vestibule of the Kv-channel pore and thereby occlude the pore (Fig. 3a). In this case, the binding of one toxin per channel may suffice to block channel activity. Alternatively, the toxin may interfere with the voltage-sensing machinery and modify Kv-channel gating. The voltage-sensing machinery is located in the periphery of the pore. As each subunit appears to have its own voltage-sensing apparatus, gating-modifying toxins may have four binding sites per Kv-channel (Fig. 3b). Characteristically, gating-modifying toxins induce a positive shift in the current–voltage relationship of Kv-channels and accelerate channel deactivation (closing of the open channel).

Many nonpeptidergic compounds are known to block Kv-channels. Where the mechanism of block is known, it is related to pore occlusion. Most frequently, nonpeptidergic drugs block the pore by interacting with amino acid residues located in the carboxy-terminal part of segment S6 (Fig. 3a). This part is particularly hydrophobic in the pore of HERG-channels (Wang and MacKinnon 2017). Apparently, it distinguishes HERG-channels from other Kv-channels and renders HERG-channels exceptionally sensitive to block by many pharmaceuticals. Since HERG-channels make an important contribution to cardiac action potential repolarization, HERG-channel block may be frequently responsible for cardiac side effects of drugs associated

Voltage-Gated K^+ Channels, Table 2 Kv-channel genes associated with heritable diseases

Gene	Trivial name	Kv-channel type	Disease
KCNA 1	Kv1.1	Shaker-family	EA
KCNA5	Kv1.5	Shaker-family	LQT
KCNB1	Kv2.1	Delayed rectifier	EIEE
KCNH 2	HERG	IKr-channel	SB, LQT
KCNE 1	MinK	Auxiliary subunit	LQT
KCNE 2	MirP1	Auxiliary subunit	LQT
KCNE 4	MirP2	Auxiliary subunit	Myopathy
KCNQ 1	KvLQT1	Delayed rectifier	LQT/JLN
KCNQ 2	–	M-channel	EIEE
KCNQ 3	–	M-channel	EIEE
KCNQ 4	–	M-channel	DFNB2

EA, episodic ataxia; EIEE, early infantile epileptic encephalopathy; LQT, long QT syndrome; SB, sinus bradycardia; JLN, Jervell–Lange Nielsen syndrome; DFNB2, deafness syndrome

with cardiac arrhythmia. Tetraethylammonium, 4-aminopyridine, and quinidine are unspecific drugs blocking Kv-channels by binding to the inner entrance of the pore. TEA may also bind to the outer entrance of Kv-channel pores.

Mutations in Human Kv-Genes Associated with Hereditary Channelopathies

Mutations in human Kv-channel genes have been detected that are associated with hereditary diseases ranging from heart arrhythmia (long QT-syndrome) and deafness to epilepsy and ataxia (see Table 2). Typically, many Kv-channel related channelopathies are correlated with a mutant phenotype that is episodic in nature and appears as a dominant hereditary trait.

Outlook

Solving crystal structures of several Kv channels represents a major breakthrough in our understanding of Kv-channel structure and function, including association of mutant Kv channels with life-threatening cardiac arrhythmias. This knowledge provides a promising basis for development of suitable drugs and personal treatment strategies.

Cross-References

- ▶ Antiarrhythmic Drugs
- ▶ ATP-Dependent K^+ Channels
- ▶ Inward Rectifier Potassium Channels

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Wall Peptidoglycan Inhibitors

► β-Lactam Antibiotics

Water Channels

► Aquaporins

Weight-Loss Therapies

► Anti-obesity Drugs

Weight-Management Pharmacotherapy

► Anti-obesity Drugs

White Plague

► Tuberculosis

Wnt Signaling

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Definition

The *Wnt*s are a group of 19 genes essentially coding for glycoproteins which have been highly conserved along the evolutionary lineage from the coelenterates to modern *Homo sapiens*. "Wnt" is a portmanteau of *Drosophila* wingless (*Wg*) and mouse *integrated-1* (*Int-1*) which stands for *wingless-related integration site.* Wnt proteins interact with serpentine Frizzled (Fzd) receptor and a wide array of co-receptors in order to execute expression of target genes regulating cellular polarity, cell motility, proliferation, cell fate determination, differentiation, as well as stem cell activity.

Basic Mechanism

Insight into Posttranslational Modification of Wnts

All of the Wnt proteins share a signal sequence required for successful secretion, and nearly 23 Cys residue-invariant patterns in their primary structure, some of which are crucial for folding into a proper secondary structure through intramolecular

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disulfide bond formation. With a number of potential N-glycosylation and fatty acylation sites, nearly all Wnts undergo huge posttranslational processing except *Drosophila*-ortholog WntD. Appending palmitoleate (C16:1) or myristoleate (C14:1) on conserved Ser residues is unique to Wnts among other secretory proteins. In addition, another selective modification including glycosylphophatidylinositol (GPI) anchor attachment to Wnt1 and Wnt3A and tyrosine sulfation on Wnt5A and Wnt11, etc. ensures "just right" Wnt signaling to be achieved.

Glycosylation of Wnts

Glycosylation might aid in proper exocytosis and extracellular spread of Wnts; yet, the role of glycosylation in Wnt secretion and function is still elusive (Ghosh et al. 2019). Interestingly, heparan sulfur proteoglycan is implicated in extracellular Wnt transportation, but its role in Wnt secretion is unclear. Wnt1 and Wnt5 are reported to bear, respectively, one and two of N-glycosylations; yet, WntD remains persistently unglycosylated. Though site-directed mutagenesis of Wnt1 and Wnt5 glycosylation sites has impaired their proper secretion to certain extents, it does not affect their activity notably. Similarly, studies showing no major change in secretion and signal transduction activity in mutant *Drosophila* Wnt wingless (*Wg*), devoid of all N-glycosylation, also postulate glycosylation to be dispensable for Wnts secretion and action.

Fatty Acylation of Wnts

Unlike glycosylation, lipidation of Wnts seems to be vital from both structural and biochemical viewpoints (Nile and Hannoush 2016). In 2003, the first report of fatty acylation at the conserved Cys77 of murine Wnt3A was documented through an experiment using radioactive ^{3}H-labelled palmitic acid (C16:0). Discovery of corresponding palmitoylated-Cys residues of Drosophila Wg and Wnt8, soon established palmitoylation to be a conserved feature across all Wnts. Afterwards, a second lipidation of murine Wnt3A was reported at Ser209 by mass spectroscopy. Indeed, the crystal structure of *Xenopus* Wnt8 in association with Cys-rich domain (CRD) of murine Frizzled 8 (Fzd8) receptor revealed the second corresponding palmitoylation at Ser187. However, the importance of Cys-acylation is quite debatable. Reports suggest that mutation of Ser209 to Ala results in retention of Wnt3A in ER; whereas, mutation of Cys77 to Ala renders Wnt3A with little to no activity without harming its secretion. Contrastingly, crystal structures have revealed that Cys77 of Wnt3A (equivalent to Cys55 in *Xenopus* Wnt8) itself engages within a disulfide bond and thus is unlikely to be acylated. In support, a biochemical experiment with non-radioactive alkyne palmitate (Alk-C16) in association with click chemistry has been performed where the presence of palmitoylation only at Ser224 and Ser209 of Wnt1 and Wnt3A, respectively, was noticed with unacylated Cys77.

Other than palmitate, fatty acids with the length of 13- to 16-carbon atoms can be acylated at Ser209 of Wnt3A through ester linkage. In contrast to S-palmitoylation of other secretory proteins which involves a high turnover of palmitate, Wnt3A doesn't undergo cycles of acylation and deacylation intracellularly. Perhaps stable O-linked acylation (ester bond), in place of labile thioester bond, has been selected to provide a greater time window for extracellular activities with endured activity.

Porcupine (PORCN), an ER-resident multi-pass membrane-bound O-acyltransferase (MBOAT)-superfamily protein, acts to lipidate Wnts. PORCN acylates Ser- and Thr-residues but doesn't modify Cys-residues on Wnts. Loss of PORCN activity abrogates Wnt secretion entirely. PORCN competently catalyzes 14- and 16-carbon fatty acylation on conserved Ser residues, whereas catalysis of 13- and 18-carbon fatty acylation was reported to be less preferable. However, PORCN has been reported to be selective for cis-Δ9-unsaturated fatty acyl Co-A over the saturated ones. Recently, it has been discovered that PORCN itself gets acylated at Cys187 in order to get regulated. Human PORCN, deficit in Cys187 palmitoylation, acylates Wnts to a slightly higher degree illustrating negative regulation of Wnt lipidation by PORCN acylation.

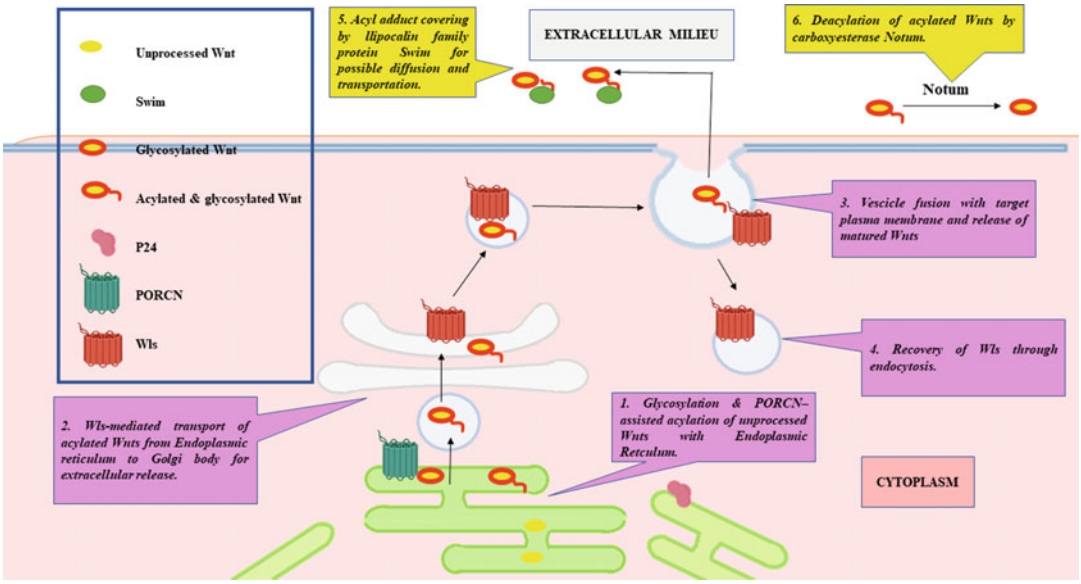
Beyond just facilitating interaction with the cellular membrane and increasing protein

hydrophobicity, fatty acylation of Wnts has other crucial jobs too. Predominantly, it eases the interaction of Wnts with its cognate Fzd receptors as unsaturated fatty acylation on Wnts enhances affinity toward the CRD of Fzd receptors. In addition, acylation might facilitate the occurrence of Wnts in active monomeric forms. Also, lipidation might enhance the interaction of Wnts with chaperones such as Wntless (Wls). Apart from that, Notum, which identifies only cis-unsaturated fatty acid adducts on Wnts, enables Wnts to be deacylated in the extracellular matrix and to be recycled.

Insight into Secretion of Wnt Proteins

Steps involving Wnt secretion seem to be controversial till date (Langton et al. 2016). However, researchers propose that an ER-resident multipass transmembrane protein Wls or evenness interrupted (Evi), to be vital for Wnt secretion in *Drosophila* (see Fig. 1). This protein corresponds to *C. elegans* Mig-14 and mouse GPR177. Other than WntD which remains unacylated, the rest of the Wnts require Wls/Evi for their secretion. However, knocking down Wls expression doesn't affect the secretion of other morphogens such as sonic hedgehog; hence, it is reported to be dedicated toward Wnt proteins only. X-ray crystal structure depicts that Wls/Evi possesses lipid-binding β-barrel in its tertiary structure which interacts with Wnts through their acylated residues. In addition to Wls/Evi, p24 performs as another cargo protein in the early Wnt secretory pathway; however, unlike Wls/Evi it appears to be optional for intracellular transport of Wnts.

Regarding Wls-assisted Wnt secretion, the simplest route is the Wls/Evi-assisted direct transport of Wnts from trans-Golgi network (TGN) to plasma membrane; yet, endosomal trafficking of Wnts in association with Wls has also been suggested. Moreover, it has been also reported that before being secreted, Wnts might undergo endocytosis along with Wls/Evi enabling Wls/Evi recycling and endosomal attachment of



Wnt Signaling, Fig. 1 Schematic representation of post-translational modification and secretion of Wnt proteins. Once translated, Wnt proteins undergo N-liked glycosylation and PORCN-mediated acylation in the endoplasmic reticulum (ER). From ER they move to the Golgi body for further modification through vesicular transport where p24 seems to be functional along with other vesicle-associated transport proteins. From the Golgi body up to the release from secretory cell occurs through vesicular transport where Wls is essential. For ongoing secretion, recycling of Wls is essential. On the other hand, once secreted, insoluble lipoprotein chiefly uses lipophorin/lipocalin family protein Swim as a shield to become soluble and eligible for transport. Otherwise, matured Wnts encounters carboxylesterase Notum in order to be deacylated in extracellular space

lipoprotein molecules for extracellular diffusion after secretion. Much complex pre-secretion transportation might take place in polarized epithelial cells. For example, in Madin-Darby canine kidney cells, different glycosylation appears to probe Wnt3A secretion basally yet Wnt11 secretion apically. Therefore, the route of Wnt secretion seems to be determined depending on the type of the secretory cells and the Wnt protein to be secreted.

An adequate pool of Wls/Evi must be maintained in order to sustain successful Wnt signaling. Thus, AP-2 and clathrin-dependent endocytosis of Wls/Evi is essential followed by retromer-mediated transport of Wls/Evi back to TGN. Lack of retromer complex results in termination of Wnt signal transduction cascade due to lysosomal degradation of Wls/Evi. Wls/Evi turnover does not depend upon classical SNX-BAR retromer-forming nexins such SNX1-2 and SNX5-6, rather SNX3-retromer complex, which retrieves Wls/Evi from early endosome through vesicular budding, seems to be the key role player. Very recently, it has been shown that Wls/Evi also backtracks from Golgi to ER through ARF-mediated COPI-coated vesicles. An ER signal at the C-terminal end of the Wls and an ER-Golgi intermediate cargo protein ERGIC2 assist in this retrograde transport.

Insight into Transportation of Secreted Extracellular Wnts

Once emitted, Wnts mostly function as a morphogen, producing a gradient and acting upon the receptor cells. Since entirely processed Wnts are unlikely to be soluble, it is essential to mask Wnts' fatty acyl adducts in order to diffuse and translocate from secretory cells to the receiving ones. Herein, a handful of subcellular structures are involved to keep Wnts soluble extracellularly (Hausmann et al. 2007). Wnts have been proposed to be shielded by lipoprotein particles (structures composed of outer phospholipid monolayer surrounding the central hydrophobic core of triglyceride and other lipids). Lipoproteins are known to stabilize through apolipoprotein insertion. In *Drosophila* imaginal disc, apolipoprotein lipophorin has been reported to engage with Wg, and knockdown of lipophorin with siRNA treatment has been shown to cut the long-range diffusion of Wg. In *Drosophila* lipophorin expression occurs in fat bodies rather than imaginal disc, which postulates the loading of Wg within lipoproteins either extracellularly or endosomally before the secretion of Wg. Besides, mammalian Wnt3A has been also reported to be incorporated in lipoprotein particles in vitro.

Other than lipoprotein particles, exosomes are known to contribute to Wnt solubility. Plasma membrane-derived micro-vesicular bodies fuse together outside the cell to form exosomes where Wnts' palmitoylated adducts are reported to be inserted within the lipid bilayer. In vitro supernatant describes the presence of Wg as well as Wnt3A-incorporated exosomes to be active. Moreover, researches have produced evidence that both Wg and Wls bear exosomes at *Drosophila* neuromuscular junction. Additionally, inhibiting expression of exosome-associated protein such RAb11, SNARE recognition protein Ykt6, has been reported to decrease Wg gradient formation, which explains the importance of exosome-mediated transportation of Wnt proteins.

Also, swim, a protein of lipocalin family, has shown to increase Wnts' solubility directly by interacting with them. Swim-knock down can deteriorate Wg long-range impact but remains passive to regulate Wg short-range gradient. Similarly, a serum glycoprotein afamin recently has been noted to keep Wnts soluble and active which dictates its great practical implications. Apart from short-range transportation, the existence of Wnt signaling has been also reported on axin-based extension of cells known as cytonemes. In zebrafish during neural plate formation, Wnt8 was shown to elicit signals at distantly located cells via cytonemes. Therefore, depending on cell types and their behavior, both close and long-range diffusion of Wnts are evident.

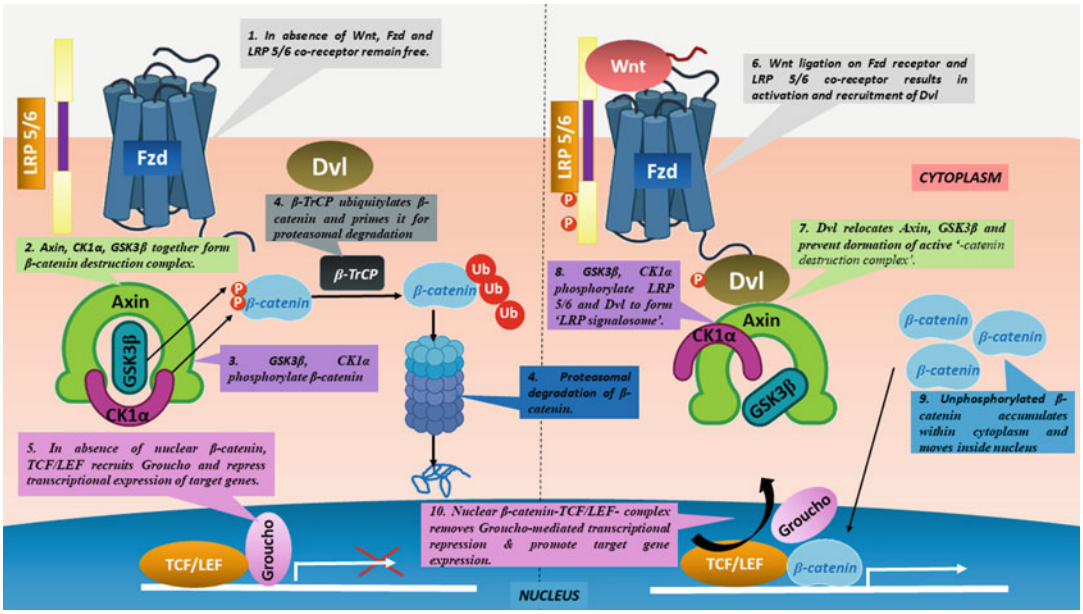
Insight into Wnt Signaling

Secreted Wnts also interact with a number of transmembrane receptors which subsequently drive the signaling towards a specific fate in a particular cellular context. Among many, Fzd is the widespread high-affinity Wnt receptor that, in

association with other co-receptors, channelizes downstream signaling into distinct branches. Co-receptors which take part in Wnt signaling include low-density lipoprotein receptor-related protein 5/6 (LRP5/6), ROR, RYK, PTK7, MUSK, etc. Depending on the type of receptor-co-receptor interaction and downstream participation of transcriptional co-activator β-catenin, broadly Wnt signaling cascade can be categorized into two types, β-catenin-dependent canonical pathway, and β-catenin-independent noncanonical pathway.

Canonical Wnt Signaling

Fzd is a group of serpentine receptors having 10 homologs in humans, all of which carry N-terminal extracellular Cys-rich domain (CRD) for ligand interaction. A three-dimensional structure of *Xenopus* Fzd8 together with mouse Wnt8 has confirmed that the palmitoylated N-terminal domain, termed as, of Wnt8 projects into a groove of Fzd8. Also, the C-terminal index finger domain of Wnt8 has shown to engage certain variable amino acids within Fzd8 CRD which possibly provides Wnt-Fzd interaction specificity. Canonical pathway is triggered when ligands such as Wnt1, Wnt3A, Wnt8, etc. (Fig. 2) (Niehrs 2012) form a ternary complex with Fzd and co-receptor LRP5/6. In absence of ligand binding, downstream "β-catenin destruction complex" (a constitute of scaffold protein Axin, adenomatous polyposis coli or APC, glycogen synthase kinase 3β or GSK-3β, and casein kinase1α or CK1α) remains active. N-terminal phosphorylation by CK1α at Ser45 followed by GSK3β-mediated phosphorylation at Ser33, Ser37, and Thr41 render β-catenin targeted by beta-transducin-repeat–containing protein (β-TrCP), a substrate recognition subunit of multimeric Skp1-Cullin-F-box (SCF) RING-type E3 ubiquitin ligase (Gao et al. 2014). Subsequently, $SCF^{\beta\text{-}TrCP}$ targets β-catenin for proteasomal degradation by adding poly-ubiquitin chain on its Lys19 and Lys49. Thus, lowered cytosolic β-catenin concentration



Wnt Signaling, Fig. 2 Schematic representation of canonical Wnt signaling pathway. Absence of Wnt ligands keeps receptor Fzd and co-receptor LRP 5/6 inactive. Cytosolic Axin, GSK3β, and CK1α together form "β-catenin destruction complex" which phosphorylates cytosolic β-catenin and aid its β-TrCp-driven ubiquitination and proteasomal degradation. But, Wnt ligation on Fzd-LRP activates Dvl which relocates Axin from "β-catenin destruction complex" and LRP-signalosome formation. Unphosphorylated β-catenins gather and enter the nucleus where they interact with TCF/LEF to wave Groucho-mediated target gene repression

prevents their nuclear gathering and, thus, represses target gene expression through repressive T-cell factor/lymphoid enhancer factor (TCF/LEF) complex and further Groucho-mediated histone deacetylase recruitment, whereas the presence of Wnt recruits disheveled (Dvl) to Fzd intracellular domain which in turn relocates Axin and GSK3β to the plasma membrane (PM). At this point Dvl itself oligomerizes inducing Wnt-Fzd complex to be internalized via endocytosis. Dvl induces type-I phosphatidylinositol-4-phosphate-5-kinase catalyzed production of phosphatidylinositol 4,5-bisphosphate (PIP_2). PIP_2 seems to be crucial for sequential phosphorylation of LRP6 intracellular domain at Ser1490, Thr1493, and Thr1479 by GSK3β, CK1α, and CK1γ, respectively. Phosphorylated PPPSP motif on LRP6 acts as the docking site for more Axin which ends up forming LRP signalosome at PM involving Fzd, LRP5/6, Axin, GSK3β, and CK1α. As a result, piled up cytosolic unphosphorylated β-catenins translocate in the nucleus where they interact with TCF/LEF in order to promote Groucho displacement and recruitment of transcription activators such as p300-CREB binding protein, BCL-9, Brahma related gene1, and Pygo, letting the downstream targeted gene expression.

Noncanonical Wnt Pathway

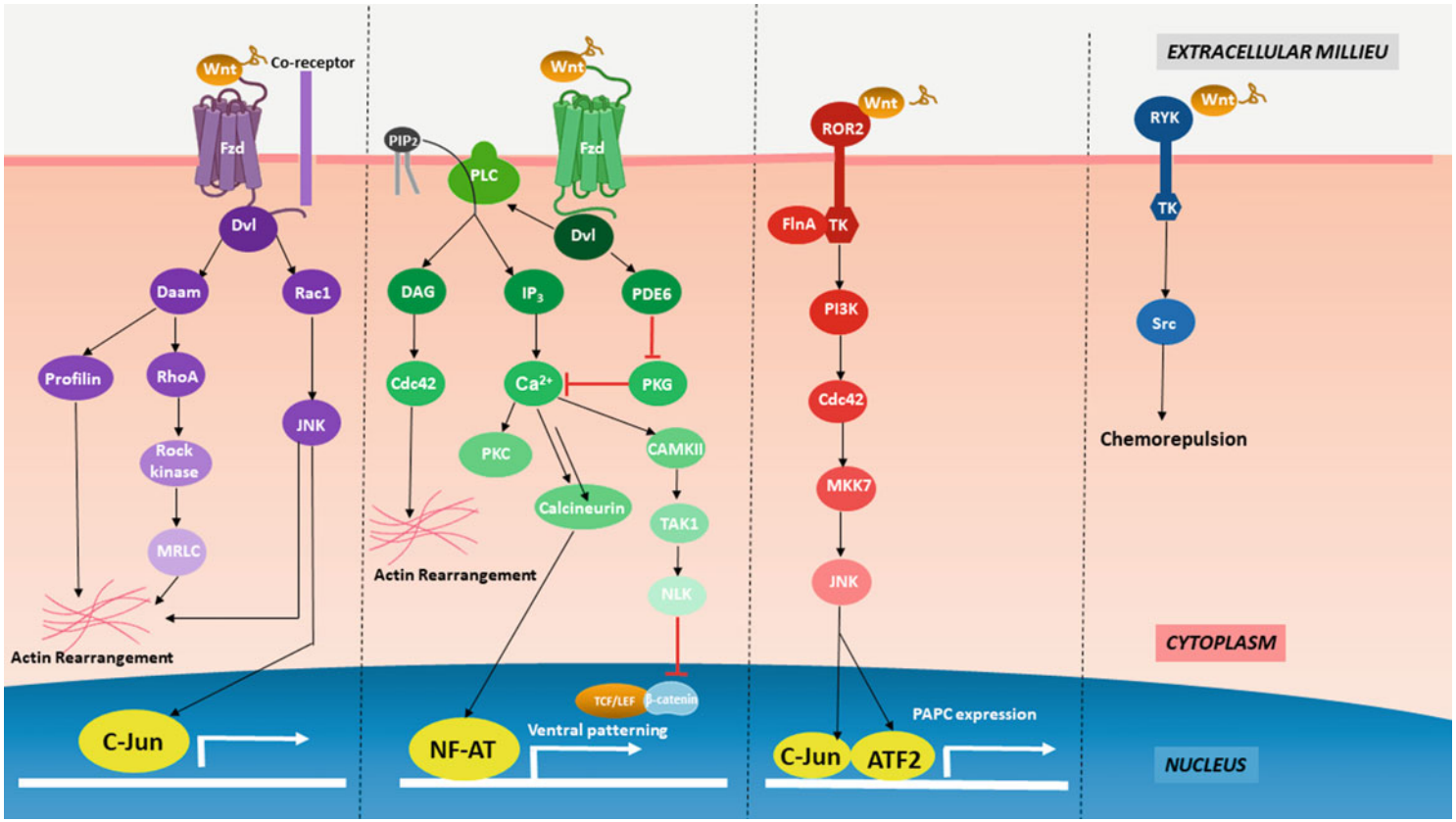
The noncanonical pathway is triggered by ligands like Wnt5A, Wnt11, etc. Among a vast array of noncanonical pathways, two categories are best understood, namely, planar cell polarity (PCP) pathway and Wnt/Ca^{2+} pathway (Niehrs 2012).

PCP signaling actuates when Wnt ligand bind with Fzd receptor along with cognate co-receptors other than LRPs. Binding initiates Dvl1 recruitment at Fzd and further stabilization by PCP factor Diego. Dvl1 in turn activates small G-protein RhoA and Rac1, parallelly activating two different pathways. In this scenario, regulation of such small GTPases occurs through the communication of Dvl with partitioning defective 6 (PAR6), Dvl-associating protein with a high frequency of Leu residues (DAPLE), DVL-associated activator of morphogenesis 1 (DAAM1), etc. RhoA in turn activates Rho kinase leading to the actin rearrangement, whereas Rac1 promotes transcriptional activation of c-Jun N-terminal kinase (JNK) in order to activate target transcription factors such as activating transcription factor 2 (ARF2) inducing target gene expression. PCP signaling is essential during embryogenic morphogenesis such as cellular movement in gastrulation, neural tube closure, and movement of stereocilia in the inner ear.

In Wnt/Ca^{2+} pathway, Wnt triggers Fzd-assisted activation of heterotrimeric G-protein which concomitantly induces phospholipase C (PLC)-mediated formation of diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3) (De 2011). Later IP_3-exterted Ca^{2+} influx results in activation of Ca^{2+} and calmodulin-dependent kinase II (CAMKII), calcineurin, phosphatase CNC, and protein kinase C (PKC). Wnt-Fzd also upregulates cGMP-specific phosphodiesterase PDE6, thus depleting cellular cGMP and inactivating protein kinase G (PKG), thereby leading to an increase in the cellular concentration of Ca^{2+}. Activated PKC further upregulates nuclear factors associated with T-cell (NFAT) promoting ventral patterning in *Xenopus*. In *Xenopus* Wnt5A-Ca^{2+}-CaMKII signaling can uplift TAK1-NLK kinases, which counteracts canonical Wnt signaling.

Depending on various receptor-co-receptor combinations, other than the aforementioned two principal pathways, there are also different modes of noncanonical Wnt signaling pathways. Some of them are discussed next (Fig. 3).

ROR1 and ROR2 are single-pass integral membrane receptor with intracellular tyrosine kinase domain and extracellular N-terminal CRD. Wnt5A binding with Fzd and ROR2 co-receptor induces ROR2 homodimerization and formation of subsequent ternary complex. Activated receptor recruits Dvl, Axin, and GSK3 which in turn incorporate actin-binding protein filamin A to activate JNK leading to fibroblast cytoskeletal rearrangement, polarization, and filopodia extension. Molecular and phenotypical resemblance of Wnt-ROR signaling with PCP signaling does not implicate their obvious overlapping. ROR1-ROR2 mutant cells were capable of JNK signaling, while mice carrying such mutation phenotypically differ from the mice mutant



Wnt Signaling, Fig. 3 Schematic representation of important noncanonical Wnt signaling pathways. Wnt5 and Wnt11 initiate noncanonical Wnt signaling pathway when they ligate with cognate Fzd receptors along with co-receptors other than LRPs. Ligand-receptor interaction activates downstream Dvl which through a number of pathways activates transcriptional co-activators ultimately expressing proteins involved in cytoskeletal rearrangement to encourage cell polarity, cell motility, morphogenesis, etc.

for Vang like-2 (a PCP pathway component). During *Xenopus* gastrulation, ROR2-Wnt5A complex activates PI3K-Cdc42-MKK7-JNK pathway leading to ATF2 and c-Jun induction and paraxial protocadherin (PAPC) expression (Semenov et al. 2007).

RYK (*Drosophila* homolog derailed) is another single-pass transmembrane co-receptor that is implicated in all three major areas of Wnt signaling through neural fate determination and providing axonal guidance in the central nervous system. RYK possesses an inactive kinase domain and is kinase-dead. Like others it also acts by Dvl recruitment probably through hiring additional Src kinase. γ-Secretase is known to cleave the intracellular domain of RYK which allows cleaved RYK to translocate in the nucleus leading to the transcriptional modification of target genes. E3 ubiquitin ligase Mind bomb1 targets RYK for proteasomal degradation which, in turn, is known to accelerate B-catenin stabilization.

Besides, PTK7 (known as off-track in *Drosophila*) is another class of single-pass transmembrane co-receptor that has been implicated only in PCP signaling through Dvl interaction. Its incomplete intracellular kinase domain seems to be kinase-dead. However, like other PCP activators, it appears to be a negative regulator of β-catenin stabilization.

Another co-receptor that acts through PCP signaling pathway is MUSK. Binding of Wnt11 to the extracellular CRD of MUSK specially at neuromuscular junction where it restricts postsynaptic acetylcholine receptors to the center of long muscle cells. Additionally, in human myocytes Wnt4, Wnt11, and Wnt9A ligation at Fzd-LRP4 lead to MUSK dimerization and concomitant phosphorylation leading to clustering of acetylcholine receptor.

Extracellular Wnt Regulation

Wnt Antagonists

Once secreted Wnt encounters with a wide variety of regulatory molecules that involves secreted as well as secreted soluble antagonists and agonists. Among them Wnt interacts with secreted extracellular inhibitors such as Wnt-inhibitory factor-1 (Wif-1), Dickkopf (Dkk), secreted Fzd-related proteins (sFRPs), bone-related SOST/scleostin, etc. Moreover, Wnt interacts with membrane-bound antagonists such as glypicans, APC down-regulated-1 (APCDD-1), Tiki1/Tiki2, etc. Dkk is a group of secreted glycoproteins with 255–350 amino acid residues. Functioning as a decoy receptor, Dkk recruits a single-pass transmembrane protein Kremen that after binding results in endocytosis of LRP5/6, diminishing canonical Wnt signaling.

Unlike Dkk, Wif-1 directly impedes Wnt signaling by sequestering Wnt ligands. Wif-1 contains one WIF domain and five epidermal growth factor (EGF)-like domains. Structural study of Wif-1 reveals 1,2-dipalmitoylphosphatidyl choline to be integral component of WIF domain. Exchange of Wif-1-linked diacyl lipid with Wnt-bound palmitoyl moiety seems to seize Wnt signaling predominantly. Besides, heparan sulfate (HS)-binding domain of EGF-like domain I-IV proposes the role of HS in co-localizing Wif-1 and Wnts together.

On other hand, sFRPs are known to harbor N-terminal CRD similar to Fzd receptors. Thus, it can be easily suggested that sFPRs dampen Wnt signal by competing with Fzd receptors for Wnt ligation.

Additionally, transmembrane Tiki1/Tiki2 acts catalytically to dampen Wnt signaling. Quantitative mass spectroscopy and protein sequencing have revealed Tiki2 functions as a protease and promote the removal of the last eight amino acid residues from the N-terminal end of the post-translationally processed mature Wnt3A and prevent their further ligation at Fzd receptor (Langton et al. 2016).

Wnt-YAP/TAZ Interplay

Two transcriptional regulators of Hippo pathway, namely, yes-associated protein/taffazin (YAP/TAZ), are reported to regulate Wnt-β-catenin pathway at the level of β-catenin destruction complex. In absence of Wnts, cytosolic YAP/TAZ forms a part of β-catenin destruction complex and thus in association with Axin1 appoint β-TrCP and sequester β-catenin for proteosomal degradation. However, Wnt ligation promotes

their displacement from Axin and further β-catenin stabilization in order to promote increased canonical Wnt signaling.

Modulation by R-spondin (RSPO)

RSPO is a group of secreted proteins all of which share common structural similarities and harbor two furin domains essential for β-catenin-mediated signaling, whereas their thrombospondin type-I domains engage in PCP signaling. High-affinity receptor syndecan binds RSPO2 and RSPO3 thrombospondin domain and enhances PCP signaling. The binding of RSPOs stimulates clathrin-assisted RSPO-receptor endocytosis by possible flocking of Wnt-receptor and Dvl.

Another class of furin-interacting RSPO receptors which drive β-catenin stabilization is known as leucine-rich repeat-containing G protein–4/5/6 (LGR 4/5/6) (Fig. 4). They are explicit markers of stem-cell renewal in intestinal epithelia. In both cases, RSPO seems to uplift Wnt signaling by downregulating membrane-bound E3 ubiquitin ligase Znrf3/Rnf43. In absence of RSPO, Znrf3/Rnf43 ubiquitylates Fzd receptors and targets a portion of it to be degraded. In presence of RSPOs, the RSPO-LGR complex assembles in a complex with Znrf3/Rnf43, causing their internalization and greater clustering of Fzd receptors on the plasma membrane to upregulate the entire Wnt Signaling (Ghosh et al. 2019).

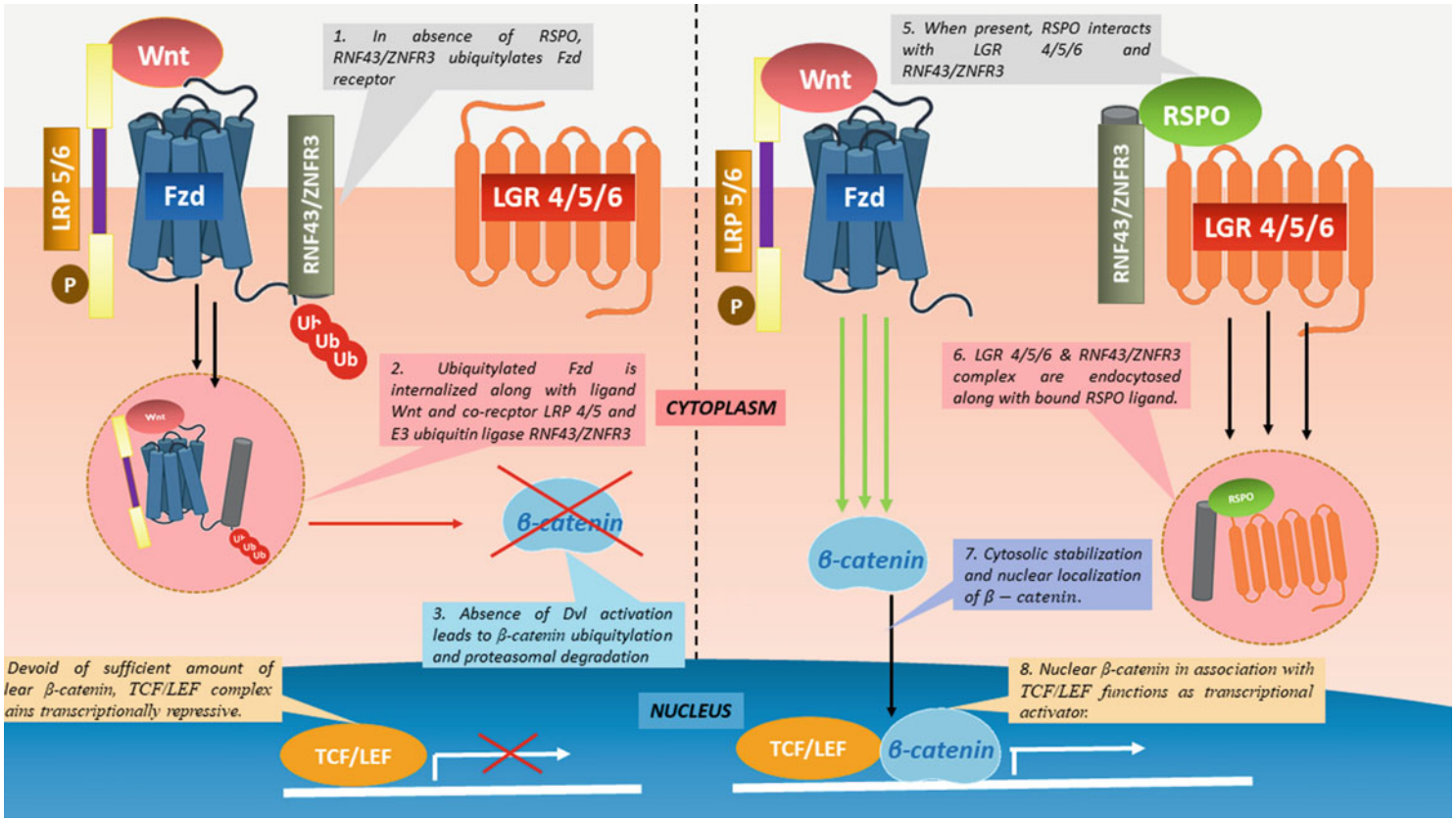
Decarboxylation of Acylated Wnts by Notum

Once secreted, acylated Wnts also act as the substrate for Notum, imposing another level of regulation on Wnt signaling (Nile and Hannoush 2016). Molecular labeling experiments and mass spectroscopic data have revealed that Notum instead functions as the carboxylesterase by hydrolyzing carboxyester linkage connecting the palmitoleate residue at Ser209 on Wnt3A. Besides, Notum forms an oxidized cluster of Wnts inhibiting the over signaling pathway. Surface plasmon resonance study of human Notum (hNotum) has been noted to interact with the glycose-aminoglycan of GPC3, and in addition, loss of dally and dlp from the outer surface of *Drosophila* imaginal disc has shown to reduce Wnt signals too; both of these findings state that glypicans tether Notum to the cell surface through their sulfated glycan moieties and seems to stimulate Wnt deacylation. Instead of modulating Wnt secretion, deacylation of Wnts has been suggested to downregulate Wnt signaling enzymatically, whereas interrupting Wnt3A-Fzd8 interaction via sequestering fatty acyl adducts seems to inhibit Wnt signaling non-catalytically. Such findings prove the activity of Notum to be entirely extracellular. Crystal structure study of both *Drosophila* and human Notum defines the presence of a conserved α/β-hydrolase fold where the catalytic triad incorporates Ser 232, Asp340, and His389. X-ray crystallographic data of enzymatically dead hNotum, complexed with palmitoleated and myristoleated human Wnt7A, illustrates that both of the substrates acquire the active site of the enzyme by entering the hydrophobic pocket of Notum through a comparatively flexible opening. At the active site, the carbonyl carbon of the fatty acyl moiety has been depicted to be placed at about 3.3 A^0 distance from the catalytic Ser232 ensuring enough place to accommodate hydroxyl nucleophilic attack. During catalysis, the interaction of palmitoleate- or myristoleate-peptide derived from acylated Wnts, with Ile291, Phe319, and P320 at the base of enzyme's hydrophobic pocket further stabilizes substrates for successful catalysis. The binding pocket of hNotum was shown to accommodate saturated fatty acid up to the length of C8-C10, beyond which the hydrocarbon tail of the fatty acid must be kinked in order to avoid steric hindrances. Therefore, Notum has been considered to show selectivity towards cis-unsaturated fatty acid explaining why Notum prefers diacylation of Wnts' palmitoleate and myristoleate residues over palmitate and myristate residues, respectively.

Drugs

Owing to the diversity of Wnt signaling, several components of Wnt signaling can be targeted for therapeutic intervention (see Table 1) (Nusse and Clevers 2017).

Wnt antagonist SOST can be targeted in several bone disorders; indeed, anti-SOST



Wnt Signaling, Fig. 4 Schematic representation of Wnt signaling pathway in presence of RSPO ligands. In absence of RSPO ligands, E3 ubiquitin ligase single-pass RNF43/ZNFR3 ubiquitylates Fzd receptor both in absence and presence of Wnt ligation. Resultant ubiquitylated Fzd receptors are targeted for cellular internalization which renders cytosolic "β-catenin destruction complex" active and β-catenin targeted for proteasomal degradation. However, RSPO ligation to receptor LGR tethers RNF43/ZNFR3 in order to promote endocytosis of RSPO-LGR-RNF43/ZNFR3 complex which deactivates "β-catenin destruction complex" and encourages β-catenin nuclear gathering for subsequent target gene transcription

Wnt Signaling, Table 1 Drugs modulating Wnt signaling pathway

Name of the drug	Phase of clinical or subclinical trial and trial number	Disease associated	Target molecule	Effect on overall Wnt signaling	Mode of action
ETC-159	Trial phase I (NCT02521844)	Solid tumor treatment	PORCN	Inhibitory	Prevents PORCN-mediated acylation of Wnt proteins
IWP-2	Preclinical –	Metastatic colorectal cancer, Notch-mutant head and neck carcinoma treatment			
LGK974	Trial phase I/II (NCT02278133 NCT02649530)	–			
CGX1321	Clinical trial phase I (NCT026759462)	Metastatic solid tumor treatment	Wnts	Inhibitory	Prevents Wnt functionality
Foxy-5	Clinical trial phase I (NCT02020291 NCT02655952)	Metastatic breast cancer, prostate cancer having lowered Wnt5A expression, and colorectal cancer			Mimics activity of Wnt5A
Ipafricept	Clinical trial phase I (NCT02069145 NCT02092363 NCT02050178)	Metastatic hepatocellular carcinoma, ovarian cancer, and stage IV pancreatic ductal adenocarcinoma			Acts as decoy receptor of Wnts through competition with Fzd receptors
OSTA101	Clinical trial phase I (NCT01469975)	Synovial sarcoma	Fzd10	Inhibitor	Yttrium90 labeled anti-Fzd10 monoclonal antibody
Vantictumab	Clinical trial phase I (NCT01973309 NCT02005315 NCT01957007)	Metastatic breast cancer, pancreatic ductal adenocarcinoma, and non-small cell lung cancer	Fzds		Humanized antibody against Fzd1, Fzd2, Fzd5, Fzd7 and Fzd8
SB-216763	–	–	GSK3 inhibitor	Activator	–
G007-LK, G244-LM	Preclinical trial phase	Colorectal cancer	Tankyrase inhibitor	Inhibitor	Prevents GSK degradation and stabilizes β-catenin
L807mts	Preclinical trial phase	Alzheimer's Disease	GSK3 inhibitor	Activator	Inhibits GSK3 by changing its catalytic site conformation
LY2090314	Preclinical trial phase	Melanoma	GSK3 inhibitor	Activator	Inhibits GSK3-mediated phosphorylation and stabilizes β-catenin
OMP121R10	Clinical trial phase I (NCT02482441)	RSPO3 positive metastatic colorectal carcinoma	RSPOs	Inhibitory	Monoclonal anti-RSPO3 antibody
Cirmtuzumab	Clinical trial phase I (NCT02222688)	Chronic lymphoid leukemia	ROR1	Inhibitory	Humanized monoclonal anti-ROR1 antibody

(continued)

Wnt Signaling, Table 1 (continued)

Name of the drug	Phase of clinical or subclinical trial and trial number	Disease associated	Target molecule	Effect on overall Wnt signaling	Mode of action
DKN01	Clinical trial phase I (NCT01457417 NCT02013154 NCT02375880)	Relapsed lung cancer, esophageal cancer, advanced biliary cancer with gemcitabine and cisplatin combination therapy	Dkk	Inhibitory	Humanized monoclonal anti-Dkk1 antibody
BHQ880	Clinical trial phase I (NCT00741377)	Multiple melanomas with zoledrinic acid combination therapy			Humanized anti-Dkk1 Ig-G1 monoclonal antibody
PRI-724	Clinical trial phase Ia (NCT01302405 NCT01764477 NCT01606579) Clinical trial phase II (NCT02413853)	Locally advanced solid tumor, pancreatic cancer with gemcitabine combination therapy, and advanced myeloid malignancy. Combinatorial therapy with bevacizumab in myeloid malignancy	CBP and β-catenin	Inhibitory	Blocks β-catenin and CBP
CWP232291	Clinical trial phase Ia/Ib (NCT0398462)	Acute myeloid leukemia, myelodysplastic syndrome	APC and TCF1	Inhibitory	Targets Sam68 through APC interaction and alternative splicing of TCF1
IWR1	Preclinical trial phase	–	Tankyrases	Inhibitory	Prevents tankyrase1 and 2 mediated PARsylation
XAV939	Preclinical trial phase	–	Tankyrase	Inhibitory	Tankyrase inhibitor
JW55	Preclinical trial phase	–	Tankyrase	Inhibitory	Same as above
NVP-PNKS656	Preclinical trial phase	–	Tankyrase	Inhibitory	Same as above
PKF115-584	Preclinical trial phase	–	β-catenin/LEF complex	Inhibitory	β-catenin/LEF inhibitor
NSC668036	Preclinical trial phase	–	Dvl	Inhibitory	Inhibits interaction of Dvl with Fzd receptor by blocking Dvl-PDZ domain
NCB-0846	Preclinical trial phase	–	Tankyrase	Inhibitory	Tankyrase inhibitor
ICG001	Preclinical trial phase	–	CBP	Inhibitory	CBP inhibitor
PKF118-310	Preclinical trial phase	–	β-catenin/TCF1 complex	Inhibitory	β-catenin/TCF1 inhibitor

monoclonal antibody romosozumab has yielded encouraging results in clinical trials. Being the central scaffold protein, Axin can be targeted widely. Tankyrases, a group of poly-ADP-ribopolymerase, ubiquitylases Axin; thus, small-molecule tankyrase inhibitors such as IWR, XAV939 etc., can be used in several types of cancer with upregulated β-catenin expression.

Another way of Wnt inhibition can be achieved by inhibiting PORCN. IWP, LGK974 etc., functions as PORCN inhibitors and thereby block Wnt acylation and subsequent secretion. Diseases with uplifted Wnt signaling can be treated by targeting Fzd receptors. Hence, monoclonal antibody OMP-18R5 has been shown to inhibit tumor formations in several xenograft studies.

Recently, soluble Wnt protein agonists have been shown to activate Wnt signaling in vivo. In addition, a number of small-molecule compounds such as Bio, L807mts, CHIR, etc. dampen GSK3 activity and thus result in target gene expression. Such drugs can be useful in treating several neurogenerative disorders involving Alzheimer's disease.

The effect of GSK3 inhibitors can be mediated by targeting the expression of gene REST which acts as the repressor of neuronal gene expression during embryonic development and seems to be protective in Alzheimer's disease.

Conclusively, we are closing in now to a point of Wnt signaling where the importance of this signaling pathway for understanding several pathological conditions is coming into focus. The efforts for finding all the interferences with Wnt signaling are yet to bloom, but there are promising ways out which hopefully will replicate soon into reality.

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X

XNPEP2

► Apelin

S. Offermanns, W. Rosenthal (eds.), *Encyclopedia of Molecular Pharmacology*,
https://doi.org/10.1007/978-3-030-57401-7

Z

Zinc-Binding

- ► Ca^{2+}-Binding Proteins
- ► S100 Proteins

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https://doi.org/10.1007/978-3-030-57401-7