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03

Volume Editors Bernard Fermini · Birgit T. Priest

Ion Channels

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Topics in Medicinal Chemistry

Ion Channels

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Preface to the Series

Medicinal chemistry is both science and art. The science of medicinal chemistry offers mankind one of its best hopes for improving the quality of life. The art of medicinal chemistry continues to challenge its practitioners with the need for both intuition and experience to discover new drugs. Hence sharing the experience of drug discovery is uniquely beneficial to the field of medicinal chemistry.

The series *Topics in Medicinal Chemistry* is designed to help both novice and experienced medicinal chemists share insights from the drug discovery process. For the novice, the introductory chapter to each volume provides background and valuable perspective on a field of medicinal chemistry not available elsewhere. Succeeding chapters then provide examples of successful drug discovery efforts that describe the most up-to-date work from this field.

The editors have chosen topics from both important therapeutic areas and from work that advances the discipline of medicinal chemistry. For example, cancer, metabolic syndrome and Alzheimer's disease are fields in which academia and industry are heavily invested to discover new drugs because of their considerable unmet medical need. The editors have therefore prioritized covering new developments in medicinal chemistry in these fields. In addition, important advances in the discipline, such as fragment-based drug design and other aspects of new lead-seeking approaches, are also planned for early volumes in this series. Each volume thus offers a unique opportunity to capture the most up-to-date perspective in an area of medicinal chemistry.

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Prof. Dr. Gunda J. Georg
Dr. John Lowe
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Preface to Volume 3

The history of ion channel research is one that is rich and fascinating. It spans many different disciplines (biology, physiology, biophysics, bioelectricity, etc.), extends over more than two centuries, and today represents a mature and exciting field. Because of their prevalence and the critical role they fulfill in virtually all tissue types and organs, ion channels play a vital role in basic physiological functions, including muscle contraction, CNS signaling and hormone secretion among many. Not surprisingly, ion channels are drug targets for a number of therapeutic agents. At the other end of the spectrum, several human diseases have been linked to mutations or dysfunction of ion channels. In some cases, elucidating the specific ion channel dysfunction underlying the disease phenotype may provide a target for therapy. The recognized importance of ion channels in health and disease, combined with the potential to develop a broad range of new drugs for the treatment of ion channel-related diseases, has fueled the need to develop more suitable screening technologies accounting for their complex structure and function, and has led to a dramatic increase in the number of medicinal chemistry programs directed at ion channel targets.

Accordingly, this volume was written to introduce medicinal chemists to the field of ion channels. Its aim is to review recent advances in the field of ion channel-related diseases, and is meant to be accessible to graduate students, teachers, biologists, chemists and many other disciplines. Following an overview chapter summarizing the current state of ion channel screening technologies, five topics covering areas such as cancer, cardiac arrhythmias, cystic fibrosis, and pain have been selected, and the current state of knowledge is presented by leading experts in their field in a way that is accessible to all. Each chapter is structured to cover the biological rational for the target, the current status in the development of agents to treat the disease, and future perspective and challenges facing each therapeutic area. Hopefully, this effort will help to foster enhanced communication and collaborations between chemists and ion channel experts. Whether we are at the verge of a golden age for ion channel drug discovery remains to be determined, but what unfolds over the coming years should be of utmost interest for anyone involved in drug development.

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Recent Advances in Ion Channel Screening Technologies

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Abstract Ion channels play a vital role in basic physiological functions such as generation of electrical activity in nerves and muscle, control of cardiac excitability, intracellular signaling, hormone secretion, cell proliferation, cell volume regulation, and many other biological processes. Because of their prevalence and the critical role they play in virtually all tissue types and organs, ion channels are also involved in a number of pathophysiological conditions. The recognized importance of ion channels in health and disease, combined with the potential to develop new drugs targeting ion channels in a broad range of diseases, has fueled the need to develop more suitable screening technologies accounting for their complex structure and function. Ion channels have been neglected as drug discovery targets because of the inability to study large number of compounds or validate large numbers of unknown or mutant ion channel genes using traditional ion channel screening technologies. Therefore, several efforts were undertaken to automate and improve the throughput of electrophysiological methods. In this chapter, we will review a number of the more standard ion channel screening technologies currently used, including: (1) radioligand binding assays, (2) fluorescent assays using membrane potential dyes, and (3) ion flux assays, and emphasize some of the advantages and shortcomings of

these different approaches. We will then discuss automated patch clamp technologies that aim to automate and dramatically increase the throughput of the standard voltage clamp method, and offer a true archetype shift in ion channel drug discovery.

Keywords Automated electrophysiology · HTS electrophysiology · Ion channels · Patch clamp · Planar patch · Screening technologies

1 Introduction

Ion channels play a vital role in basic physiological functions such as generation of electrical activity in nerves and muscle, control of cardiac excitability, intracellular signaling, hormone secretion, cell proliferation, cell volume regulation, and many other biological processes. Because of their prevalence and the critical role they play in virtually all tissue types and organs, ion channels are also involved in a number of pathophysiological conditions. Diseases involving dysfunction of ion channels in humans and animals are termed channelopathies, defined as inherited diseases caused by mutations in the genes encoding a multitude of ion channels. Channelopathies can arise in a number of different ways, ranging from complete loss of function of the channels through dominant-negative suppression, to discrete disturbances in channel function, often resulting in major health issues. They manifest themselves as prominent genetic and phenotypic heterogeneity related to the mutations affecting the different channels. Today, a multitude of human disorders have been linked to ion channel dysfunction including epilepsy, episodic ataxia, long QT syndrome, Bartter's syndrome, and cystic fibrosis (see the chapter from Dr. Van Goor et al. in this volume). A summary of a number of channelopathies associated with human disease can be found elsewhere [1].

Ion channels are also drug targets for a number of therapeutic agents developed for the treatment of various diseases (covered in this volume). Several of these agents are ion channel modulators that provide beneficial effects in the treatment of diseases, not by attenuating the effects of mutated channels, but rather by having direct effects on the channels in a more physiological context. For example, Na^+ channel blockers such as lidocaine and lamotrigine are used as local anesthetic and to treat epilepsy, respectively. Calcium channel blockers such as verapamil and nifedipine are used for the treatment of cardiac arrhythmias and hypertension, while ATP-dependent K^+ channel inhibitors such as tolbutamide and glibenclamide are effective in the treatment of type II diabetes. The recognized importance of ion channels in health and disease, combined with the potential to develop new drugs targeting ion channels in a broad range of diseases, has fueled the need to develop more suitable screening technologies accounting for their complex structure and function. In this chapter, we will review some of the recent advances in ion

channel screening technologies that offer a true paradigm shift in ion channel drug discovery.

2 Ion Channels as Drug Targets

Ion channels are broadly described in two major groups (Table 1): (1) voltage-gated channels, such as sodium, calcium, and potassium channels that respond to changes in membrane potential and are found in many different tissues including nerves and the heart; and (2) ligand-gated channels, which are regulated by various extracellular or intracellular ligands. These channels are often named according to the ligand they bind. For example, the nicotinic acetylcholine receptor (nAChR) is a prototypic ligand-gated channel. It is activated by the endogenous ligand, acetylcholine, and the drug nicotine. Stretch-activated, stretch-gated and/or mechano-sensitive channels represent another class of ion channels that open and close in response to mechanical stimuli, changes in membrane tension, or hypo-osmotic shock. Although our understanding of these channels has increased significantly over the past few years [2, 3], relatively little is known about them when compared to voltage-gated or ligand-gated channels, and they will not be covered in this section.

Table 1 Classification of the two major groups of ion channels found in the plasma membrane of mammalian cells

Ion channel	Selectivity	Activator
Voltage-gated channels		
Potassium	K^+	Membrane potential
Sodium	Na^+	Membrane potential
Calcium	Ca^{2+}	Membrane potential
Chloride	Cl^-	Membrane potential
HCN	Na^+, K^+	Membrane potential
Ligand-gated channels		
nAChR	Na^+, K^+, Ca^{2+}	Ach, nicotine
$GABA_{A,C}$	Cl^-	GABA
Glycine	Cl^-	Glycine, strychnine
$5\text{-}HT_3$	Na^+, K^+, Ca^{2+}	Serotonin
AMPA	Na^+, K^+, Ca^{2+}	Glutamate, AMPA
Kainate	Na^+, K^+, Ca^{2+}	Glutamate
NMDA	Na^+, K^+, Ca^{2+}	Glutamate, NMDA
CNG	Na^+, K^+, Ca^{2+}	cAMP
IP_3R	Ca^{2+}	IP_3
P2X, P2Z	Na^+, Ca^{2+}, Mg^{2+}	ATP

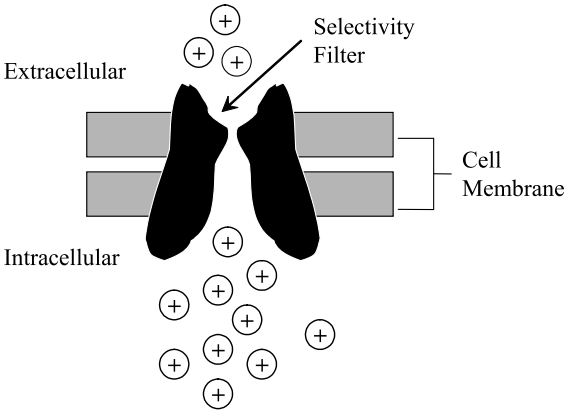

Fig. 1 Schematic of a voltage-gated ion channel

Tremendous progress has been achieved in elucidating the structure of several ion channels, and such discoveries have increased our understanding of the properties underlying their ionic selectivity, conductance, and gating [4]. Ion channels are protein complexes that span the cell membrane lipid bilayer, allowing charged ions to pass across a naturally impermeant barrier (Fig. 1). The core membrane-spanning domain of the channel forms the pore, allowing ions such as Na^+, Ca^{2+}, K^+, or Cl^- to flow through down their electrochemical gradient (Table 2). Ion channels are usually formed of an α-subunit that comprises the pore and the selectivity filter for that channel, as well as the gating apparatus that allows for channels to open and close in response to various stimuli. Often associated with α-subunits are multiple auxiliary subunits that have been shown to play a number of roles, including but not limited to modifying the function of the α-subunit, or playing a role in trafficking of the channel to the plasma membrane [5]. Most ion channels show selectivity, allowing only some inorganic ions of appropriate size and charge to pass through them. In order to do so, permeating ions need

Table 2 Typical concentrations of five major ions inside and outside mammalian cells

Ion	Ionic concentration (mM) Extracellular	Intracellular
Na^+	145	12
K^+	4	155
Ca^{2+}	1.8	0.001 (rest)–0.1 (active)
Mg^{2+}	1.5	0.8
Cl^-	123	4.2

to shed most of the water molecules associated with them in order to pass in single file through the narrowest portion of the channel, often referred to as the selectivity filter [6]. Most ion channels allow ions to move through them in either direction, but the direction of the current flow through a specific channel is dictated by the electrochemical gradient (Table 2). Moreover, channels are not continuously open, but rather undergo conformational changes from closed to open states. These properties distinguish channels from aqueous pores, transporters, and pumps. Once open, channels allow the passage of ions at rates of about 100 million ions per second. Yet some, like sodium channels, stay open for less than a millisecond at a time before closing again. Ion channels operate in virtually every cell, whether electrically excitable or not.

The diversity of ion channels is significant, especially in excitable cells of nerves and muscles. Of the more than 400 ion channel genes currently identified in the human genome [7], about 170 encode potassium channels, 38 encode calcium channels, 29 encode sodium channels, 58 encode chloride channels, and 15 encode glutamate receptors. The remaining are genes encoding other channels such as inositol triphosphate (IP_3) receptors, transient receptor potential (TRP) channels and others [8]. Currently, the potential for ion channel-directed drugs is relatively untapped. Only about 5% of marketed drugs target ion channels [9]. Given the number of genes identified in the human genome project it would appear that there remains a significant pool of unexploited targets within the ion channel field for successful discovery projects. Moreover, taking into consideration specific distribution of ion channel expression within and between tissues, heterogeneity in channel assembly and association, "splice variations" of the basic subunits, and potential state-dependent drug actions (i.e., open vs. closed), it is easy to envision the tremendous opportunities for selective modulation of ion channels and the re-emergence of drug discovery programs for the treatment of ion channel-related diseases. Yet, in spite of their historical success as therapeutic targets, and the considerable investment in this target class by the pharmaceutical industry, very few small molecule ion channel drugs have been submitted, or approved, by regulatory agencies in the past 10 years [10], and none were submitted for approval in 2007 [11]. A major constraint in developing new ion channel drugs has been the difficulty in screening ion channels in a cost-effective manner with validated functional screens that are relevant to clinical outcome at the throughput currently required by modern chemistry efforts. However, with the recent development of automated ion screening technologies, this is about to change.

The aim of this volume is to review, for the non-specialist, recent advances in the field of ion channel-related diseases. Six topics covering diseases such as cancer, cardiac arrhythmias, cystic fibrosis, and pain have been selected to reflect a number of different therapeutic areas, and each chapter is structured to cover the biological rational for the target, the current status in the development of novel agents to treat the disease, and the potential status of

clinical trials and future prospects. This first chapter provides an introduction into the world of ion channels, and covers new technologies that have allowed breakthroughs in the field of ion channel screening.

3 Historical Perspective

In order to fully appreciate the current advances in ion channel screening technologies, it is important to highlight some of the major discoveries that have directly or indirectly led to the current state in this field. The history of electrophysiology is rich and fascinating. It spans many different disciplines and fields (biology, physiology, biophysics, bioelectricity, etc.), extends over more than two centuries, and cannot be appropriately covered in this short review. Therefore, readers are referred to more comprehensive material on the subject matter [12–16].

The concept of ions and cell membranes dates back to the late 1800s. Carlo Matteucci (1811–1868) was an Italian physicist and neurophysiologist who was a pioneer in the study of bioelectricity. Using a galvanometer, an instrument that can detect and measure small amounts of currents, Matteucci was able to prove that injured excitable biological tissues generated direct electrical currents. He was the first to demonstrate that it was possible to induce muscle contraction by means of an action potential, and that action potentials were associated with depolarization of the muscle resting potential. His work in bioelectricity influenced directly the research developed by Emil du Bois-Reymond (1818–1896), a nineteenth century German physiologist, described by some historians [13] as the founder of modern electrophysiology. Using a galvanometer, du Bois-Reymond detected the flow of charges through all muscular and nervous tissue. He developed the view that a living tissue, such as muscle, might be regarded as composed of a number of "electric molecules" having certain electric properties, and that the electric behavior of the muscle as a whole in varying circumstances was the outcome of the behavior of these native electric molecules. We now know that these are sodium, potassium, and other ions that are responsible for electric membrane phenomena in excitable cells. His research established electrophysiology as a scientific discipline.

Following the discoveries by du Bois-Reymond, Sidney Ringer (1836–1910), a British clinician and pharmacologist, serendipitously discovered that Ca^{2+} was active in the heart, and performed a completely novel function: it carried the signal that initiated heart contraction. Ringer was able to show that adding small amounts of potassium chloride to a normal solution of sodium chloride allowed isolated organs to stay functional for longer periods of time. Ringer's papers published in the *Journal of Physiology* in the early 1880s are rightly acknowledged as the starting point for the development

of modern understanding of the role of calcium in the contraction of the heart [14]. In 1902, a German physiologist named Julius Bernstein (1839–1917) correctly proposed that excitable cells were surrounded by a membrane selective to K^+ ions at rest, and that during excitation the membrane became permeable to other ions. His hypothesis and research laid the foundation for understanding conduction of the nerve impulse and electrical transmission of information in the nervous system. In 1907, the British physiologist John Newton Langley (1852–1925) introduced the concept of receptor molecules on the surfaces of nerve and muscle tissue, in an attempt to explain the specific and potent actions of certain chemicals on muscle and nerve cells. Langley's theories were much debated at the time, and receptors remained theoretical until their discovery in the 1940s [15]. Then in 1937, John Zachary Young (1907–1997) was one of the first to make use of squid neurons to study ionic currents. The ease of working with large neurons made important experiments possible for the first time, including the first intracellular recordings of the nerve cell action potential, as well as the first measurements of the underlying ionic currents that produce them. His discovery and work with giant squid axons eventually led to the award of the Nobel Prize to Alan Hodgkin and Andrew Huxley (see below).

The appearance of true cellular electrophysiology followed the introduction, in 1949, of intracellular glass microelectrodes by Gilbert Ling and Ralf Gerard [17]. With this invention, it became possible to detect and measure the resting potential of a cell by impaling its membrane, without destroying the cell. Microelectrodes quickly became the technique of choice for electrophysiological recordings from all types of tissues and cells. Not long after this, using squid neurons, Alan Lloyd Hodgkin and Bernard Katz (1949) removed sodium ions from outside the neuron and were able to conclude from their data that sodium ions were responsible for the formation of the action potential [18]. The next improvement in instrumentation took place around the same period when Kenneth Cole and George Marmont described the concept of the voltage clamp method [19, 20]. This approach consisted of placing a second glass electrode inside the cell in order to stabilize or "clamp" the membrane potential of neurons for experimental purposes. Voltage clamping allowed measurements of the effect of changes in membrane potential on the conductance of this membrane to various individual ion species.

In the early 1950s, Hodgkin and Huxley characterized the time and voltage dependency of the ionic conductances that underlie an action potential in the squid giant axon, using the voltage clamp technique, and developed a mathematical model that accurately predicted the waveform of the action potential [21]. For this determining work on neuronal excitability, Hodgkin and Huxley received the Nobel Prize in Physiology or Medicine in 1963.

However, the ionic currents measured using the voltage clamp technique were the result of fluxes through an ensemble of membrane channels. Until the 1970s, it had only been possible to study ion channels as macroscopic cur-

rents, because there was simply no experimental method that could be used to isolate and characterize individual ion channels. Then, in 1976, Erwin Neher and Bert Sakmann pressed a smooth electrode tip on the surface of an isolated skeletal muscle fiber, electrically isolating a patch of membrane and reducing extraneous electrical noise so low that picoampere currents flowing through a single ion channel could be measured directly [22]. The patch clamp technique was born, and it quickly became the backbone of modern electrophysiology. It has since been referred to as the single most important development in ion channel research in the last half of the twentieth century. In 1991, Neher and Sackmann were rewarded the Nobel Prize for Physiology or Medicine for the development of the patch clamp technique.

However, this technique still had limitations because of the relatively low seal resistance (MΩ) between the recording pipette and the cell membrane. But all of this changed in 1980 with the discovery of the high resistance seal (GΩ) between the micropipette tip and the cell membrane [23]. This discovery turned out to be one of the most important revolutions in the world of electrophysiology. The incredible stability and tightness of the gigaseal's interaction between the pipette and the cell membrane allowed a complete isolation of a patch of cell membrane and an entirely new type of electrophysiological experiment (Fig. 2, part A). The gigaseal formed also allowed for three different configurations of the technique to be used:

1. Cell-attached patch, where the electrode remains sealed to the patch of membrane, allowing for the recording of currents through single ion channels in that patch of membrane
2. Inside-out patch, where the electrode is quickly withdrawn from the cell when the gigaseal is formed, thus ripping the patch of membrane off the

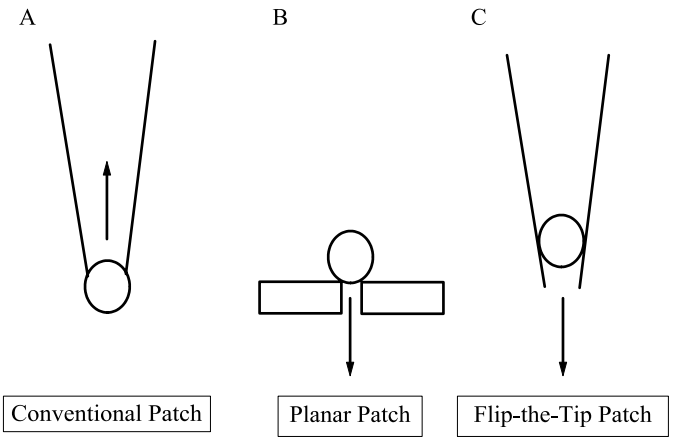

Fig. 2 Configuration of the different patch clamp methods. The *arrows* indicate the direction of suction

cell, leaving the patch of membrane attached to the electrode, exposing the intracellular surface of the membrane to the external media

3. Whole-cell recording or whole-cell patch, where the electrode is left in place, but more suction is applied to rupture the portion of the cell's membrane that is inside the electrode, thus allowing access to the intracellular space of the cell, and allowing the long-sought full control of the ionic driving forces, even with very small cells [24].

The patch clamp technique suddenly became the workhorse of modern electrophysiology, and the whole-cell configuration became one of the most crucial and popular techniques for the biophysical and pharmacological study of ion channels.

Breakthroughs in other scientific areas allowed further development of the ion channel field. For example, even though there would be no known sequence of an ion channel until the 1980s, Clay Armstrong in 1973 proposed that the structure of sodium channels in the squid neuron allows for opening and closing of their pores by a "ball and chain" model. He showed that there was a portion of the channel that could be cleaved off by a protease enzyme injected directly into the squid giant axon, acting as a kind of "ball", which was present just below the channel and could come up and plug the pore when the voltage on the cell membrane changed. When the ball was not present, the channel remained in the open configuration at all times [25].

The next major revolution in ion channel research occurred because of the advent of recombinant DNA technology in the mid to late 1970s. This approach provided the means for obtaining sequence information about genes and therefore the proteins they coded for, as well as the production of large amounts of the protein in easy-to-grow organisms such as the bacteria *E. coli*. With the amino acid sequences known, it was possible to begin predictions of what ion channels should look like in three-dimensional space. Another important advantage of being able to work with the gene of an ion channel was that the sequence could be changed deliberately (site-directed mutagenesis) in order to understand how it worked. Cloning of the first ion channel (nicotinic acetylcholine receptor, nAChR) occurred in 1982 [26], and the first channel to be sequenced was the Na^+ channel from the electric organ of the electric eel *Electrophorus electroplax* [27], followed by the Ca^{2+} channel from rabbit skeletal muscle [28]. Soon thereafter, the first K^+ channel was characterized from the *Shaker* behavioral mutant in *Drosophila* [29–32]. Introduction of recombinant expression of ion channels provided a well-defined, replenishable source of cells expressing a variety of human ion channels, opening the way to a number of biochemical assays to study ion channel function.

The first high resolution crystal structure of an ion channel (3.2 Å resolution), the potassium channel *KscA* from a bacteria, was provided by

MacKinnon and his group [4]. This meant that it was now possible to determine the exact positions of nearly all of the individual atoms in the protein. For this astonishing breakthrough, MacKinnon was awarded the Nobel Prize in Chemistry in 2003. His work also revealed for the first time the inner workings of an ion channel at the atomic level. It provided tremendous insight into the selectivity of potassium channels by revealing rings of carboxyl oxygen atoms in the narrow selectivity filter that stabilize the potassium ions as they travel in the water-filled cavity in the center of the channel, and shed their water molecules [6].

At about the same time, MacKinnon published the crystal structure of the *KscA* channel, and a report [33] reviewed the technologies available to screen ion channels as drug targets. The techniques included radioligand binding, cell-based fluorescence, radiotracer assays, and electrophysiological patch clamping techniques. However, patch clamping had not yet evolved into a high throughput screening (HTS) process, and further automation was still awaited.

Finally, in 2003, the first automated parallel patch clamp system rolled off the assembly line, revolutionizing the HTS approach to ion channel drug discovery [34]. Solutions to the problem of internal perfusion in patch clamping led to the first prototype of planar electrodes, pioneered in the Bogolometz Institute of Physiology in Kiev [35]. However, in this initial version the seal resistances achieved were rather low, and the appearance of the apertures in planar substrates in cellular electrophysiology remained a short episode until it resurfaced when the appropriate technology allowed microchip fabrication (for more details see [35]). Planar patch electrophysiology uses a planar arrangement of micron-sized holes in a glass or plastic substrate to replace the glass electrode or pipette used in conventional patch clamping. The resurgence of this technology in the mid–late 1990s also coincided with an increased focus on drug-induced or acquired Long QT syndrome (LQT), and a potential link with the inhibition of the cardiac human Ether-A-Go-Go Related Gene (hERG) potassium channel by a variety of non-cardiac drugs. This prompted regulatory agencies around the world to require preclinical testing of all new drugs to determine their propensity to block hERG channels, and induce LQT [36]. The necessity to evaluate all novel chemical entities for their activity on hERG channels fueled the need to develop automated and HT technologies for ion channel screening.

There is little doubt that combining the information and knowledge acquired from electrophysiology, molecular biology, crystallography, and other techniques has greatly improved our understanding of ion channel structure and function, and heightened the interest in developing novel ion channel modulators. It seems nonetheless remarkable that only a decade after the initial concept, planar patch clamping is already an established technology with numerous commercially available devices producing high quality data in HTS format.

4
Methods for Evaluating Ion Channels

Although it was first described in 1981 [23], the whole-cell configuration of the patch clamp technique remains the definitive or "gold-standard" method for the study of ion channel function and drug effects. It allows precise characterization of the biophysical and pharmacological interactions of drug molecules with channels of interest. It shows high sensitivity, can detect currents in the picoAmpere (pA) range, and allows time resolution in the millisecond range. It also offers some flexibility in the ability to manipulate the solution composition on either side of the cell membrane. Finally, it has a wide applicability, allowing the study of all channel types. However, for all of its glory, the patch clamp method is laborious, it has a low throughput even for an experienced electrophysiologist (10–20 cells per day), and a relatively modest success rate amenable only to low throughput screening. Furthermore, this approach requires the use of precision micromanipulators, high-powered microscopes, and vibration damping equipment, requirements that are not amenable to HTS. Consequently, in an era when compound libraries are readily available and HTS has moved from 96- to 1536-well plates, ion channels have been neglected as drug discovery targets because of the inability to study large number of compounds or validate large numbers of unknown or mutant ion channel genes using this technique. Therefore, several efforts were undertaken to automate and improve the throughput of electrophysiological methods. In this section we will review some of the more traditional ion channel screening technologies including (i) radioligand binding assays, (ii) fluorescent assays using membrane potential dyes, and (iii) ion flux assays, and highlight some of the advantages and shortcomings of these different approaches. We will then discuss automated patch clamp technologies that aim to automate and dramatically increase the throughput of the standard voltage clamp method, using cells that express the ion channel target of interest. Approaches such as fluorescence-based ion sensitive dye methods that measure intracellular ionic concentrations will not be covered in this chapter.

4.1
Radioligand Binding Assays

Developed in the early 1960s, radioligand binding assays have been used extensively to screen for several different targets, including ion channels. Binding assays ideally require the use of a high-affinity ligand, labeled with a radioactive tracer that binds to the site of interest on the ion channel. Binding of the labeled ligand can be displaced by an unlabeled compound if it binds to the same site on the channel as the ligand. The activity of the unlabeled compound can be quantified in a dose-dependent manner by its ability

to compete with the labeled ligand. Binding assays can be performed in 96-, 384- and 1536-well format with a throughput of 50 000–100 000 compounds per day with reasonable reagent costs [37, 38].

While relatively straightforward, binding assays suffer from a number of drawbacks. This method is prone to false negatives because it can only detect compounds that affect the specific site where the ligand is binding and not those that bind at other sites on the channel but can still modify the channel activity. The sensitivity of the assay is also determined by the affinity of the ligand. Consequently, high affinity ligands may prevent the identification of weak binders while low affinity ligands may lead to increased sensitivity of non-specific binding. High specific activity of the ligand is also important to allow reliable measurement of radioactivity at low concentrations, since in most binding assays high affinity sites become saturated in the nanomolar concentration range and only a small fraction of the added radioligand is bound. The advantage is that high specific activity (for example > 2000 Ci/mmol for ^{131}I-labeled compounds) can be used when the size of the sample is limited. The major disadvantages include a relatively short half-life, the possibility of radiation-induced damage to the preparation studied and, of course, possible biological hazard to personnel.

Binding assays are not considered functional assays because they do not reflect the affinity of a compound for different states of the channel (open or closed) and do not identify a compound as an agonist or antagonist of channel activity. Finally, it is not generally possible to screen ion channels with this approach because the binding site linked to the desired modulatory effect on the channel is often unknown, or high-affinity ligands are not available. Consequently, only few voltage-gated ion channel targets are suitable for radioligand binding studies in HTS.

4.2 Fluorescence Assays Using Membrane Potential Dyes

The membrane potential of a cell is determined by the difference in the concentration of ions on either side of the membrane, and the permeability of the membrane to these ions. Activation of ion channels and the ion flux resulting from this change in permeability will lead to a change in membrane potential. This is the principle behind the use of "voltage-sensing" fluorescent dyes that can detect changes in membrane potential, and provide a measure of ion channel activity. These dyes do not measure ionic current directly, but rather measure changes in the membrane potential as a result of ionic fluxes through channels. In general, if the ion channel of interest contributes significantly to setting the resting potential of a cell, than opening or closing of these channels should lead to a measurable change in the resting potential. However, in actuality, the membrane potential is determined by the sum of the activity of all ion channels present in the membrane (Na^+, Ca^{2+}, K^+, Cl^-, etc). Therefore,

measurement of changes in the membrane potential can be a very indirect measure of the activity of the ion channel of interest. Nonetheless, with the use of cell lines stably over-expressing channels of interest or by altering the biophysical properties of the channels studied using mutation that shift membrane potential to more favorable values, the use of fluorescent assays allows large-scale screening of ion channels.

Voltage-sensing dyes measure changes in the membrane potential either by redistribution of a dye, such as the anionic oxonol $DiBAC_4(3)$ that partitions across the cell membrane according to the membrane potential [38], or by fluorescence resonance energy transfer (FRET) between a dye pair [39]. In this latter approach, the FRET donor is a coumarin dye linked to a phospholipid that inserts itself into the outer leaflet of the cell membrane, and the FRET acceptor is a negatively charged oxonol derivative that can distribute across the cell membrane. Under resting conditions, excitation of the coumarin dye using the appropriate wavelength produces FRET and excites the oxonol derivative, which emits a fluorescent signal. When the cell membrane is depolarized, the oxonol derivative moves into the inner layer of the cell membrane, thereby increasing the physical distance between the two dyes and disrupting the FRET. Under these circumstances, the emission from the coumarin is increased, while the emission of the oxonol is reduced. The changes are quantified as changes in the ratio of emission detected from the FRET donor and acceptor [39]. The changes measured are proportional to the changes in membrane potential. The signals are measured using multiwell plate readers, such as the Voltage/Ion Probe Reader (VIPR) from Aurora Biosciences (acquired by Vertex Pharmaceuticals), or the Fluorometric Imaging Plate Reader (FLIPR) from Molecular Devices, which allows a throughput of 35 000–50 000 compounds per day [40].

One of the major drawbacks of this approach is that it suffers from a lack of sensitivity. By the nature of the assay, the membrane potential is not controlled, and changes in membrane potentials can lead to a non-linear readout lacking sensitivity. Furthermore, in cases where the ion channel of interest determines in large part the membrane potential, it may be necessary to inhibit a large majority of channels before a small change in membrane potential may be observed. For this reason, fluorescent assays are approximately one-log value less sensitive during the screening of certain compounds, when compared to patch clamp recording [40–43]. In cases where the pharmacology of the channel is voltage-dependent, results obtained using this approach become more difficult to interpret because the membrane potential is not controlled. Moreover, high numbers of false positive compounds are identified using this approach owing to the indirect nature of the readout, compound autofluorescence, and compound–dye interactions. Finally, the temporal resolution of this assay is significantly slower (subseconds) than that of the ion channels under study (microseconds), because it reflects the redistribution of the dye across the membrane. Therefore, while fluorescence-

based voltage-sensitive assays offer high throughput, they are limited in the quality of the data they provide.

4.3 Ion Flux Assays

Ion flux assays measure the movement of radioactive or non-radioactive tracer elements through the open configuration of specific ion channels. Common radioactive tracers used to permeate through ion channels include $^{86}Rb^{+}$, $^{22}Na^{+}$, and $^{45}Ca^{2+}$. Based on the concentration gradients and the conductivity of the different ion channels, Na^{+} and Ca^{2+} tracers are used to measured ion influx, whereas $^{86}Rb^{+}$ is measured as efflux through potassium channels. Typically, cells expressing the channels of interest are incubated with buffer containing the tracer for several hours before they are washed and incubated with an agonist (to stimulate the channels to open), allowing efflux or influx of the tracer through these channels. The cells and supernatant are then collected for radioactive counting [33]. Because of the required tracer loading and wash steps, this method can only offer a medium throughput. Furthermore, its use is now limited because of the required use of a radioisotope, which is associated with high costs, safety concerns, and environmental issues. For example, the $^{86}Rb^{+}$ routinely used in studies looking at the effects of compounds on hERG channels has a half-life of 18.6 days.

The recent development of a commercially available non-radioactive Rb^{+} assay for potassium channels has greatly enhanced the ease of using this technique [44, 45]. This approach utilizes flame atomic absorption spectrometry (FAAS) for the determination of the Rb^{+} flux. Similarly to the radioactive assay, cells are loaded with Rb^{+} by replacing the external or bath KCl with RbCl. Following a series of wash steps to remove the extracellular Rb^{+}, K^{+} channels are activated using elevated external K^{+} concentrations to depolarize the cells, leading to Rb^{+} efflux measured in the supernatant and cell lysate by FAAS. This approach is easily integrated into an HTS platform. For example, Aurora Biomed has recently developed the Ion Channel Reader, currently available in 96/384- (ICR8000) or 96/384/1536-well plates (ICR12000) with throughputs of 5000–10 000 wells/day depending on the system used [46]. Granted, one of the major advantage of this approach is the fact that is does not require the use of radioisotopes. However, flux assays in general do not allow control of the membrane voltage of the cells, leading to inadequate control of the gating of the ions channels studied and altered channel pharmacology when compared to conventional patch clamp data [47, 48]. Also, many channels produce a signal that is too small, or to transient, to be accurately measured using this approach. Another important issue is the fact that a tracer is required for each ion channel type studied and these might not be available or detectable with spectroscopy at relevant concentrations. Finally, because of the

limitations of this approach, hits identified in flux assays require post-testing validation using more standard electrophysiological studies.

5
Automated Electrophysiology Technologies

Initial efforts to develop automated patch clamp approaches were deployed by NeuroSearch (now Sophion Biosciences) by using robotics to mimic the steps used by a human operator to obtain patch clamp data [49]. Their system, called the Apatchi-1, was a pipette-based system that reproduced the procedures a patch clamper would perform during an experiment. Cells seeded on cover slips were identified and selected for patch clamping by an imaging routine based on user-defined selection criteria such as size and geometry. Up to 18 patch pipettes could be loaded into the system and used without the involvement of the operator, and GΩ seals and whole-cell formation were obtained using operator-defined pressure protocols. The system also incorporated fluid handling. Although quite ingenious, this approach provided neither an increase in throughput (20–50 data points per day) nor data quality over conventional patch clamping, and was plagued with technical issues. Another pipette-based automated patch system, the AutoPatch, was developed at NeuroSearch. Unlike the Apatchi-1, it required no microscope or vibration isolation but rather used a technology called interface patch clamping, where cells were suspended in a liquid medium and patch clamping was accomplished by patching onto the cells held at a liquid–air interface. Nevertheless, the AutoPatch was never commercialized.

Over recent years, most of the limitations concerning automated electrophysiology have been addressed with the introduction of planar array electrophysiology, a technology that offers most of the benefits of the conventional patch clamp approach, but with a HTS format. Planar array electrophysiology replaces the conventional single glass micropipette with a planar substrate embedded with an array of microapertures that allows currents to be recorded from individual cells, under voltage clamp conditions. This technology is rapidly replacing the traditional patch clamp electrode, allowing hundreds to thousands of compounds to be screened daily. In this section we will review these emerging technologies for ion channel screening that are based on voltage clamp methods. A summary of some of the advantages and limitations are presented in Table 3.

5.1
Automated Electrophysiology-Planar Patch Technology

Recent development of the planar patch technique has allowed the development of high throughput electrophysiology. The planar patch clamp process

Table 3 Comparison of automated patch clamp systems

Company/website	Product	Assay format	Estimated throughput data points/day	Pro	Contra
Molecular Devices Corp Now part of MDS Analytical Technologies Sunnyvale, CA USA http://www.moleculardevices.com/home.html	PatchXpress 7000A; Planar patch system	16-well glass sealchip	2000	Voltage control; GΩ seal; high temporal resolution; variable success rate but occasionally high depending on cell type studied	Low throughput overall, low success rate
Molecular Devices Corp Now part of MDS Analytical Technologies Sunnyvale, CA USA http://www.moleculardevices.com/home.html	IonWorksHT Planar patch system	384-well plastic PatchPlate	3000	Voltage control; variable success rate but occasionally high depending on cell type studied	Low seal resistance; Low throughput overall, low success rate
	IonWorks Quattro		12 000	High success rate; recordings from up to 64 cells per well	Low seal resistance
Sophion Biosciences Ballerup, Denmark mailto:info@sophion.com	Qpatch16 Planar patch system	16-well glass QPlate; perfusion incorporate in QPlate	250–1200	Integrated cell preparation facility; integrated fluidics in recording plate	
	QPatchHT	48-well glass QPlate	1500–7000		

Table 3 (continued)

Company/website	Product	Assay format	Estimated throughput data points/day	Pro	Contra
Nanion Technologies GmbH Munich, Germany http://www.nanion.de/	Port-a-Patch Planar patch NPC16	Single-well borosilicate glass chip 16-well	20–50 250	Fluid exchange intracellular and extra-cellular side	Low throughput
Flyion GmbH Tübingen, Germany http://www.flyion.com/	FlyScreen 8500	Glass micropipette; embedded in plastic jacket (Flip Tips)	100–500	Voltage control; GΩ seal; Flip-the-Tip seal formation similar to traditional patch clamping	Low throughput

reverses the traditional sequence of patch clamping and moves the cell to the patch pipette, consisting of a fine pore opening (1–2 microns) in a flat surface made of glass, silicon, quartz, or plastic that constitutes the bottom of the multiwell array (Fig. 2, part B). Suction is used to attract and position the cells over the hole to form a high-resistance seal. The membrane within the hole is then disrupted either mechanically (negative pressure) or chemically using a perforating agent such as an ionophore (amphotericin or a detergent) to obtain the whole-cell configuration. It is the ability to fabricate these planar patch plates, combined with the use of multiple independent amplifiers and the development of microfluidic systems that has allowed the emergence of automated patch clamp systems. The planar geometry offers a variety of advantages compared to the classic experiment. For example, the use of microfluidics enables automatic compound application for ion channel screening, and allows perfusion of the intracellular side. Moreover, non-adherent cells, such as blood cells, can be studied using this system.

On the other hand, these systems share similar drawbacks. For example, the suspension of cells expressing the ion channel of interest needs to be homogenous, because cells cannot be selected on size or any other selection criteria, unlike the conventional patch clamp technique. Also, despite extensive miniaturization and greatly increased throughput, the cost of purchase and cost per measurement using the planar approach remains significantly higher than with the conventional whole-cell voltage clamp (see below).

5.2 IonWorks

IonWorks was the first available automated patch clamp system on the market that utilized planar electrode technology [50]. It was developed by Essen Instruments, and commercialized by Molecular Devices (Sunnyvale, CA). It was introduced on the market in 2002. It uses a planar 384-well disposable patch plate with a single laser-drilled hole in the center of each well, fitted above a common reservoir serving as signal ground for the entire plate. Electrodes connected to a 48-channel voltage clamp amplifier allow sequential clamping of eight different regions of a 384-well patch plate (8×48) at a time. All of this is accomplished without the need for micromanipulators, microscopes, vibration isolation, and perfusion apparatus. Cells and solutions are added to each individual well from above. Negative pressure is used to pull the cells to the hole to form a high-resistance seal. Whole-cell recording is obtained using a membrane perforating agent, such as amphotericin, simplifying the need for complex fluidics and pressure control for each well, as is the case when negative pressure is used to break open the membrane. This method, also described as “perforated patch”, limits the diffusion of intracellular subunits

required for channel function to leak out of the cells. Using the IonWorks system allows approximately 2000–3000 cells to be patch-clamped in a day with a 60–90% success rate [38].

When compared to the conventional patch clamp approach, the seal resistances that are obtained with the IonWorks are low (50–600 mΩ, compared to 1–10 GΩ), and compensation for capacitance artifacts and errors that arise from voltage drop across the electrodes cannot be compensated. This can lead to variability in the data obtained, and errors in the evaluation of drug effects. For example, significant shifts in the potency of validation compounds (compounds being less potent in IonWorks) have been reported using this system, when compared to conventional patch clamp data [47]. This shift may be attributed, at least to some extent, to the hydrophobic nature of some compounds potentially causing them to stick to the substrate that the patch plates are made of. Also, addition of compounds to the wells requires the transient removal of the amplifier headstage from the plates, a period during which the cells are not clamped. The potential consequences of this discontinuous voltage control have not been studied carefully but have the propensity to introduce significant variability in the data. Nonetheless, the ability to increase the throughput 100-fold over the traditional patch clamp approach makes this instrument a valuable screening tool, not only for pharmacological studies, but also for other usages such as the selection of clonal cell lines [51].

5.3
The IonWorks Quattro – Population Patch Clamping

The second generation enhancement of the IonWorks, the IonWorks Quattro, incorporates a novel approach designated the Population Patch Clamp (PPC), whereby multiple (64) apertures are machined in each well, enabling the recording of up to 64 cells in parallel in each well, instead of a single cell, as done with the first generation model [52]. The main advantage of the PPC approach is that it averages whole-cell currents, thereby greatly reducing biological variations such as cell health, channel expression levels, and seal formation, and allowing better consistency of the measurements from each well [53]. The fact that successful recordings can be obtained from up to 384 wells (success rate > 95%) dramatically increases the throughput to a level that has never been attained previously with conventional electrode technology, or with the planar patch clamp approach. Moreover, a fourfold increase in throughput is also claimed over the first generation system.

The Quattro system allows recording of signals in real time, corresponding to channel function. However, the higher throughput limits the complexity of the protocols that can be used, as well as constrains the number of compound additions to a two-step discontinuous clamp process, as with the first generation system.

5.4 PatchXpress 7000A

The PatchXpress 7000A is another commercially available automated planar patch clamp system from Molecular Devices. It was introduced on the market in 2003 by Axon Instruments, now part of Molecular Devices. The PatchXpress allows patching up to 16 cells simultaneously on a planar array disposable glass chip. Unlike the IonWorks systems, whole-cell voltage clamp on the PatchXpress is achieved by negative pressure that is individually adjusted to each well to obtain the highest possible seal (GΩ seals), providing long-lasting whole-cell recordings. The system allows continuous voltage control, measurement, and compensation for series resistance and high temporal resolution, leading to high quality recordings similar to those obtained with the conventional voltage clamp technique. Continuous current measurement also allows detailed studies of the functionality and pharmacology of ion channels, including the study of ligand-gated channels. The system incorporates relatively fast fluidics, allowing for the addition of multiple drug concentrations to each cell while continuous voltage clamp control is applied. As a result, concentration response curves can be generated from a single cell to produce IC_{50} values for each compound studied. A good correlation between data obtained on this system and that obtained with the conventional patch clamp has been reported in several studies [54, 55]. A throughput of approximately 2000 data points per day is reported [56]. This represents a two- to tenfold increase over conventional patch clamp studies. The PatchXpress system offers numerous advantages for testing compounds when quality control parameters need to be monitored and optimized during continuous recording of data.

Nonetheless, the use of this system is greatly impacted by its relatively low success rate (20–70%), the high costs of its consumables, and its relatively low throughput when compared to the IonWorks systems.

5.5 QPatch

A planar patch system developed by Sophion Bioscience, the QPatch16, entered the market in 2004. Its design offers improved and some unique features over the IonWorks and PatchXpress systems. The QPatch16 automatically clamps 16 cells in parallel with the same high quality as conventional patch clamping using glass electrodes, at least for some specific cell lines and protocols. The 16-channel electrode array, called the QPlate, includes silicon-based glass-coated electrodes and microfluidic channels, potentially preventing compound absorptions to the sides of the plate, and providing more reliable and accurate IC_{50} value determinations. The system has four independent pipetting heads for more efficient liquid handling, and includes series resistance compensation for more accurate voltage control and high quality

recordings. One of the unique features of the QPatch system is that it includes an automated cell preparation station and storage unit, allowing several hours of unattended operation. The system has the ability to maintain and eventually dispense the cells in the 16 chambers of the QPlate, at the optimal cell concentration. Cells are pulled to the hole using suction, and negative pressure is used to rupture the membrane for whole-cell access. Integrated fluidics permits multiple rapid fluid additions and exchanges, allowing the study of both voltage and ligand-gated channels. Sophion claims that the QPatch16 has a throughput of approximately 250–12 000 data points per day, with a success rate of approximately 40–95%, depending on the cell line tested, and a good correlation with conventional patch clamp data [57, 58]. Sophion Bioscience recently introduced the QPatch HT, a 48-channel gigaseal patch clamp system that can be applied to both voltage-gated and ligand-gated ion channels. The system includes 48 individual low-noise patch clamp amplifiers and pressure controllers. Independent pressure control (i.e., suction on each cell individually) allows for GΩ seal formation. The QPatch HT has eight pipettes attached to the fluid handling robot to keep pace with the liquid handling demand of up to 48 simultaneous patch clamp experiments. The HT has a reported potential daily throughput of approximately 750–3500 data points [59].

5.6
Port-A-Patch

The Port-a-Patch is a planar-based automated electrophysiology workstation by Nanion Technologies (Munich, Germany) that allows patch clamping of a single cell at a time. It is a bench-top instrument, described by Nanion as the world's smallest patch clamp setup, that can produce whole-cell and single channel recordings with rapid fluid exchange on both the intracellular and extracellular side, with temperature control. It is a self-contained unit with integrated amplification, suction control unit, and amplifier with corresponding electrophysiology software. As a stand-alone unit, it has a throughput of approximately 20–50 data points per day. A more elaborate 16-well instrument, the Patchliner, is also now available. This system is a fully automated patch clamp station that can record from two, four, or eight chambers at a time. The glass chips used in this system also incorporate microfluidic channels for precise application of compounds to the cell's external or internal side. While the data is of high quality, the throughput of the Patchliner is low with approximately 250 data points obtainable per day [60].

5.7
Non-planar System

Among other designs available for automated patch clamping is the Fly-Screen 8500 patch clamp robot, by Flyion (Tübingen, Germany). This is a fully

automated whole-cell patch clamp system. It uses conventional glass pipettes, but defies conventional patch clamping by injecting a cell internally into the pipette tip, described as Flip-the-Tip technology, establishing a high-resistance GΩ seal (Fig. 2, part C). A true whole-cell configuration is achieved by applying suction pulses to the cell sealed inside the tip of the electrode. Suction towards the tip disrupts the membrane area facing the tip, allowing whole-cell recording. Compounds can be perfused into the pipette to mimic extracellular perfusion, without displacement of the cell. High quality recordings can be obtained with this system, comparable to those obtained with the standard voltage clamp. On the other hand, it is designed to record from a maximum of up to six independent electrodes, and therefore has a limited throughput reported at approximately 100–500 data points per day [61].

6 Future Perspectives and Challenges

In combination with methods such as fluorescence and flux assays, the new automated patch clamp electrophysiology systems have revolutionized the way in which ion channels are now screened. However, the future is not without major challenges, and important limitations of these technologies remain.

For example, none of the non-patch methods such as ligand binding, flux, or fluorescence assays allow appropriate voltage control of the ion channel studied, thereby precluding the identification of state-dependent blockers. This state dependency can be important and often conveys functional selectivity to some compounds if the conformation to which the drug binds is observed more often in the disease state. For example, this is the case with several clinically used Na^+ channel blockers [62]. This may result in significant false negative rates. Many channels also generate a signal that is either to small or too transient to be recorded using these approaches. Finally, non-patch methods as well as automated patch methods require the generation of stable cell lines with robust expression of the ion channel of interest. Given the channel type, many different lines with various combinations of α and auxiliary subunits may have to be examined, and their activity validated against the biologically relevant ion channel of interest. Finally, the increased throughput of the non-patch approaches may lead to a downstream bottleneck in the validation of hits and lead optimization if automated electrophysiology systems are not available.

Likewise, important issues remain with the automated patch clamp technology. There is no doubt that this approach has filled the niche between HT low information content assays, such as fluorescence and binding assays, and low throughput high quality content, such as conventional patch clamp electrophysiology assays. However, this technology comes at a tremendous price. At an approximate cost of US$ 400 000 each, these systems are expensive

to purchase, operate, and maintain, and are usually not within the financial realm of academic institutions. Presently, fluorescent imaging assays, run in HT mode, cost only about 20 ¢ per data point [57], in sharp contrast to the current cost of US$ 1.60–3.80 per data point [63] for the automated voltage clamp assays. These costs are mostly related to the purchase of the planar patch electrode arrays, which, unlike standard 96- or 384-well plates, require special manufacturing processes. This makes the use of this technology in HT mode prohibitive. Nonetheless, it is expected that the technologies for fabrication of the electrode arrays should continue to improve over the coming years, potentially driving down the cost of consumables.

Another important challenge that remains to be addressed is the relatively low success rate in obtaining useable and consistent high quality patch clamp data from most of these instruments. The success rate currently stands at around 40–60% for most systems [64], excluding the IonWorks Quattro that incorporates population patch clamping and claims a success rate of > 95% [53]. Some of the factors that contribute to this lack of success include stable positioning of the cells over the pore of the planar electrode, lack of adequate suction control in some cases, and the inability to achieve high resistance seals or electrical access.

Finally, the issue of throughput remains to be considered. The daily throughput of the automated electrophysiology systems are not currently considered HTS, which begins at 10 000 data points per day [63]. However, increased throughput will result in restrictions on flexibility of the voltage protocols used, as well as the ability to use multiple compound additions to generate dose–response relationships. Nonetheless, even if these issues are addressed, the pricing per data point still remains a major issue.

Automated patch clamp systems have only become available for the past few years, and their impact on ion channel drug discovery is, therefore, just beginning. Whether we are at the verge of a new golden age for ion channel drug discovery remains to be determined, but what unfolds over the coming years should be of utmost interest for ion channel drug discovery.

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Role of Kv7 and Cav3 Ion Channels in Pain

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Abstract Pain sensation is encoded in the firing patterns of a variety of neurons throughout the body, and much of this signaling is dependent on the complement of expressed ion channel proteins. Ion channels, therefore, present excellent molecular targets for designing new therapeutic molecules to manage pain. In this review we discuss two classes of channels which are currently of interest to the pharmaceutical community in this area. The first are Kv7 potassium channels which encode the M-current. Opening of these channels tends to dampen neuronal excitability and pain signaling; therefore, agonists are of potential interest to treat pain. The second class is Cav3 calcium channels which encode T-type calcium currents. The opening of these channels typically promotes neuronal excitability and inhibitors could also be useful to treat various types of pain. The following review discusses the properties of theses two channel types along with recent advances in the identification of small molecule modulators.

Keywords Cav3 · KCNQ · Kv7 · Neuropathic pain · T-type calcium

1 Introduction

Pain is typically divided into two categories, nociceptive and neuropathic. Nociceptive pain occurs following tissue injury or inflammation and is part of the normal pain signaling systems which help individuals avoid injury and/or prevent further injury. As the injury or inflammation resolves, the pain dissipates, although there is a need to manage pain in cases of chronic inflammatory diseases. In general, this type of pain is treated adequately by existing therapeutic agents; however there are instances where nerve fibers which do not normally encode pain become sensitized leading to chronic conditions that are more difficult to manage clinically. Neuropathic pain differs in that it is associated with direct injury to nerves by a wide variety of insults and can persist even after the initial cause has resolved. Neuropathic pain involves aberrant processing of what would otherwise be normal tactile or thermal signals of pain and is much more difficult to manage using existing therapies. It is estimated that neuropathic pain may affect as many as 26 million people worldwide (Butera 2007).

Mechanistically, pain signaling is encoded by a system of nerves, including peripheral nociceptors, second-order spinal neurons, and central neurons synapsing within pain centers in the brain. A variety of ion channels regulate the electrical activity of these neurons and, consequently, modulate both the normal and pathophysiological processing of pain signals. In this review we focus on two classes of ion channels, the Kv7 and Cav3 families, which recent data suggest are key regulators of neuronal pain signaling in a variety of systems. These examples also serve to illustrate how activation of one ion channel type can decrease neuronal excitability and pain signaling, whereas the second gene family signals in the opposite direction such that activation increases the neuronal excitability of nociceptive neurons. Both channel types have recently been characterized using molecular and pharmacological tools, and the data indicate that each gene family includes interesting molecular targets for new pain therapies.

2 Kv7.x Channels

Kv7.x channels encompass a gene family consisting of 5 distinct members denoted Kv7.1–Kv7.5. Kv7.1, also known as KvLQT1, is expressed primarily in non-neuronal tissues, including cardiac tissue where it contributes to repolarization of the cardiac action potential. Kv7.1 loss-of-function mutations are associated with long QT syndrome that can lead to a rare ventricular arrhythmia, torsades de pointes (Wang et al. 1996). The other members of the Kv7 family encode related potassium channels that are widely expressed

in neurons, including hippocampal, cortical and dorsal root ganglion neurons. Each gene encodes a six transmembrane subunit of a voltage-gated potassium channel and the individual subunits contain a charged S4 region and single P-loop. These subunits self-assemble as tetramers to form a voltage-dependent, K+-selective ion channel. The assembled tetramers include homomeric and heteromeric combinations that contribute to Kv7 channel diversity. As noted above, Kv7.1 plays a prominent role in cardiac-electrophysiology, but is not directly relevant to the topic of discussion in this chapter which is the role these channels play in pain signaling and processing. Similarly Kv7.4 is largely restricted to cells and tissues associated with auditory processing (see below) and to date has not been directly linked to neurons involved in pain signaling. The other members of the family, Kv7.2, 7.3 and 7.5; however, are all important signaling proteins and drug targets for potential new therapies to treat pain and disorders of neuronal excitability (Jentsch 2000).

Interest and excitement in the role of Kv7.x channels in neuronal excitability was triggered by molecular studies demonstrating that Kv7.2 and Kv7.3 hetero-multimers encode the neuronal M-current (Wang et al. 1998). The M-current, which was first identified by Brown and Adams (1980) stabilizes the resting membrane potential and contributes to the integration of synaptic inputs to determine the level of neuronal excitability (Marrion 1997). Activation of G-protein coupled receptors, such as the muscarinic receptor, inhibits M-currents, but the specific mechanism by which this occurs has been difficult to assess. A breakthrough was made when pharmacological modulators of phosphoinositide turnover were demonstrated to affect G protein receptor-coupled inhibition of Kv7.x channels (Suh and Hille 2002). In follow-up studies, using a clever system of specifically designed translocatable enzymes, Suh et al. (2006) demonstrated Kv7.2/Kv7.3 channel activity to be dependent upon the availability of membrane-associated PI(4,5)P. Depletion of membrane-associated PI(4,5)P is sufficient to suppress Kv7 currents in the absence of changes in other candidate signaling molecules. This decrease in functional Kv7 density stimulates neuronal excitability in those cells where M-currents play a prominent role in setting resting membrane potential. The discovery that Kv7.2, 7.3 and, subsequently, Kv7.5 genes encode the M-current allowed studies to be initiated regarding expression and genetics of the Kv7 gene family which further elucidate the role of these channels in neuronal excitability and pain signaling (see Wickenden et al. 2004).

2.1 Kv7.x Genetics

The majority of Kv7.x genetic research has been directed toward CNS disorders, such as benign familial neonatal convulsions (BFNC), a rare epilepsy. BFNC is characterized by unprovoked partial or generalized clonic convul-

sions beginning approximately 3 days after birth and remitting 3–10 weeks later. The association between BFNC and the M-current led to the cloning of Kv7.2 and Kv7.3 from patients with this disorder, and thus the identification of the underlying deficit in membrane currents (Wang et al. 1998). To date this seizure disorder has been linked to over 35 missense, frameshift and splice-site mutations in Kv7.2 or Kv7.3 that compromise but do not eliminate channel function. Consequently, even though these mutations are not dominant-negative, the decrease in overall current density is still sufficient to cause increased excitability leading to BFNC and an increased risk of seizures later in life. A reduction in M-current density as low as 25% has been associated with neonatal epilepsy (Schroeder et al. 1998), emphasizing the importance of Kv7.x channels in modulating the excitability of CNS neurons.

The genetic association of Kv7 channel mutations and neuronal hyperexcitability suggests that Kv7.x channels may also modulate the excitability of neurons involved in pain processing since many currently used anti-epileptic drugs which affect excitability are also used to treat pain. Kv7 channels are known to be expressed at multiple levels of pain pathways, including thalamic, spinal superficial dorsal horn and dorsal root ganglion neurons. Thus, small molecule openers of Kv7 channels may not only diminish the neuronal hyper-excitability associated with epilepsy, but may also be effective in treating pain conditions.

In addition to the CNS neuronal excitability deficits linked to BFNC, there are a few reports of Kv7 disorders linked to peripheral nerve hyperexcitability. Mutations in a specific residue of Kv7.2, manifests as a form of myokemia, an irregular twitching of the muscle (Dedek et al. 2001), and peripheral nerve hyper-excitability (PNH). Recently, a second mutation at the same position in Kv7.2 has been reported which also shows a similar peripheral nerve phenotype (Wuttke et al. 2007). Both mutations target arginine 207, which is thought to form part of the voltage-sensor in these channels. A positively charged arginine residue is mutated to either tryptophan (R207W) or glutamine (R207Q), resulting in depolarizing shifts in the activation curves of Kv7.2/7.3 channels and a slowing of activation rates. Dedek et al. (2001) postulate that the slowing of activation will result in a dominant-negative effect which is rate-dependent such that neurons firing at slower rates are more dramatically affected while greater firing rates tend to normalize the difference between mutant and wild-type channels. The shift in gating is greater for the R207W mutation than for R207Q (Wuttke et al. 2007), which could explain why the patient with the R207W mutation presented with BFNC and PNH, while the patient with the R207Q mutation presented with PNH alone and no history of neonatal seizures. The genetic results suggest that modulation of Kv7 channels would be expected to affect neuronal firing in a frequency-dependent manner although this speculation remains to be rigorously validated.

2.2 Distribution of Kv7 Channels

With respect to the potential involvement of Kv7 channels in pain signaling, Kv7.2, 7.3 and 7.5 genes are expressed in the appropriate regions of the brain and peripheral nervous system to play a role. Each of these three gene products has been reported in many of the same cell types, including thalamic, spinal superficial dorsal horn and dorsal root ganglia neurons (Yang et al. 1998; Cooper et al. 2000; Rundfelt and Netzer 2000). As noted previously, Kv7.4 has a more restricted distribution in neurons related to auditory signal processing (Kubisch et al. 1999; Kharkovets et al. 2000) and therefore is unlikely to be involved in pain processing. Consistent with this notion, Kv7.4 loss-of-function mutations lead to progressive hearing loss in man, but have not been reported to be associated with epilepsy or disorders of the peripheral nervous system.

Passmore et al. (2003) first demonstrated the expression of Kv7.2, 7.3 and 7.5 channels in dorsal root ganglia neurons at both the protein and functional level. Interestingly, these authors report heterogeneous expression of the Kv7.2, 7.3 and 7.5 in DRG neurons with some cells expressing all three subunits, while other cells appear to express only Kv7.2 and Kv7.3. The functional significance, if any, of this selective expression pattern remains to be determined. Given that hetero-multimeric channels have different properties from homo-multimeric channels (e.g., greater current density), the heterogeneous expression could have implications for the involvement of these channels in peripheral nociceptive signaling as well as for the efficacy of Kv7 openers with varying selectivity profiles across different pain conditions.

2.3 Biophysical Properties of Kv7 Channels

A typical M-current from a nociceptive DRG neuron is shown in Fig. 1 illustrating the tonic activation of current at the holding potential and the slow kinetics of deactivation following a hyperpolarizing voltage step. The current is blocked by a selective Kv7 channel blocker XE-991 (see below). Kv7.2–Kv7.5 channels exhibit similar biophysical properties in that they all are non-inactivating and activate and deactivate with relatively slow kinetics. Kv7.2–7.5 channels are K^+-selective with relatively low single channel conductances of 2–9 pS in symmetrical K^+. The maximum open probability at depolarized potentials has been reported to vary for the different homomeric and heteromeric channel combinations ranging from 0.07 to 0.89. This variation in maximum open probability across channel sub-types may be influenced by variations in second messenger modulation. Voltage-dependent activation of Kv7.2–7.5 channels occurs at a threshold of –60 to –50 mV (<5% of maximal current) with a midpoint of –25 to –15 mV. Open probability is maximal at membrane potentials more depolarized than 0 mV.

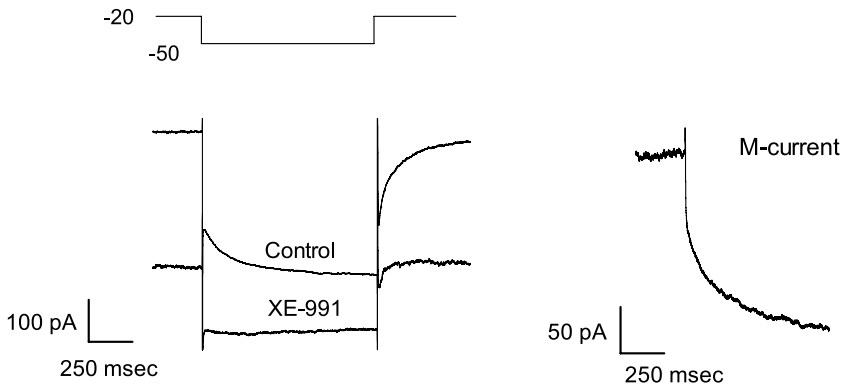

Fig. 1 Illustration of a representative M-current in nociceptive dorsal root ganglion neurons. Currents were generated during voltage steps from –20 mV to –50 mV as shown in the *inset*. XE-911 was added to block the Kv7 (M) current and the difference record is shown on the *right*

The non-inactivating nature of Kv7 channels is fundamental to their involvement in the stabilization of the neuronal resting potential. Because neuronal resting membrane potentials are typically at the threshold for Kv7 activation, only a fraction of the total available Kv7 conductance is required to maintain Vm. Because of the large resting reserve of Kv7 conductance, Kv7 openers are attractive agents for the treatment of neuronal hyper-excitability disorders such as epilepsy and neuropathic pain. Conceptually, one would expect Kv7 openers to hyperpolarize the neuronal resting Vm with an associated decrease in membrane resistance. The likely effect on neuronal firing would be to increase the stimulus intensity required to reach action potential threshold resulting in reduced excitability.

Upon neuronal depolarization, Kv7 channel activity increases with relatively slow kinetics. This biophysical property allows Kv7 channels to shape neuronal bursting patterns. For example, in hippocampal neurons, activation of Kv7 causes a time-dependent increase in inter-spike interval within a train of action potentials ultimately resulting in burst termination. When Kv7 channels are pharmacologically inhibited, the action potentials fail to adapt resulting in a longer and higher frequency burst. In contrast, Kv7 openers facilitate spike frequency adaptation resulting in less action potentials within a burst. Thus, in addition to an effect on the resting membrane potential, Kv7 channels are uniquely suited to modulate neuronal firing patterns.

2.4 Pharmacological Agents to Modulate Kv7 Channels

Figure 2 illustrates the structures of several agents which are known to block or activate Kv7.2–7.5 channels. These modulators have been useful experi-

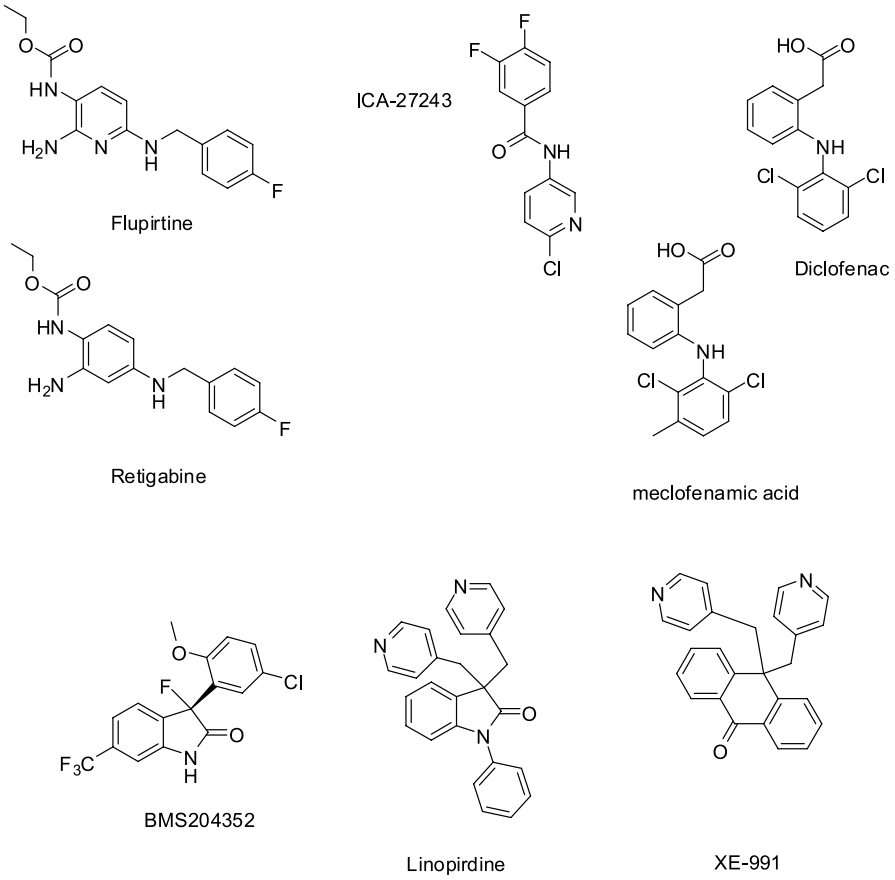

Fig. 2 Structures of selected Kv7.x channel modulators

mental tools in dissecting the role of these channels in different systems. Data generated in pre-clinical models and in man using each of these agents are discussed in subsequent sections.

Flupirtine was the first compound identified to affect KCNQ channels and has been used in man to treat pain. However, only recently has this drug been shown to be an activator of Kv7 channels (see Munro and Dalby-Brown 2007). The clinical efficacy of flupirtine was originally postulated to occur through receptor-dependent mechanisms, but as noted by Munro and Dalby-Brown (2007) it is likely that Kv7 channel activation occurs at clinically relevant exposure levels. Retigabine, a flupirtine analog, is currently in clinical trials for the treatment of epilepsy and pain and has been the most widely used tool to explore both the in vitro and in vivo effects of Kv7 activation. This compound was initially shown to increase K^+ channel activity in a neuroblastoma cell line (Rundfeldt 1997; see also Rundfeldt 1999) and subsequently was

demonstrated to have direct effects on Kv7 channels (Rundfeldt and Netzer 2000; Main et al. 2000; Wickenden et al. 2000). Recently, Wuttke et al. (2005) and Schenzer et al. (2005) demonstrated that retigabine interacts with a putative gating-hinge region in the channel and that mutation of a single amino acid, W236, completely eliminates the agonist activity of retigabine on Kv7.2 channels. Similarly, retigabine was ineffective versus the related Kv7.3 mutant (Schenzer 2005). ICA-27243 is a structurally distinct Kv7 opener which has been reported to activate recombinant Kv7.x channels, DRG M-currents and to alleviate pain in pre-clinical models (Roeloffs et al. 2005; Gerlach et al. 2006). ICA-27243 shows greater activity against Kv7.2/7.3 channels compared to other heteromeric or homomeric forms and is the only subtype selective opener reported to date (Wickenden et al. 2008). Other reported activators are meclofenamic acid, diclofenac (Peretz et al. 2005) and BMS-204352 (Schroder et al. 2003; Dupuis et al. 2002), but these latter compounds have not been widely used as tools to assess Kv7 activity because of activity on other molecular targets (i.e., COX enzymes for meclofenamic acid/diclofenac and KCa1.1 potassium channels for BMS-204352). Two other widely used tool compounds are linopirdine (Aiken et al. 1995; Lamas et al. 1997; Schnee and Brown 1998) and its more potent analog XE-991, which are both blockers of Kv7.x channels. These compounds have been instrumental in delineating the role of Kv7.x channels in native systems and to confirm the selectivity of Kv7 openers. Several recent reviews provide examples of additional compounds noted in the patent literature (Wu and Dworetzky 2005; Munro and Dalby-Brown 2007).

2.5 Effects of Kv7 Channels on Electrical Activity of Sensory Neurons

While it is clear from a number of studies that Kv7 currents regulate membrane potential in central neurons, the data supporting a similar role in primary sensory afferents are not as well established. Several studies, however, do support a similar role for Kv7 channels in sensory neurons.

As noted above, Passmore et al. (2003) were the first to demonstrate that Kv7.2, 7.3 and 7.5 are expressed at the protein level in sensory neurons and also that functional M-currents can be measured under voltage-clamp. They were further able to demonstrate that pharmacological activation of Kv7 channels by direct application of retigabine to the spinal cord inhibited both C fiber and Aδ fiber-mediated signaling. This was true when the afferent fibers were excited by either electrical or tactile stimuli. Moreover, "wind-up", an increased sensitivity of the stimulus/response relationship to repetitive stimuli, is also inhibited. These results demonstrate a clear role of Kv7 channels in sensory neuron processing. Gerlach et al. (2006, 2007) have also reported direct effects of ICA-27243 on both M-currents and membrane potential in isolated DRG neurons. Activation of the M-current by ICA-27243

resulted in a concentration-dependent increase in the stimulus threshold for action potential firing consistent with a decrease in input resistance associated with the opening of Kv7.x channels.

Carlsson and Jurna (1987) used flupirtine before it was known that this compound activated Kv7 channels and demonstrated that i.v. administration inhibited neuronal activity following nerve stimulation. Responses were measured from both C and Aδ fibers with reductions in spontaneous activity in both populations and also a reduction of evoked C-fiber activity. The authors go on to demonstrate that the activity of flupirtine in these experiments is likely due to a spinal/supraspinal mechanism which does not involve opiate receptors. Considering these results in context of more recent data on the mechanism-of-action for flupirtine, this study is one of the first reports suggesting pharmacological modulation of Kv7 channels can directly affect C-fiber activity. Rivera–Arconada et al. (2004) further demonstrated effects on nociceptive neuronal reflex activity using retigabine with little effect on non-nociceptive neurons. The activity on nociceptive reflex loops was long lasting and reversed by the Kv7 blocker XE-991. The same authors (Rivera–Arconada and Lopez–Garcia 2006) went on to show that retigabine hyperpolarizes the membrane potential of primary afferents and increases the stimulation threshold. The authors conclude from their studies that Kv7 channels are involved in both dorsal and ventral horn elements responsible for sensory and motor nociceptive responses and therefore, M-current modulators could potentially be useful analgesics. Finally, Wladyka and Kunze (2006) demonstrated expression of Kv7 channels in visceral sensory neurons from nodose ganglion and show depolarization upon block of Kv7 channels with XE-991 and hyperpolarization following administration of retigabine. These results are consistent with other reports demonstrating functional expression of Kv7 currents in sensory neurons. Interestingly, they do differ in that block of Kv7 channels often has little effect on resting membrane potential in many DRG neurons suggesting little constitutive activation. It is possible that the basal activity in visceral neurons differs and that Kv7 channels show greater activation at the resting potential. Additional experiments will be necessary to determine whether such quantitative differences exist across peripheral nerve populations. In general, however, one can conclude from these studies that there is significant functional expression of Kv7 channels in the peripheral nervous system and that modulation of these channels affects neuronal firing patterns involved in pain signaling.

2.6
Evidence for a Role of Kv7 Channels in Pain: Pre-Clinical in Vivo Studies

The aggregate results from genetic data, expression profiles, and biophysical studies suggest Kv7 channel activation to be beneficial in reducing or elimi-

nating pain signaling. One of the best means for testing this hypothesis is to identify selective small molecule activators which can be dosed in pre-clinical pain models to determine efficacy. Despite significant interest in this area, there have been relatively few studies reported in peer-reviewed journals testing the effects of Kv7 openers in such animal models and the majority of these studies have used retigabine (see below). Despite the small number of reports, the results clearly support the role for Kv7 channels in pain signaling and by extension a potential role for Kv7 openers as pain therapeutics.

Results with retigabine have been reported from pre-clinical models of neuropathic pain, including the spinal nerve ligation (SNL or Chung), chronic constriction injury (CCI or Bennett) and the spared nerve injury models (SNI or Seltzer). In the SNL model, retigabine was effective in reversing tactile allodynia with a minimum effective dose (MED) of 10 mg/kg po and was also effective on tactile hyperalgesia in the SNL model (Dost et al. 2004). This effect was completely reversed by the Kv7 blocker linopirdine consistent with an on-target effect at Kv7 channels. Roeloffs et al. (2005) reported similar efficacy on tactile allodynia in the SNL model using a chemically distinct compound, ICA-27243. Results from these two studies suggest that Kv7 activators should be effective in reducing allodynia associated with neuropathic pain.

In the other surgically-induced neuropathic pain models, however, the results are less clear. Blackburn–Munro and Jensen (2003) found no effect on tactile allodynia (assessed with von Frey filaments) in the CCI model, but did observe an effect on tactile hyperalgesia induced by pin prick, following administration of retigabine. Cold allodynia was also reduced. The same group reported similar effects in the spared nerve injury (SNI) model, where retigabine was not effective on tactile allodynia, but did show efficacy against tactile hyperalgesia. In the SNI model, however, there was no effect on cold allodynia.

Overall, the results in neuropathic models suggest that Kv7 activation will reduce pain although the specific modality affected is somewhat model-dependent. These results are consistent with the effects on neuronal properties noted by Passmore et al. (2003) in the SNL model.

A variety of other pre-clinical pain models have also been used to examine the effects of activating Kv7 channels. Both retigabine (20 mg/kg po) and ICA-27243 (30 mg/kg po) are efficacious in the formalin model, particularly in reducing phase II responses, which are thought to result from central sensitization and occur with a delay following formalin administration. Passmore et al. (2003) reported efficacy of retigabine in the carrageenan model of inflammatory pain using weight bearing as an endpoint at a dose of 5 mg/kg (po). The effect was completely reversed by XE-991 consistent with an on-target effect of retigabine at the doses administered. Additional examples of in vivo efficacy with retigabine include visceral pain induced by intracolonic injection of capsaicin (Hirano et al. 2006) and acid-induced muscle pain (Nielson et al. 2004).

One additional point of consideration when interpreting the above studies is to note that in pre-clinical models, retigabine and to a lesser extent ICA-27243 also affect locomotor activity. However, in general there is a separation between the MED for efficacy in pain models and the lowest dose which shows locomotor side-effects, supporting the hypothesis that Kv7 agonists are effective in pre-clinical models of pain independent of inhibiting motor function. These results in conjunction with those reported in studies on sensory neurons indicate that Kv7 activation inhibits nociceptive neuronal firing and behavioral endpoints in pre-clinical pain models.

2.7 Experience in Man

As noted above, retigabine has been the most extensively studied Kv7 agonist in pre-clinical pain models, but there are currently no reports on the effectiveness of this compound in treating pain in man. Results have, however, been published regarding efficacy and tolerability in epilepsy. Porter et al. (2007) reported the results of a Phase III study testing the efficacy and safety of retigabine as adjunctive therapy in patients with partial-onset seizures. Doses were 600, 900 and 1200 mg/day (tid). The authors note that retigabine was well tolerated and reduced the frequency of seizures in a dose-dependent manner. While these results do not bear directly on the effect of retigabine in pain, they do indicate that a reduction in CNS excitability via Kv7 activation is tolerated warranting additional studies of Kv7 activators in man. Perhaps a better example of a Kv7 agonist which has been widely used in man is the predecessor to retigabine, flupirtine. A number of studies have been published on the clinical effects of flupirtine when this compound was marketed as a non-opiate, centrally-acting analgesic with muscle relaxant properties. Freidel and Fitton (1993) summarize these studies and reported efficacy against pain due to surgery, traumatic injury, dental procedures, headache/migraine and abdominal spasms. Short-term investigations are also noted where flupirtine was effective against neuralgiform pain, dysmenorrhea, soft tissue rheumatism and cancer pain. Subsequently, Ringe et al. (2003) have reported efficacy against osteoarthritic pain.

Collectively, the results suggest that Kv7 activators are efficacious in disorders of hyper-neuronal excitability including pain. Exclusively ascribing the activity in man to Kv7 activation is difficult since flupirtine is the only real example noted and is not quite selective enough to convincingly support this conclusion. However, it does appear that at plasma levels achieved in clinical studies Kv7 channels should be activated and, based on the pre-clinical body of data, are likely to contribute to the in vivo activity observed. Additional studies with a wider array of compounds will be needed to clarify these points.

3
Cav3 Channels

Following the invention and application of patch-clamp technology (Hamill et al. 1981), a large number of electrophysiology studies were performed in the earlier 1980s and among the currents investigated was a new class of voltage-gated calcium channels in sensory neurons. Carbone and Lux (1984) described the channels as "low-voltage activated calcium channels", which we know today as T-type or Cav3 channels. The super-family of voltage-gated calcium channels is complex and includes at least ten different α subunits distributed among Cav1, Cav2 and Cav3 groups. The Cav3 sub-family is unique with respect to gating properties and pharmacology and channels formed in this group are generally thought to be simpler in composition than those from Cav1 and Cav2 complexes. Several different subunits in addition to the α subunit are required for complete Cav1 and Cav2 complexes while properties of Cav3 channels are largely reconstituted by expression of a primary α subunit. There have been reports indicating that both γ and $\alpha 2\delta$ subunits can associate with Cav3 α subunits (Gao et al. 2000; Klugbauer et al. 2000), although this association is not yet firmly established in native tissues. Indirect evidence of Cav3 type channels existed prior to the definitive studies by Carbone and Lux (1984), since a calcium-dependence to initiation of burst firing in certain neurons (Llinas and Yarom 1981) suggested an underlying ion channel. This hypothetic channel differed from known calcium channels and appeared to be inactivated at normal resting potential, but could re-activate following hyperpolarization and provide a boost of current stimulating action potential bursting. Subsequent molecular approaches led to identification of the Cav3 channel family which has exactly the properties required to support this type of electrical activity.

3.1
Cav3 Genetics and Molecular Approaches

The identification of specific genes encoding Cav3 channels allowed the initiation of a series of genetic/molecular approaches to address the physiological role these channels play, including the processing of pain signals. One of the first studies reported was knockout of the Cav3.1 gene (Kim et al. 2001) in mouse with a primary focus on signaling in the CNS related to seizure activity. Consistent with a role of Cav3.1 channels in regulating burst firing, thalamic relay neurons were found to be deficient in this type of electrical activity following stimulation of $GABA_B$ receptors and to be resistant to seizure induction. Notably, knockout animals were overtly normal with no notably behavioral differences when compared to wild-type controls. No deficits were observed in development of the brain or other organs under histological examination, suggesting Cav3.1 channels do not play a critical

role in developmental or homeostatic function in the CNS. Following the initial study, the same group went on to investigate the role of Cav3.1 channels in pain (Kim et al. 2003). Elimination of Cav3.1 was found to have no effect on responses to noxious mechanical or thermal pain and no differences compared to wild-type littermates were observed for thermal hypersensitivity in a pre-clinical model of inflammatory pain (i.e., complete Freund's adjuvant). However, the authors did report a hypersensitivity to visceral pain induced by intraperitoneal injection of either acetic acid or $MgSO_4$. Through a variety of experimental approaches they concluded that the effect on visceral pain was mediated centrally and that the Cav3.1 –/– animals had deficits in sensory-gating within the thalamic pain centers. Mechanistically Cav3.1 channels may be involved in sensory gating which normally dampens visceral pain signaling. Consequently, elimination of Cav3.1 in the knockout animals leads to visceral hypersensitivity.

Subsequent to the studies by Kim et al. (2001, 2003), Bourinet et al. (2004) utilized an anti-sense approach to address the question of whether peripheral knockdown of Cav3 activity indicated a role in pain pathways and, if so, could one attribute activity to a specific subtype of Cav3 channel. Unlike the Cav3.1 knockout studies described above, pan anti-sense knockdown (i.e., non-selective among Cav3.1, 3.2 and 3.3 subtypes) increased the threshold to both noxious mechanical and thermal pain in normal animals, indicating an analgesic effect. Similarly, in the CCI model of neuropathic pain, pan-Cav3 antisense administration reversed the post-surgical thermal hyperalgesia and increased the nociceptive threshold to noxious mechanical stimuli. The subtype of Cav3 channel(s) involved was further investigated by administration of specific anti-sense molecules to Cav3.1, 3.2, and 3.3 and an increase in the nociceptive threshold was only observed with Cav3.2 knockdown. This effect was seen in both normal and neuropathic animals. The behavioral phenotype correlated with near complete elimination of low voltage-activated calcium currents in dorsal root ganglia neurons by the Cav3.2 antisense treatment. In addition to nociceptive thresholds, further studies with Cav3.2 antisense treatment demonstrated that tactile allodynia in neuropathic animals was also reversed.

The specific role of Cav3.2 channels in regulating and/or signaling pain has been further established by Choi et al. (2007a), who reported experimental results with Cav3.2 knock-out animals. Similar to results with Cav3.1 –/– animals, general behavior of the Cav3.2 (–/–) animals was normal; however, knockout animals showed decreased responses to a variety of pain stimuli, including acute mechanical, thermal, capsaicin-induced, and formalin-induced pain. Visceral pain responses to both acetic acid and $MgSO_4$ were attenuated, opposite to what was observed for the Cav3.1 (–/–) animals, suggesting opposing Cav3.1- and Cav3.2-dependent mechanisms may be involved in regulating visceral sensory signaling. Finally, responses to neuropathic pain in the SNL model were investigated in the knockout animals. Contrary to the

results observed by anti-sense knockdown of Cav3.2 in the CCI model, the mechanical and thermal responses in the Cav3.2 (–/–) animals were indistinguishable from wild-type littermates.

A larger data set is needed before definitive conclusions may be drawn, but the above results would suggest that peripheral knockdown of Cav3.2 leads to an increase in nociceptive thresholds and an anti-hyperalgesic response. Moreover peripheral Cav3.2 knockdown appears sufficient to increase nociceptive thresholds and reverse allodynia in at least one model of neuropathic pain; however, the result from Choi et al. (2007a) in the SNL model leave open the question of whether Cav3.2 inhibition is effective against all types of neuropathic pain.

Despite the normal appearance and behavior of both Cav3.1 and Cav3.2 knockout animals, one additional question that arises is whether deficits are observed under more detailed analysis. Mangoni et al. (2006) have noted cardiovascular changes in the Cav3.1 knockout and specifically bradycardia and slowing of atrioventricular conduction. These effects were observed in unrestrained animals at rest, but upon activity maximal heart rates were not different from control, suggesting other mechanisms can compensate for the loss of Cav3.1. The ECG data reported for Cav3.2 (–/–) were normal, suggesting that in the mouse the Cav3.1 channel is the dominant gene family member in the heart. Based on the observation that normal heart rates could still be achieved and that there was no evidence of arrhythmias in the Cav3.1 (–/–) mice, Mangoni et al. (2006) state that "T-type (Cav3) channel inhibition would have no deleterious consequences on cardiac physiology" and speculate about the potential utility of Cav3 blockers in cardiovascular applications as well as those for epilepsy and pain. Another observation with respect to the cardiovascular system involves the role of Cav3 channels on vascular tone. Chen et al. (2003) reported Cav3.2 knockout animals showed constitutively constricted coronary arterioles and also had focal myocardial necrosis. The knockout animals were also smaller at 8 weeks of age compared to their wild-type litter mates. The observation that elimination of Cav3.2 leads to increased coronary vascular contraction is at first counter-intuitive, but may reflect functional coupling between Cav3.2 and calcium-activated potassium channels in vascular smooth muscle (Nelson et al. 1995). Elimination of Cav3.2 effectively would remove the calcium activation signal for these channels and lead to a vasoconstrictive phenotype. Whether this phenotype is observed in species beyond the mouse remains to be determined.

Other studies demonstrate a role for Cav3 in sleep and the onset of action for volatile anesthetics and therefore should be considered when targeting Cav3 channels as molecular targets for pain indications. Anderson et al. (2005) have reported that thalamic deletion of Cav3.1 results in fragmented and reduced sleep which is consistent with a role in determining thalamocortical oscillatory frequencies. Petrenko et al. (2007) studied Cav3.1 knockouts and report that while the in vivo doses required for effects of

volatile anesthetics were not altered, there was a significant delay in the onset of anesthesia induction. This observation appears on the surface to be consistent with the increased state of wakefulness noted by Anderson et al. (2005) and, again, may be a contraindication when advancing new Cav3 blockers through pre-clinical models and into clinical trials.

3.2 Cav3 Distribution

Cav3 channels are expressed in a wide variety of neurons involved in pain signaling and processing. Across the three members of the Cav3 gene family, Cav3.3 has the most restricted neuronal expression (Lee et al. 1999) while Cav3.1 and Cav3.2 are more broadly distributed. Channels with Cav3 properties have also been identified in non-neuronal tissues, including heart, kidney, smooth and skeletal muscle, sperm and endocrine tissue (see Perez-Reyes 2003 for review) and as such these are possible systems to consider for side-effects when one targets Cav3 channels in the treatment of pain. One of the most detailed expression studies for Cav3 gene family members in rat neuronal tissue has been published by Talley et al. (1999). In CNS and PNS regions relevant to pain, both Cav3.1 and Cav3.3 were found to be expressed in thalamic neurons while all three subtypes were present in sensory ganglia, with Cav3.2 detected at the highest level. In general, similar results have been reported in human neuronal tissue for all three members of this gene family (Williams et al. 1999; Monteil et al. 2000a,b). These results are consistent with earlier reports of Cav3 currents in thalamic relay and reticular neurons (Coulter et al. 1989; Huguenard and Prince 1992) as well as isolated dorsal root ganglia neurons (Scroggs et al. 1992). Changes in Cav3 channel expression have also been investigated in pre-clinical models of pain. In a model of diabetic neuropathy, Cav3 current density increased by two-fold in medium sized dorsal root ganglia neurons (Jagodic et al. 2007). These changes correlated with increased excitability in this population of neurons and are consistent with a primary or adaptive role in the development and/or maintenance of the neuropathy.

3.3 Biophysical Properties

The T-type family of calcium channels is unique in their biophysical properties compared to the other voltage-gated calcium channels. To varying degrees the Cav3 channel activation curve (m_∞) overlaps the inactivation curve (h_∞), creating a "window current" near –60 mV, the normal resting potential of peripheral neurons. At this potential, Cav3.2, for example, is constitutively open and therefore able to influence neuronal activity in neurons where the resting membrane potential is near –60 mV. Endogenous agents

present in pathological conditions can potentiate Cav3.2, such as L-cysteine (Todorovic et al. 2001), and can dramatically increase neuronal activity. This may be a physiological mechanism by which Cav3.2 modulates pain transmission (see below). Another property of Cav3.x channels that facilitates control of neuronal activity is a low voltage threshold for channel activation, as seen in Fig. 3. A rat dorsal root ganglion cell was voltage-clamped at –65 mV and ramped from –100 mV to +40 mV in 500 ms. Threshold for Cav3.x activation is near –50 mV with peak current at –35 mV. The second peak is a high voltage-gated calcium channel that begins to activate at –20 mV with peak current near 0 mV. Since the normal resting potential of a sensory neuron is near –60 mV, this experiment illustrates that a minor depolarization of the resting membrane potential can activate Cav3.x, while it would take a large membrane depolarization to activate a high voltage-gated calcium channel.

The biophysical properties of recombinant Cav3.1, 3.2, and 3.3 channels expressed in mammalian cells have been compared in a number of studies. Chemin et al. (2002) provide a nice review of the influence that the differences in the gating properties might play on neuronal excitability. Table 1 summarizes the relative kinetic properties for members of the Cav3 family.

The slow deactivation kinetics of Cav3 channels make this family well suited for allowing calcium entry into an excitable cell, such as a sensory neuron. During the duration of a typical action potential (i.e., 4 ms), the repolarization of the membrane causes Cav3.2 to deactivate with a prolonged time-course. This allows a significant influx of Ca^{2+} into the cell, which may influence the activity of other cellular proteins, including calcium-activated potassium channels such as KCNN3 (SK). This coupling of calcium influx through Cav3 and activation of SK has been demonstrated to be important in dopaminergic (DA) neurons present in the midbrain. SK mediated after-

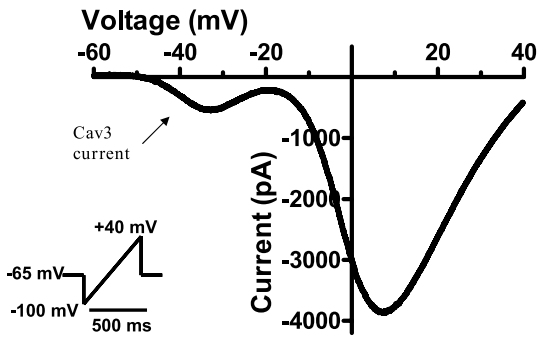

Fig. 3 Illustration of Cav3 currents in dorsal root ganglion neurons compared to high-voltage activated currents. Currents were generated in voltage-clamp using the ramp protocol illustrated in the inset. Cav3 currents activate at more negative potentials that other calcium channels observed in these neurons as noted by the label

Table 1 Cav3 Biophysics

	Cav3.1 (α1G)	Cav3.2 (α1H)	Cav3.3 (α1I)
Inactivation τ (ms)	30[a], 19.9[b]	47[a]	137[a], 272.9[b]
Deactivation τ (ms)	1.7[a]	2.7[a]	1.0[a], 1.2[b], 1.25[c]
Recovery, long-term inactivation (ms)	1806[a]	2587[a]	611[a]
Recovery, short-term inactivation (ms)	117[a]	352[a]	395[a], 297[b]

[a] Klöckner et al. (1999) *Inactivation* Holding potential –90, step to –45 mV, *Deactivation* 25 ms step to –30 mV, step to –120 mV
[b] Monteil et al. (2000b) *Inactivation* Holding potential –110, step to –50 mV, *Deactivation* 35 ms step to –25 mV, step to –80 mV
[c] Lee et al. (1999) *Deactivation* Step to –25 mV, repolarize to –100 mV

hyperpolarizing (AHP) currents in DA neurons were insensitive to blockers of high voltage-gated calcium channel antagonists, but were highly sensitive to nickel and mibefradil at concentrations which are consistent with blocking Cav3 (Wolfart and Roeper 2002). In addition, the authors report that the activity of the DA neurons could switch from a single-spike firing to burst activity when Cav3 was blocked by Ni^+, indicating a close association with neuronal excitability and Cav3 activity. However, a study in the Cav3.1 (–/–) mouse showed a lack of bursting activity in the thalamic-cortical relay neurons (Kim, et al. 2001), indicating Cav3.1 activity is required to support bursting. These apparently contradictory results could be due to the different neurons studied (thalamic and DA) and suggest a complex role for Cav3 regulating CNS neuronal activity.

3.4 Pharmacological Agents which Modulate Cav3 Channels

The agents currently available to investigate the role of Cav3 channels in vivo exhibit varying degrees of selectivity among ion channels; therefore, it is difficult to make conclusive statements based on individual studies. However, analyzing results for a variety of agents which inhibit Cav3 channels, including synthetic small molecules, toxins and endogenous compounds, a conservative approach is to consider the data in aggregate to investigate the role of Cav3 channels in pain pathways. The structures for selected molecules used in published studies are shown in Fig. 4, including three which have been used in man. Mibefradil is perhaps the most widely cited compound to be used as a modulator of Cav3 channels. Originally identified as an atypical calcium channel antagonist that showed selectivity for Cav3 channels over other calcium channel subtypes (see Clozel et al. 1997 for review), mibefradil was used for the treatment of cardiovascular disorders in man, but withdrawn due to drug-drug interaction problems as a consequence of cytochrome P450 in-

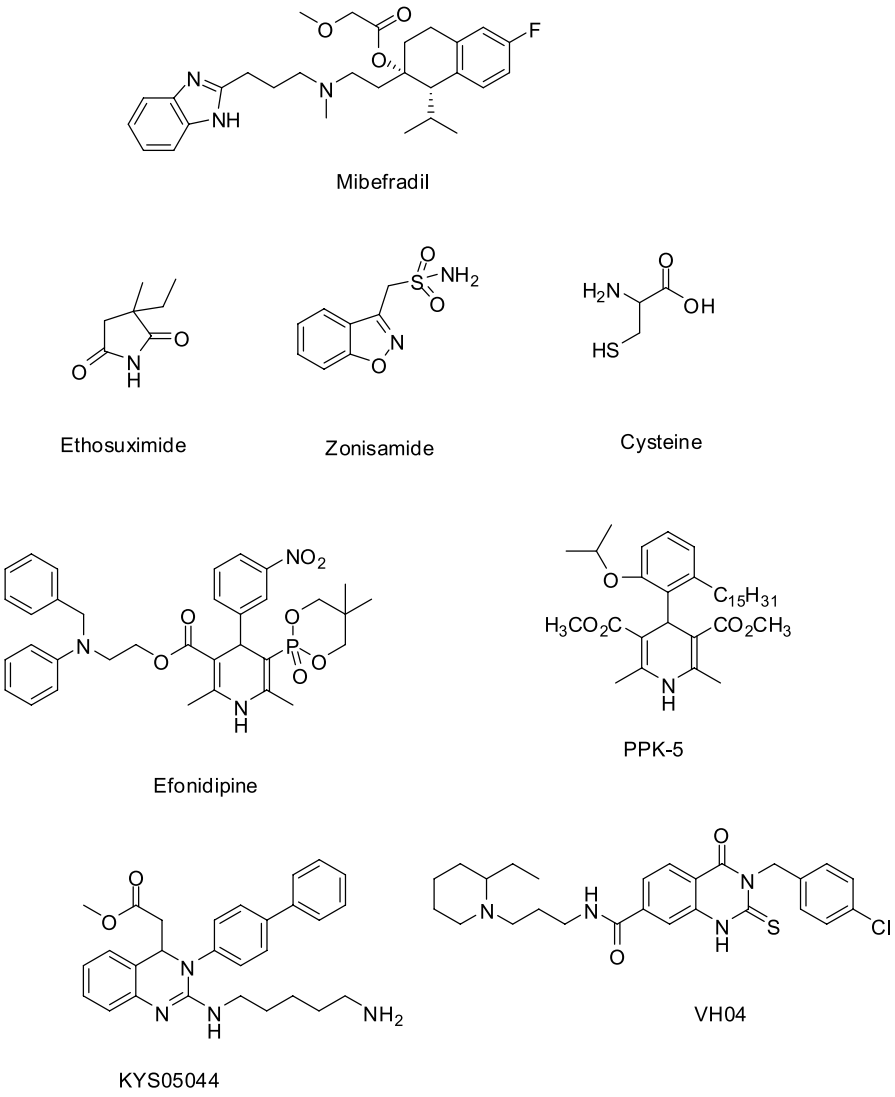

Fig. 4 Structures of selected Cav3 calcium channel modulators

hibition. Nevertheless, mibefradil has been tested in pre-clinical studies to investigate the role of Cav3 channels. Martin et al. (2000) reported the effects of mibefradil on cloned Cav3 channels and found that the most potent inhibition was observed for Cav3.1 and Cav3.2 compared to Cav3.3. Mibefradil is approximately tenfold selectivity for Cav3 block compared to L-type calcium channel block and this selectivity ratio has been generally reported across investigators. Martin et al. (2000) noted that the potency of inhibition differed with the divalent charge carrier or when the membrane potential

was depolarized to induce steady-state inactivation. Conversely, block decreases as temperature increases. These investigators did an excellent job of summarizing results in the literature across different charge carriers and temperatures (see Table 1, Martin et al. 2000) in an effort to draw quantitative conclusions regarding the potency and selectivity of mibefradil for Cav3 channels and to allow interpretation of the effects of mibefradil in vivo. For example, mibefradil blocks Cav3.2 with an IC50 value of 69 nM when calcium is the charge carrier and membrane potential is set to inactivate ~50% of the channels. If the compound is ~5-fold more potent at physiological temperatures, then the in vivo potency of mibefradil for Cav3.2 is approximately 350 nM. This is an oversimplification since other factors may need to be considered in vivo. One of these is that mibefradil generates a metabolite in vivo reported to be a potent L-type calcium channel blocker which may complicate mechanism of action interpretations, particularly in cardiovascular studies. Nevertheless, mibefradil clearly is a Cav3 blocker and with proper caveats one can begin to interpret in vivo results in the context of on-target activity.

Ethosuximide is a another compound which has been reported to block Cav3 channels and has been used in vitro and in vivo to investigate the role these channels play in pain signaling. Ethosuximide is used for the treatment of absence epilepsy in man and the mechanism of action has been hypothesized to be Cav3 dependent. Gomura et al. (2001) investigated the effects of ethosuximide along with related compounds and observed block of Cav3 channels with *Ki* values ranging from 0.3–1.2 mM; consistent with the exposure levels observed for this compound in man (Bauer et al. 1982). The authors note that block was state-dependent and "window" or maintained currents were particular sensitive to inhibition by ethosuximide (IC50 value ~0.6 mM). It has been noted, however, that ethosuximide blocks other ion channels at similar concentrations and that in vivo results with respect to mechanism of action need to be interpreted with caution (Perez-Reyes 2003; Leresche et al. 1998).

Recently a series of quinoxalinone derivatives have been published which show excellent selectivity for Cav3 channels over N-type calcium channels. 3,4-Dihydroquinazolinone derivatives, typified by KYS05044, have been reported with approximate IC50 values of 1 μM for Cav3.1 and Cav3.2, but no appreciable block of N-type calcium channels at 10 μM (Park et al. 2006; Rhim et al. 2007; Choi et al. 2007b). Doddareddy et al. (2007) reported a related chemical series identified through a virtual screening approach and highlighted compound VH04 which blocks Cav3.1 with an IC50 value of 0.1 μM and shows 20-fold selectivity against N-type calcium channels. These studies serve to illustrate that novel chemotypes are currently being identified which potently and selectively block Cav3 channels. Interestingly, more established chemotypes which are known calcium channel blockers have also been investigated to determine whether it is feasible to achieve Cav3 selectiv-

ity. Furukawa et al. (2004) showed that efonidipine can block Cav3 channels in a stereoselective manner while Kumar et al. (2002) demonstrated that derivatives of nifedipine, which are generally non-selective between Cav3 and L-type channels, can be identified with selectivity ratios up to 40-fold (see PPK-5, Fig. 4). Therefore, both novel chemistries and modifications of known calcium channel blockers have the potential to identify selective Cav3 compounds.

Beyond the small molecules noted above, there are two other classes of pharmacological agents that deserve mention as Cav3 blockers. Nickel (Ni^+) has been particularly useful as a tool for selectively blocking certain classes of Cav3 currents, particularly in sinoatrial nodal cells and sensory neurons (Hagiwara et al. 1988; Todorovic and Lingle 1998). Lee et al. (1999) examined the effects of Ni^+ on Cav3 currents expressed in recombinant cell lines from the three different gene family members and found Cav3.2 is approximately 20-fold more sensitive to block by Ni^+ than either Cav3.1 or Cav3.3. As such, Ni^+ has been used as a tool in both in vitro and in vivo studies where Cav3.2 currents are under investigation.

Another particularly exciting area of research with respect to pharmacological modulation of Cav3 channels involves potentiation of current observed in sensory neurons by redox agents. Todorovic et al. (2001) first reported that L-cysteine, an endogenous reducing agent, enhances Cav3 channels in nociceptive neurons. This sensitization affects the threshold for firing in nociceptors and induces bursting behavior consistent with a pronociceptive response. Several independent lines of research have recently come together to suggest that the effector for this response may be H_2S, which is produced by the action of cystathionine lyase with L-cysteine as the substrate (Kawabata et al. 2007). H_2S is currently under consideration as an endogenous signaling molecule in pain sensation (Szabo 2007) and direct application of NaHS as an H_2S donor to the paw of the rat produces hyperalgesia (Kawabata et al. 2007).

Other agents are also available which modulate Cav3 activity with varying degrees of selectivity, including toxins (Sidach and Mintz 2002; Chuang et al. 1998), nitrous oxide (Todorovic et al. 2001), ascorbate (Nelson et al. 2007), and anti-convulsants (Todorovic and Lingle 1998). Additional research should clarify the usefulness of these agents as pharmacological tools for elucidating the role of Cav3 channels in pain.

3.5 Effects of Modulating Cav3 Channels in Pre-Clinical Models of Pain

As noted above, in vivo pharmacological studies with selective Cav3 blockers have not yet been reported extensively. Consequently, most of the results reported to date use either mibefradil or ethosuximide and while the data are solid, the on-target mechanistic interpretation of the results must be

approached with caution. Nevertheless, the available pharmacological data generated to date would suggest that Cav3 blockers are effective in pre-clinical models of pain (see McGivern 2006).

Mibefradil has been reported to be effective in reversing both tactile allodynia and thermal hypersensitivity in the SNL model following both i.p. and intraplantar administration (Dogrul et al. 2003). However, no effect was observed following intrathecal administration suggesting a peripheral site of action for mibefradil. Todorovic et al. (2004) reported no effect on thermal hypersensitivity following intraplantar administration in the same model so there appear to be laboratory-dependent differences for certain effects in specific models. Following systemic exposure, mibefradil (i.p.) reduces pain related signals in a writhing model (Kim et al. 2003), although intracisternal administration potentiated pain in the same model. This result may reflect differential effects with respect to visceral pain when blocking either Cav3.1 or Cav3.2 as was observed in the knockout studies (see above). Systemically, though, mibefradil is also effective in acute mechanical and thermal pain models (Todorovic et al. 2002). Following either intrathecal or intracisternal administration mibefradil has also been reported to be efficacious in both phases of the formalin model (Barton et al. 2005; Cheng et al. 2007), so there appears to be a range of models where this particular compound is active across a number of routes of administration.

Less data are available with ethosuximide in pain models, but following i.p. dosing efficacy has been reported in the capsaicin, formalin, tail flick, and SNL models (Dogrul et al. 2003; Barton et al. 2005). Efficacy has also been reported against tactile and cold allodynia in a taxol-induced peripheral neuropathy model (Flatters et al. 2004). Ethosuximide (i.t.) is generally less efficacious in reported studies perhaps due to the weak activity of this compound and was not active in either the SNL (Dogrul et al. 2003) or formalin models (Cheng et al. 2007). However, Matthews et al. (2001) did find that intrathecal ethosuximide does inhibit electrical activity in spinal neurons following SNL surgery, although behavioral endpoints were not assessed.

There have been several other in vivo studies which suggest that Cav3 channels play a role in pain signaling and that these channels represent reasonable therapeutic targets. Pathirathna et al. (2005a,b) investigated the actions of different neurosteroids which show varying degrees of both Cav3 block and $GABA_A$ activity in the CCI model of neuropathic pain. In addition, these investigators synthesized neurosteroids which inhibit Cav3, but showed no $GABA_A$ activity (Todorovic et al. 1998). One such compound produced an anti-allodynic effect in the CCI model and an antinociceptive effect in sham animals with the effect in the neuropathic animals reaching statistical significance at lower doses. Nickel (i.t.) has also been shown to be effective in the formalin model (Cheng et al. 2006), possibly suggesting a role for Cav3.2 given the greater sensitivity of this channel to block by Ni^+. Finally, Nelson

et al. (2007) reported that local application of L-cysteine induces thermal sensitization in a Cav3-dependent manner since the effect was not observed in the Cav3.2 knockout mouse, suggesting this channel subtype is the dominant player among the Cav3 family members in regulating of thermal responses in the periphery.

3.6 Pharmacological Modulation of Ca3.x Channels in Man

Two approved drugs which block Cav3 channels, zonisamide and ethosuximide, are currently used to treat absence epilepsy in man. There have been several anecdotal observations suggesting these compounds may be effective in treating different types of pain, as well as, one small randomized, controlled clinical trial. Eggers and Moreno (2002) reported 3 patients with migraine-induced pain who had failed prophylactic treatment with other drugs, but responded favorably to ethosuximide. Similarly, Drake et al. (2004) tested zonisamide in an open label trial for refractory migraine pain and reported statistically significant reductions in severity, duration and frequency of headache. Finally, Takahashi et al. (2004) reported 2 patients with centrally mediated poststroke pain where treatment with zonisamide was effective. These patients had thalamic lesions and the effectiveness of zonisamide is at least consistent with modulation of thalamocortical firing patterns which are regulated by Cav3 channels.

The above studies focus on pain that is exclusively centrally mediated. There have also been additional reports on the effectiveness of zonisamide in neuropathic pain and painful diabetic neuropathy. Backonja (2002) summarized unpublished results from an open label study with zonisamide, where marginal improvements in pain scores were observed although it was noted that a small number of previously unresponsive patients did achieve noticeable pain relief in the study. The only randomized, controlled trial reported to date, however, was by Atli and Dogra (2005) in patients suffering from painful diabetic neuropathy. This was a small study with 13 patients on zonisamide and 12 on placebo. Trends toward improvements in pain scores were observed in the treatment groups, but statistical significance was not achieved. The authors suggest that a larger study is required to definitively establish efficacy and tolerability in this patient population.

In all of the studies noted above, ethosuximide and zonisamide were reported to be well tolerated and, by extension, one could hypothesize that Cav3 block is generally well tolerated. Notably no cardiovascular side-effects were observed. Since the compounds used to date are not highly selective, additional studies with newer compounds will likely be required to answer the question of whether selective Cav3 blockers are effective for the treatment of pain in man.

4 Summary

The existing data for both Kv7 and Cav3 channels indicate that these gene families encode members which play a key role in regulating pain signaling in pre-clinical animal models and likely in man. Knockout and anti-sense studies have validated these gene families in a variety of pain models, and use of an array of pharmacological agents suggests that modulation of these channels in man would be expected to show therapeutic benefit. Definitive conclusions will have to await future studies and clinical trial results, but it remains possible that both Kv7 and Cav3 modulators will find their way into the pharmacopoeia of pain therapeutics in the not too distant future.

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Potassium Channels: Oncogenic Potential and Therapeutic Target for Cancers

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Abstract Cell proliferation and cell death are two counterparts in sharing the responsibility for maintaining normal body function, and the delicate balance between the two coordinates developmental morphogenesis, cell homeostasis, and tissue modeling in organisms. Abnormally enhanced proliferation and/or impaired cell death often cause loss of control of cell growth leading to tumorigenesis or cancer formation. Several fundamental steps need to be fulfilled at the cellular level for tumorigenesis and these steps can be roughly viewed as characteristic alterations of some physicochemical processes: cell volume, intracellular Ca^{2+}, and intracellular pH. Evidence has rapidly emerged indicating a pivotal role of K^+ channels in controlling these fundamental biological processes and a deregulated expression of potassium channel protein-coding genes, as well as malfunction of K^+ channels as an important step in the development and progression of cancers. Herein, the role of K^+ channels in cancer progression will be introduced by presenting the data obtained over the past 25 years, beginning with the evidence for K^+ channels as cancer markers, followed by the data linking K^+ channels to neoplastic growth and cancer metastasis. The potential of targeting K^+ channels for cancer therapy will then be discussed by outlining the promising approaches and strategies including inhibition of K^+ channel activities using pharmacological agents and downregulation of K^+ channel expression using various nucleic acids (siRNA, decoy ODN and miRNA). Some unanswered questions and unsolved problems with respect to K^+ channels and cancer are discussed in the final section.

Keywords Biomarker · Cancer cells · K^+ channels · Gene therapy · Oncogenesis

1 Introduction

Cell proliferation and cell death are two counterparts in sharing the responsibility for maintaining normal body function, and the delicate balance between the two coordinates developmental morphogenesis, cell homeostasis, and tissue modeling in organisms. Abnormally enhanced proliferation and/or impaired cell death often cause loss of control of cell growth leading to tumorigenesis or cancer formation. Several fundamental steps need to be fulfilled at the cellular level for tumorigenesis and these steps can be roughly viewed as characteristic alterations of some physicochemical processes: (1) cell volume, (2) intracellular Ca^{2+}, and (3) intracellular pH (Schreiber 2005).

The regulation of cell volume is a fundamental function of healthy cells for maintaining constant size. Tumor cells in proliferation and invasion both undergo characteristic changes of cell volume. Cell swelling is a prerequisite for cell division whereas cell shrinking is required for cell migration. In neuroblastoma and glioma cells (Rouzaire-Dubois et al. 2000, 2004, 2005; Dubois and Rouzaire-Dubois 1998) and in the MCF-7 human breast cancer cell line and A549 human lung cancer cell line (author's unpublished data), the rate of cell proliferation changes with cell volume in a bell-shaped manner, being optimal within a cell volume window. Beyond the optimal cell-volume window either with over-swelling or over-shrinking, the rate of cell proliferation is deemed to diminish, indicating a growth control by a low and a high cell size checkpoint (Fig. 1). Biologically, cell proliferation and invasion depend on turning-on and switching-off intracellular growth and division pathways. Cell swelling leads to dilution and cell shrinkage to concentration of cellular constituents or proteins involved in growth and division pathways. Relatively small changes in macromolecule concentration can result in large changes in their activity, because conformation and activity of macromolecules are critically determined by their crowding and the ratio of hydration versus osmotically active wa-

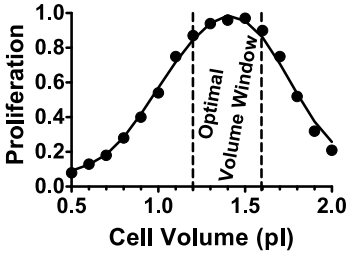


Fig. 1 Illustration of the relationship between cell proliferation and cell volume. The *dashed lines* define the optimal volume window for cell proliferation. Outside the window, either cell shrinking or swelling, cell proliferation decreases. *y-axis*: Proliferation: cell numbers normalized to the maximum value; *x-axis*: Cell volume in pico-liter (pl)

ter (Schreiber 2005). Macromolecules in the cell sterically "crowd" each other in their cohabited aqueous space and this steric interaction can be enhanced by the preferential hydration of molecules. In an isotonic environment, cell volume changes require the respective alterations of ion transport across the cell membrane; ion movements are among the earliest signals that could play important roles in cancer cell proliferation and metastasis.

Ca^{2+} acts both as a ubiquitous allosteric activator and inhibitor of intracellular enzymes in the cytosol, organelles and nucleus. Depletion of intracellular Ca^{2+} can lead to inhibition of DNA synthesis, protein synthesis and nuclear transport (Greber and Gerace 1995; Short et al. 1993). Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is normally maintained at ~100 nM in resting conditions and changes of $[Ca^{2+}]_i$ are associated with progression through the cell cycle. $[Ca^{2+}]_i$ can rise through several mechanisms stimulated by growth factors and mitogens, and it is also critically controlled by cell membrane potential. In excitable cells, membrane potential controls $[Ca^{2+}]_i$ by modulating the activity of voltage-dependent Ca^{2+} channels (Nelson et al. 1990; Tsien and Tsien 1990); membrane depolarization (intracellularly more positive) opens voltage-gated Ca^{2+} channels, increasing Ca^{2+} influx. In non-excitable cells, membrane hyperpolarization (intracellularly more negative) increases the driving force (electrical gradient) for Ca^{2+} entry thereby increasing $[Ca^{2+}]_i$ (Fig. 2). It was demonstrated that depolarization by voltage clamp decreased and hyperpolarization increased $[Ca^{2+}]_i$, illustrating a transmembrane flux of Ca^{2+} following its electrochemical gradient in human melanoma cell (Lepple-Wienhues et al. 1996). Consistently, blockade of K^+ channels inhibits Ca^{2+} influx in colon cancer cells (Yao and Kwan 1999). Indeed, oscillation of membrane potential is required for progression of the cell cycle. Terminally differentiated G0 cells display a hyperpolarized value of their membrane potential, whereas tumor cells are quite depolarized. The membrane potential in early G1 phase is depolarized and the transition from G1 to S phase during mitosis is accompanied by hyperpolarization of the membrane potential (Wonderline 1995, 1996).

The growth of many tumors requires acidic and hypoxic micro-environments; the average extracellular pH (pH_0) values ranging from 6.5–6.9 for tumors and values of 7.0–7.5 for normal cells. On the other hand, the intracellular pH (pH_i) of tumor cells is generally more alkaline than that of normal cells. Alkaline pH_i appears to be necessary for various mechanisms involved in cell proliferation and higher pH_i generally correlates with higher rate of metabolism and of cell proliferation (Wakabayashi et al., 1997). This is because the syntheses of protein, RNA and DNA necessary for cell proliferation are optimal at an alkaline pH micro-environment. In addition, similar to membrane potential-Ca^{2+} entry coupling there also exists a cross-talk between pH_i and Ca^{2+} homeostasis; low pH_i reduces $[Ca^{2+}]_i$ and cell proliferation leading to growth arrest. Moreover, pH_i is also regulated by membrane potential. Among the main acid/base transporters in mammalian cells are the

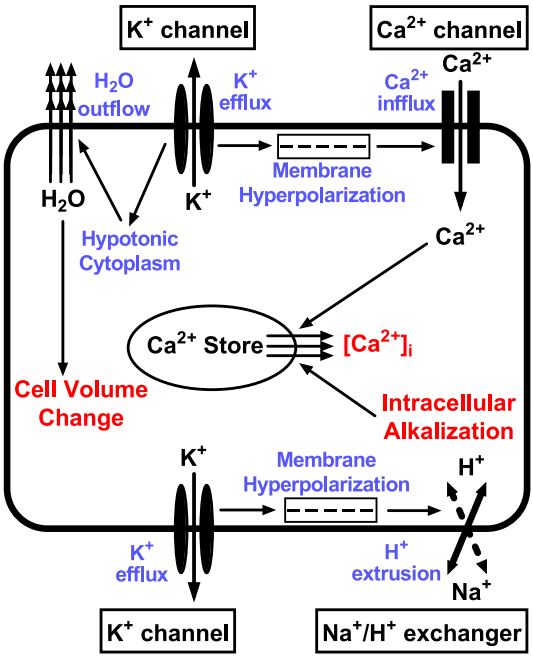


Fig. 2 Schematic illustration of the possible role of K^+ channels in $[Ca^{2+}]_i$, cell volume regulation and intracellular alkalization in tumor cells. Activation of K^+ channels causes membrane hyperpolarization that in turn increases Ca^{2+} entry by increasing the driving force for Ca^{2+} and causes H^+ extrusion by enhancing Na^+/H^+ exchanger activity leading to intracellular alkalization; increased Ca^{2+} entry and intracellular alkalization induce Ca^{2+} release from Ca^{2+} store, leading to an increase in $[Ca^{2+}]_i$. Activation of K^+ channels also causes H_2O outflow due to hypotonic cytoplasm as a result of K^+ efflux, leading to cell volume regulation

Na^+/H^+ exchangers (NHE) (Grinstein et al. 1989; Shrode et al. 1997), and the operation of NHE requires low intracellular Na^+ concentrations and hyperpolarized membrane potentials (Kahl and Means 2003; Wonderline et al. 1995). Activation of the NHE and resulting cellular alkalinization is a key mechanism in oncogenic transformation and is necessary for the development and maintenance of the transformed phenotype (Reshkin et al. 2000).

Notably, all these characteristic alterations are conferred by cell membranes particularly the cytoplasmic membrane that is basically a lipid-bilayer structure imbedded with certain proteins. The lipid-bilayer forms a barrier to surround and protect the cell contents and the transmembrane proteins are responsible for the cell communications with the environment. For example, receptor proteins mediate the growth signals produced by growth factors and mitogens and any other stimuli. Ion channel proteins control the flux of ions across the cytoplasmic membrane to regulate membrane potential, osmolarity (or cell volume), etc.

Evidence has rapidly emerged indicating a deregulated expression of ion channel protein-coding genes, as well as ion channel malfunction as an important step in the development and progression of cancers (Conti 2004; Wang et al. 2004; Pardo 2004; Arcangeli 2005). Several ion channels have been related to cell proliferation and/or cancer including Ca^{2+}, Cl^- and Na^+; however, the ion channels most commonly related to cell proliferation and cancer are the K^+ channels (Conti 2004; Wang et al. 2004; Pardo 2004; Arcangeli 2005; Kuhlmann 2005). The reasons that K^+ channels play a critical role in cancer cell proliferation and migration are obvious. First, K^+ channels are the major determinant of the cytoplasmic membrane potential that regulates Ca^{2+} influx and pH_i. Ion concentrations and membrane potential are interconnected via electrochemical gradients. Potassium-dependent alterations in membrane potential play a pivotal role in the proliferation of many types of cells and tumor cell lines. Second, K^+ ions constitute the majority (130–150 mM) of ion species in the cell and are the major determinant of intracellular osmolarity thereby cell volume. Activation of K^+ channels induces regulatory volume decrease. Third, K^+ channel activity regulates the concentration of intracellular solutes critical for cell metabolism. Finally, K^+ channel activity might serve to maintain permissive membrane potentials at critical cell cycle checkpoints.

2
Role of K^+ Channels in Cancer Progression

K^+ channels of different subfamilies have been correlated with tumor proliferation, including Ca^{2+}-activated K^+ channels (K_{Ca}), Shaker-type voltage-gated K^+ channels (Kv), the ether-*a-go-go* (*EAG*) family of voltage-gated K^+ channels, and the two-pore domain (2PD) K^+ channels (27, 28, 41, 42). Expression and channel activity studies have highlighted the tight association between K^+ channels and cancer; furthermore, modulation and pharmacological experiments have provided important clues for the potential therapeutic use of K^+ channels in cancer treatment. The best known K^+ channels that elicit mitogenic response or bear oncogenic potential include *Shaker*-type voltage-gated K^+ channel Kv1.3 (*KCNA3*), Ca^{2+}-activated K^+ channel K_{Ca} (SK4, *KCNN4*), *ether-a-go-go* K^+ channel EAG (Kv10.1, *KCNH1*), human *ether-a-go-go* related K^+ channel HERG (Kv11.1, *KCNH2*), and two-pore inward rectifier K^+ channel TASK-3 (K2P9.1, *KCNK9*).

2.1
K^+ Channels as Cancer Biomarkers

To be a cancer biomarker, a protein must have one of the following features: mutated gene, deregulated expression and altered function that pro-

duce phenotypes corresponding to the transformed cells. Overexpression of K^+ channels seems to match the criteria, whereas malignant phenotypes resulting from K^+ channel mutations have not yet been reported. Some K^+ channels are either not expressed at all or expressed only at low levels in healthy tissues/cells but become prominent or even predominant in terms of their expression and function in cancerous cells (Conti 2004; Wang et al. 2004; Pardo 2004; Arcangeli 2005; Kuhlmann 2005). More importantly, the increase in expression of these K^+ channels predicts transformation of cells. These K^+ channels might at least be used as biomarkers for early diagnosis of cancers, with appearance of these K^+ channels indicating carcinogenesis.

In several tumor cells lines including phaeochromocytoma (Conforti and Millhorn 1997), prostate cancer (Fraser et al. 2003), Jurkat T-lymphoma (Lampert et al. 2003), and gliomas (Preussat et al. 2003), Kv1.3 overexpresses and is the predominant component of K^+ channel subunits. The expression of Kv1.3 in 60 human breast cancer specimens was surveyed using immunohistochemistry by Abdul et al. (2003). Eighteen (30%) breast cancer specimens showed high, 35 (58%) moderate and 7 (12%) low Kv1.3 staining in the epithelial compartment. Another study demonstrated that the K^+ currents in Daudi human B lymphoma cells was carried by Kv1.3 because the current was strikingly suppressed by treatment with antisense oligonucleotide of human Kv1.3 gene (Zhou et al. 2002).

Recently, Mu et al. (2003) have reported that the *KCNK9* potassium channel (TASK) gene is amplified and overexpressed in breast cancers, lung and prostate cancers. Moreover, overexpression of *KCNK9* was found in 57 (46.0%) of the 124 patients with colorectal carcinomas, but not in the patients with colorectal adenoma (Kim et al. 2004). Interestingly, overexpression of KCNK9 promotes tumor formation and induces resistance to both hypoxia and serum deprivation.

Increased expression of SK4 channels, a member of the Ca^{2+}-activated K^+ channel superfamily, is found in all primary pancreatic cancer tissue tested and inhibition of SK4 activity suppresses proliferation of pancreatic cancer cells (Jager et al. 2004). This channel positively regulates cell proliferation in response to mitogenic activation of the Ras/ERK signaling pathway (Pena et al. 2000). Small-conductance K_{Ca} channels (SK3) are expressed in a highly metastasizing mammary cancer cell line MDA-MB-435s and in tumor breast biopsies but not in non-tumor breast tissues (Potier et al. 2006).

EAG has a rather restricted distribution in healthy tissues; it expresses nearly exclusively in brain, slightly in placenta and transiently in skeletal muscle (Occhiodoro et al. 1998; Pardo et al. 1999). In contrast to its restricted distribution in normal tissues, expression of EAG has been found in several human somatic cancer cell lines including HeLa cervix carcinoma, MCF-7 and SHSY-5Y neuroblastoma derived from breast tumor, melanoma IGR1, rhabdomyosarcoma, etc. (Meyer 1998, 1999; Crociani 2003). Most notably, EAG mRNA expression was found in 100% of the human cervical cancer sam-

ples in one study and only in 33% of the normal control samples (Farias et al. 2004). In addition, EAG has also been found in several other human tumors including mammary gland, liver, prostate, uterine cervix, ovary, endometrium, colon, and thyroid (Camacho 2006; Pardo 2004). Because of their oncogenic properties, their restricted distribution in normal tissue and ubiquitous expression in tumor cells (Pardo et al. 1999), EAG K^+ channel has become one of the most attractive candidates as a potential tumor marker.

HERG channels are ubiquitously distributed even in healthy subjects; transcripts can be found in heart, brain, kidney, liver, testis, uterus, prostate, etc., however the level of HERG expression is consistently increased when these tissues become cancerous. Indeed, HERG at both mRNA and protein levels has been found overexpressed in tumor cell lines of differing histogenesis (Bianchi et al. 1998), as well as in primary human cancers, such as endometrial adenocarcinomas (Cherubini et al. 2000), hematopoietic cells (Burkitt's lymphoma, chronic myelogenous leukemia, acute lymphoblastic leukemia and acute promyelocytic leukemia) (Pillozzi 2002; Smith et al. 2002), retinoblastoma, mammary adenocarcinoma (Wang et al. 2002), Barrett's esophagus (Lastraioli et al. 2006) and colorectal cancer (Lastraioli et al. 2004). Investigations in human gliomas have also been made (Patt et al. 2004) that suggest a differential expression of HERG and EAG depending on the malignancy grade and nature of tumor cells; such expression seems to be inversely related to the malignancy of the tumor.

Overexpression of K^+ channels in cancerous cells indicates their potential as cancer biomarkers. A question to ask is how a cell during transformation turns on the expression of K^+ channels. Answering this question should aid us to understand the mechanisms. In general, there are at least five ways through which gene expression can be enhanced; they are somatic mutation, DNA reduplication, gene hypomethylation, genomic amplification, and transcription activation by mitogens or oncogenes. Which of these mechanisms are used by a cell to promote K^+ channel expression is at present poorly understood. The currently available data indicate that different mechanisms may operate for different K^+ channels.

We have recently identified and characterized the promoter regions (genomic DNA sequences responsible for initiating gene transcription) of the *EAG* and *HERG* genes. We found that *EAG* is transactivated by E2F, an oncoprotein acting as a transcription factor (unpublished observation) and the transcription of *HERG* is controlled by another oncoprotein Sp1 (stimulating protein 1) (Lin et al. 2007). Moreover, transcription of the *HERG* gene is also significantly enhanced by other oncoproteins such as NF-κB and Nkx3.1 (Lin et al. 2007).

The mechanism by which the *KCNK9* gene overexpresses in human colorectal cancers is a good example of chromosomal gain or genomic amplification. Mu et al. (2003) have identified *KCNK9* as a new protooncogene

demonstrating increased copy number at chromosome 8q24. The finding represents the first indication that genetic alteration of an ion channel gene can play a direct role in cancer. Indeed, genomic amplification, an increase in gene copy number, is one of the predominant manifestations of the genetic instability that typifies cancer cells.

2.2 K^+ Channels and Neoplastic Growth

As already described above, some of the K^+ channels are more abundantly expressed in transformed cells or cancerous cells than in the healthy cells of the same histological origins. The subsequent question to ask is whether this gain-of-expression in tumor cells is merely a bystander or one of the multifactorial processes during tumorigenesis. Evidence has been accumulating pointing to a common role of various K^+ channels in cell-cycle progression and cell proliferation of various tumor cells.

During progression from the G1 to S phase, many cells undergo changes in membrane potential, cell volume, cytoplasmic pH, and ion content, that, in principle, could arise from differing K^+ channel expression. K^+ channels differentially regulate the electrical potential of the plasma membrane during proliferation. It has been known that cancer cells are on average more depolarized than non-tumor cells (Arcangeli et al. 1995), although a transient hyperpolarization is required for the progression of the early G1 phase of the cell cycle (Wonderlin et al. 1995). Thus, blockade of K^+ efflux, which leads to depolarization, should interfere with proliferation by inhibition of such transient hyperpolarization. Indeed, progression through the cell cycle is dependent on K^+ channels, and blocking these channels causes inhibition of proliferation (Wonderlin and Stroble 1996). Inhibition of K^+ channels leads to membrane depolarization and cell cycle arrest in early G1 phase in the MCF-7 human breast carcinoma cell line (Wang et al. 1998). Conversely, activation of K^+ channels promotes the cell cycle progression from the G0/G1 to S phase (Wonderlin et al. 1995; Ouadid-Ahidouch et al. 2001; Chittajallu et al. 2002).

Treatment of endometrial cancer (EC) cells with clotrimazole and TRAM-34, two agents known to inhibit SK4 channels, suppressed the proliferation of EC cells and arrest the EC cell cycle at the G0/G1 phase. Similarly, downregulation of SK4 by siRNA inhibited EC cell proliferation and arrested its cell cycle at the G0/G1 phase (Wang et al. 2007). Moreover, clotrimazole also inhibits proliferation of human prostate cancer cell lines, such as LNCaP and PC-3 cells (Parihar et al. 2003).

Overexpression of KCNK9 promotes tumor formation and induces resistance to both hypoxia and serum deprivation. Furthermore, wild-type KCNK9 confers a growth advantage to cells, whereas the inactivating mutant has no effect on cell growth, suggesting that KCNK9 is directly involved in cell proliferation and has oncogenic properties (Pei et al. 2003).

Antisense oligodeoxynucleotide against EAG decreased significantly DNA synthesis in the tumor cell lines. In a following communication, the same group reported that inhibition of EAG (either expression or channel function) by small molecule, blocking antibody or short interfering RNA led to a reduction in DNA synthesis and proliferation in several human tumor cell lines (Pardo et al. 1999). Similarly, inhibition of EAG-mediated currents by imipramine has been suggested to decrease cell proliferation in IGR1 cells (Gavrilova-Ruch et al. 2002). EAG was identified in the breast cancer cell line MCF-7 (Gavrilova-Ruch et al. 2002). MCF-7 cells arrested in G0/G1 were depolarized and EAG density was small compared to cells progressing in the G1 phase. Transient activation of EAG K^+ channels induced a hyperpolarization of the membrane potential and progression through the early G1 phase. Extracellular perfusion of astemizole inhibited both EAG and cell proliferation (Gavrilova-Ruch et al. 2002). On the other hand, cells transfected with *EAG* are able to grow in the absence of serum, lose contact inhibition, and induce aggressive tumors when injected into immune-depressed mice (Pardo et al. 1999). In sharp contrast, cells expressing non-conducting EAG channels fail to induce tumor formation when injected into immune-depressed mice.

Blockers of HERG channels such as dofetilide, E-4031, cisapride, etc., all have been shown to inhibit tumor cell proliferation (Wang 2004; Arcangeli 2005). Conversely, we have shown that HERG K^+ channel expression facilitates tumor cell proliferation caused by tumor necrosis factor α (TNF-α) at concentrations <1 ng/ml (Wang et al. 2002). The effect is observed only in HERG-expressing cells such as SK-BR-3 (human mammary gland adenocarcinoma cells), SH-SY5Y (neuroblastoma cells) and HL-1 (rat atrial tumor cells), but not in tumor cells without endogenous HERG (human lung cancer cell A549 and skin cancer SK-Mel-28 cells).

2.3 K^+ Channels and Cancer Metastasis

The acquisition of the capacity of cancerous cells to invade surrounding tissues confers a more malignant phenotype and is necessary for the establishment of metastases. A study using a series of light microscopic and electron microscopic images, capturing glioma cells in the process of cell invasion, revealed that glioma cells shrink as they invade. It has therefore been proposed that the cell shrinkage is a prerequisite of many migratory cells. Similar to cell cycle progression, cell invasion requires Ca^{2+} influx and membrane hyperpolarization. It is then conceivable that enhanced activities of K^+ channels due to overexpression on one hand mediate efflux of K^+ in concert with water outflow, leading to cell shrinking or cell volume decrease, and on the other hand hyperpolarize the cytoplasmic membrane, promoting Ca^{2+} influx by increasing the driving force for Ca^{2+} entry. Indeed, participation of K^+ channels in

cancer cell metastasis is another evidence for the role K^+ channels in cancer progression.

Large-conductance K_{Ca} (BK) channel expression has been shown to be upregulated in human glioma biopsies, and expression levels correlate positively with the malignancy grade of the tumor. Iberiotoxin inhibits glioma proliferation. Small-conductance K_{Ca} (SK3) channels are expressed in a highly metastasizing mammary cancer cell line, MDA-MB-435s.

A study from Arcangeli's laboratory demonstrated that the activity of HERG channels regulates cell invasiveness in colorectal cancers, and that a direct correlation exists between the amount of HERG protein on the plasma membrane and the invasion capacity of cancer cells (Arcangeli 2004). Moreover, both the *HERG* gene and HERG protein were expressed in a high percentage of primary human colorectal cancers, with the highest incidence occurring in metastatic cancers, whereas no expression could be detected either in normal colonic mucosa or in adenomas. The regulatory role of HERG channels in cell invasion may be conferred by the functional association between HERG channels and $\beta 1$ integrins in neoplastic cell lines (Hofmann et al. 2001). HERG K^+ channels are involved in the establishment of an invasive phenotype in colorectal cancer cells both in vitro and in vivo (Lastraioli et al. 2004).

3 Targeting K^+ Channels for Cancer Therapy

Overexpression and the ability of K^+ channel inhibitors and modulators to impair cancer cell proliferation in vitro and to counteract cancer progression in vivo makes them viable targets for anti-cancer therapy, though rigorous scientific verification of the possibility at a pre-clinical level has not yet been rationally performed. Several strategies can be used for the purpose. A straightforward approach is to use the compounds that have K^+ channel inhibitory actions (K^+ channel blockers) or any other modulators that produce negative effects on K^+ channel activities. The second approach is to downregulate expression of K^+ channels to reduce K^+ channel activities, using currently available nucleic acid techniques. The former refers to the classical small-molecular-weight pharmaceutical agents, belonging to chemical therapy (chemotherapy). The latter is considered as gene therapy (genotherapy, or more accurately, nucleic acid therapy), which utilizes a short stretch of nucleotides (normally 20 to 30 bases in length) with sequence base-pairing with the target (thereby being gene-specific). Immunotherapy, targeting membrane K^+ channel proteins using antibodies (since these membrane proteins are extracellularly accessible), or targeting K^+ channel protein synthesis (intracellular trafficking) should also be considered as alternative approaches for K^+ channel-related anticancer therapy.

3.1 Shutting Down the Function of K^+ Channels

In 1996, Wonderlin and Strobl (1996) published an exhaustive review that has become a mandatory reference in the field; in that paper, they provide a thorough list of cell lines in which non-specific K^+ channel blockers such as 4-aminopyridine, quinidine, and TEA have anti-proliferative effects: lymphocytes, brown fat cells, Schwann cells, melanoma, breast carcinoma, neuroblastoma, small-cell lung cancer, and bladder cancer cells. The list of cell types has grown since that publication, e.g., neuroendocrine cells (Kayser et al. 1998), hepatocytes (Liu et al. 1998), GH3 pituitary cells (Warmke et al. 1991), endothelial cells (Faehling et al. 2001), keratinocytes (Mauro et al. 1997; Wohlrab and Markwardt 1999) corneal epithelium (Roderick et al. 2003), retinal pigment cells (Hoffman et al. 1998), chondrocytes (Wohlrab et al. 2002), myeloblastic leukemia (Xu et al. 1996; Xu et al. 1999), prostate cancer (Skryma et al. 1997), hepatocarcinoma (Zhou et al. 2003), mesothelioma (Utermark et al. 2003), colon cancer cells (Abdul and Hoosein 2002; Lastraioli et al. 2004).

The selective HERG channel blocker, E-4031, reduced proliferation of human leukemia CEM, U937 and K562 cell lines (Crociani et al. 2003). Cisapride, a gastroprokinetic agent, could inhibit the growth and clonogenicity of human gastric cancer lines by blocking HERG channels, and the effect was time- and dose-dependent (Shao et al. 2005). Imipramine, a tricyclic antidepressant, and the antihistamine astemizole block both ERG and EAG and either of them can impair cell proliferation in several human tumor cell lines (Ouadid-Ahidouch et al. 2001; Gavrilova-Ruch et al. 2002).

Strikingly, tamoxifen, an anticancer agent in clinical use, significantly inhibited K^+ currents and proliferation of mouse neuroblastoma cells NG108-15. The effect of tamoxifen on the cell proliferation was well correlated with the effect of tamoxifen on the resting K^+ flux, indicating that the antitumor action of tamoxifen could be due to its interaction with K^+ channels (Rouzaire-Dubois and Dubois 1990).

On the other hand, K^+ channel activators such as valinomycin (Rouzaire-Dubois et al. 1993) and minoxidil (Abdul et al. 2003) do the opposite: promoting tumor cell proliferation.

Table 1 summarizes the available data on the effects of K^+ channel blockers and activators on cell proliferation.

From the available studies, we could reach the following conclusions:

1. If K^+ channels are inhibited, cancer cell proliferation is impaired, whereas if K^+ channel activities are enhanced, cancer cell growth is promoted. One exception is quercetin; it has been reported to block Kv channels (Rouzaire-Dubois et al. 1993) and to activate BK_{Ca} as well (Cogolludo et al. 2007; Kuhlmann et al. 2005). Pharmacological tools opened the way toward the understanding of the role of K^+ channels in cell proliferation

Table 1 Compounds that reportedly inhibit tumor cell proliferation and/or migration

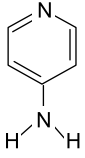
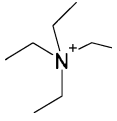
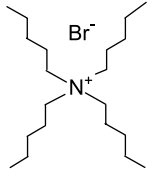
Compound name	Formula	Chemical structure	Target ion channel	Target tumor cell
K^+ Channel inhibitors				Inhibition of growth
Barium		Ba^{2+}	Inward rectifier	Melanoma cell line (Lepple-Wienhues et al. 1996)
4-Aminopyridine	$C_5H_6N_2$		A-Type	Small-cell lung cancer (Pancrazio et al. 1991); Ovarian cancer cell line (Zhanping et al. 2007); Leukemic cell (Renaudo et al. 2004); Hepatocarcinoma cells (Zhou et al. 2003)
Tetraethyl-ammonium	$C_8H_{20}NCl$		Non-specific	Neurofibromas; peripheral nerve sheath tumor cell (Wonderlin and Strobl 1996); melanoma cell; Leukemic cell (Renaudo et al. 2004); Prostate Cancer cell (Skryma et al. 1997)
Tetrapentyl-ammonium	$C_{20}H_{44}NBr$		Non-specific	Neurofibromas; peripheral nerve sheath tumor cell Ovarian cancer cell line (Zhanping et al. 2007); Hepatocarcinoma cells (Zhou et al. 2003); Melanoma cell line (Lepple-Wienhues et al. 1996)

Table 1 (continued)

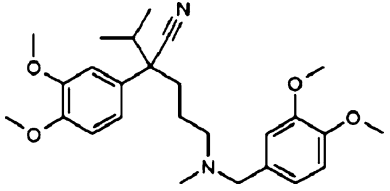
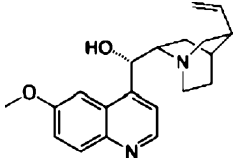
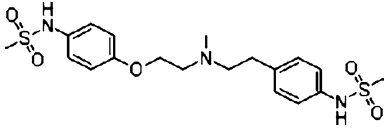
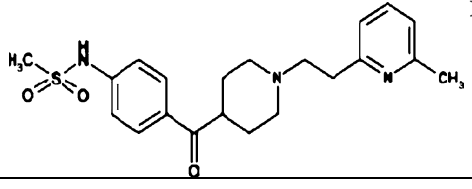
Compound name	Formula	Chemical structure	Target ion channel	Target tumor cell
K^+ Channel inhibitors				Inhibition of growth
Verapamil	$C_{27}H_{38}N_2O_4$		Non-specific	Small-cell carcinoma of the lung (Pancrazio et al. 1991); Prostate cancer cells (Rybalchenko et al. 2001; Fraser et al. 2000); Ovarian cancer cell line (Zhanping et al. 2007)
Quinidine	$C_{20}H_{24}N_2O_2$		Non-specific	Colonic carcinoma cells (Spitzner et al. 2007)
Dofetilide	$C_{19}H_{27}N_3O_5S_2$		HERG	Breast cancer and neuroblastoma (our unpublished observation)
E-4031	$C_{21}H_{27}N_3O_3S$		HERG	Breast cancer and neuroblastoma (our unpublished observation); leukemias, Neuroblastoma cell (Crociani et al. 2003); Breast cancer and neuroblastoma (our unpublished observation)

Table 1 (continued)

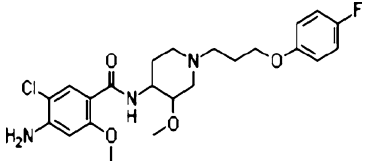
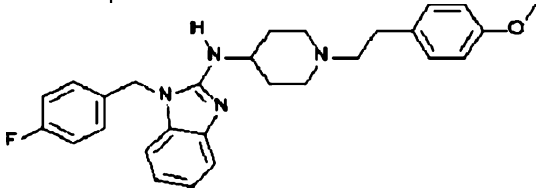
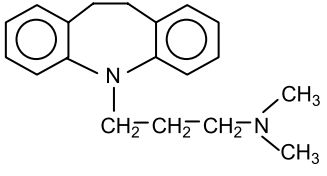
Compound name	Formula	Chemical structure	Target ion channel	Target tumor cell
K^+ Channel inhibitors				Inhibition of growth
Cisapride	$C_{23}H_{29}ClFN_3O_4$		HERG	Breast cancer and neuroblastoma (our unpublished observation); Gastric cancer cells (Shao et al. 2005)
Astemizole	$C_{28}H_{31}FN_4O$		HERG EAG	Breast cancer cell (Ouadid-Ahidouch et al. 2001)
Imipramine	$C_{19}H_{24}N_2$		HERG EAG	Melanoma and neuroblastoma cells (Gavrilova-Ruch et al. 2002)

Table 1 (continued)

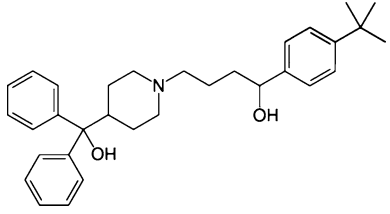
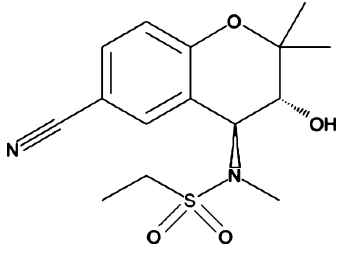
Compound name	Formula	Chemical structure	Target ion channel	Target tumor cell
K^+ Channel inhibitors				Inhibition of growth
Terfenadine	$C_{32}H_{41}NO_2$		HERG EAG	Colonic carcinoma cells (Spitzner et al. 2007)
Chromanol 293B	$C_{15}H_{20}N_2O_4S$		KCNQ1	Colonic carcinoma cells (Spitzner et al. 2007)

Table 1 (continued)

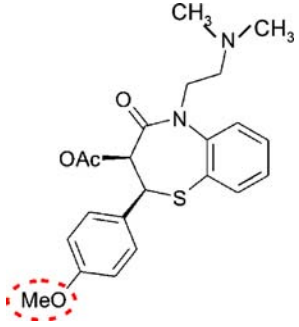
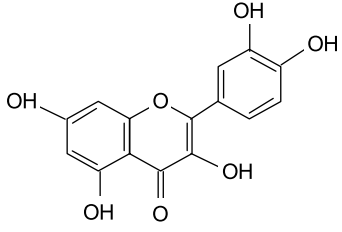
Compound name	Formula	Chemical structure	Target ion channel	Target tumor cell
K^+ Channel inhibitors				Inhibition of growth
Diltiazem	$C_{22}H_{26}N_2O_4S$		HERG	Colon cancer cell line (Yao and Kwan 1999)
Quercetin	$C_{15}H_{10}O_7$		Kv channels	neuroblastoma x glioma hybrid cells (Rouzaire-Dubois et al. 2005)

Table 1 (continued)

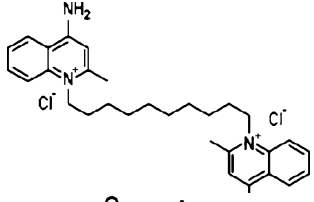
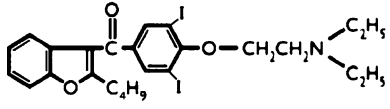
Compound name	Formula	Chemical structure	Target ion channel	Target tumor cell
K^+ Channel inhibitors				Inhibition of growth
Dequalinium	$C_{30}H_{40}Cl_2N_4$		Kv channels	Breast, prostate, and colon cancer cell (Abdul and Hoosein 2002)
Amiodarone	$C_{25}H_{29}I_2NO_3$		HERG	Human breast, prostate, and colon cancer cell (Abdul et al. 2003)
Iberiotoxin		QFTDVDCSVSKECWSVCKDLFG VDRGKCMGKKCRCYQ **Toxin region** **Disulfide**	BK_{Ca} (large-conductance)	Human endometrial cancer (Wang et al. 2007)
Charybdotoxin	$C_{176}H_{277}N_{57}O_{55}S_7$	MKILSVLLLALIICSIVGWSEA QFTNVSCTTSKECWSVCQRLHN TSRGKCMNKKCRCYS **Signal peptide** **Toxin region** **Disulfide**	BK_{Ca}, SK4, Kv1.3	Human endometrial cancer (Wang et al. 2007)

Table 1 (continued)

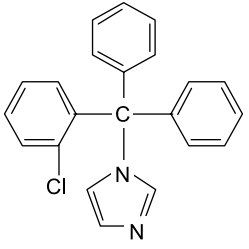
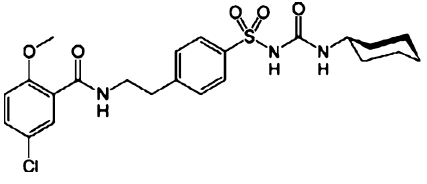
Compound name	Formula	Chemical structure	Target ion channel	Target tumor cell
K^+ Channel inhibitors				Inhibition of growth
Clotrimazole	$C_{22}H_{17}ClN_2$		SK4 (intermediate conductance)	Human endometrial cancer (Wang et al. 2007); human pancreatic cancer cell (Jager et al. 2004);
Glibenclamide	$C_{23}H_{28}N_3ClO_5S$		K_{ATP}	Hepatocarcinoma cells (Zhou et al. 2003); Colon cancer cell (Abdul and Hoosein 2002)

Table 1 (continued)

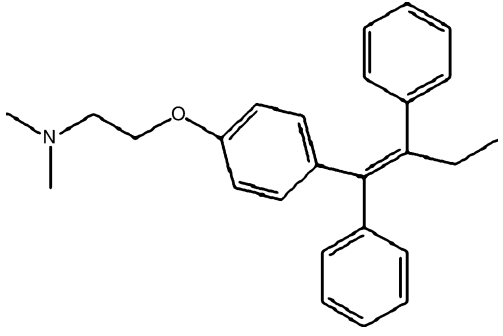
Compound name	Formula	Chemical structure	Target ion channel	Target tumor cell
K^+ Channel inhibitors				Inhibition of growth
Tamoxifen	$C_{26}H_{29}NO$		Non-specific	Neuroblastoma (Rouzaire-Dubois and Dubois 1990)

Table 1 (continued)

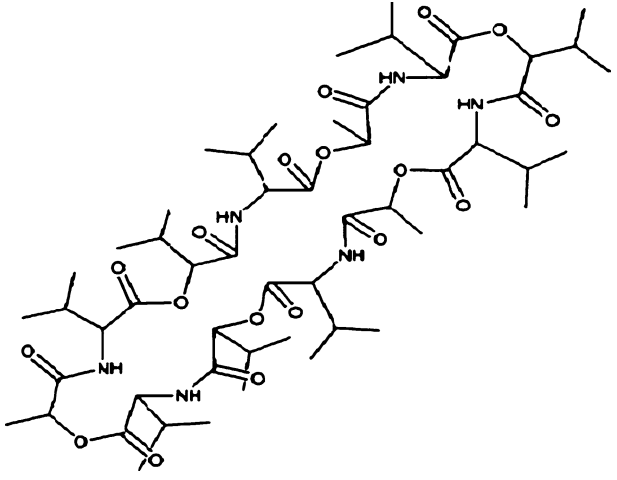
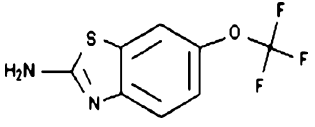
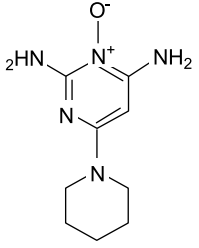
Compound name	Formula	Chemical structure	Target ion channel	Target tumor cell
K^+ Channel activators				Promotion of growth
Valinomycin	$C_{54}H_{90}N_6O_{18}$		K^+ channel ionophore	A variety of tumor cell growth (Rouzaire-Dubois et al. 1993)
Riluzole	$C_8H_5F_3N_2OS$		SK4 (inter-mediate conductance)	Colonic carcinoma (Spitzner et al. 2007)

Table 1 (continued)

Compound name	Formula	Chemical structure	Target ion channel	Target tumor Cell
K^+ Channel activators				Promotion of growth
Minoxidil	$C_9H_{15}N_5O$		K_{ATP} activator	Human breast cancer cell (Abdul et al. 2003)

(Note that this is not an exhaustive collection of all related studies and the purpose of this table is merely to indicate the types of K^+ channels involved in cancer cell growth and the types of chemical structures that are able to alter the growth of various types of cancer cells by changing K^+ channel activities)

and also indicate the possibility of K^+ channels as targets for anticancer therapy. However, the compounds are generally limited by their lack of K^+ channel specificity. Some K^+ channel inhibitors require high doses to show effects on proliferation, which might produce effects on unrelated proteins. Additionally, even if we accept that the effects of such agents are specific on K^+ channels, there are very few available specific inhibitors for a certain K^+ channel type, with the exception of some toxins.

2. In a given cancer cell line, there might be multiple distinct K^+ channels; therefore, a vast variety of chemical structures acting on different K^+ channels may affect proliferation in that given cell line. For example, TPeA (non-specific), quinidine (non-specific), chromanol 293B (KCNQ1), dequalinium (Kv), glibenclamide (K_{ATP}), amiodarone (HERG), terfenadine (HERG), etc., can all inhibit the proliferation of human colonic carcinoma cells.
3. On the other hand, a given K^+ channel may well express in multiple cancer cells of distinct histological origins; therefore even a relatively specific K^+ channel blocker might inhibit proliferation of several distinct cells. For instance, cisapride can inhibit proliferation of breast cancer cells, neuroblastoma cells, gastric cancer cells, etc., simply because HERG is ubiquitously expressed in human tissues.
4. Even though there is a general lack of cell type-restricted expression of K^+ channels and of specificities of K^+ channel blockers, different tumor cells demonstrated different sensitivities to growth modulation by the K^+ channel blockers. In T84 human colonic carcinoma, cells numerous K^+ channels are expressed, but only voltage-gated K^+ (Kv) channels seemed to influence proliferation, since Kv channel blockers 4-aminopyridine, tetrapentylammonium and verapamil produce similar inhibitory effects on cell proliferation, but iberiotoxin, a selective inhibitor of BK_{Ca} channels, and glibenclamide, a potent inhibitor of K_{ATP} channels, had no effect (Spitzner et al. 2007). In SW1116, LoVo, Colo320DM and LS174t human colon cancer cell lines, dequalinium and amiodarone caused marked growth-inhibition while tetraethylammonium and 4-aminopyridine did not have significant growth-suppressive effects. In contrast, in another colon cancer cell line DLD-1, 4-aminopyridine, tetrapentylammonium, tetraethylammonium, verapamil and diltiazem inhibited cell proliferation, whereas charybdotoxin, iberiotoxin and glibenclamide did not have any effects (Yao and Kwan 1999). These experiments suggested a critical role of voltage-gated K^+ channels in the proliferation of colon cancer cells. In the human breast cancer cell line MCF-7, however, both Kv channels and K_{ATP} channels are involved in growth regulation and K_{Ca} channels seem to play minimal role, as evidenced by the fact that quinidine, glibenclamide, linogliride (K_{ATP} channel inhibitor), 4-aminopyridine, and tetraethylammonium produced a concentration-dependent inhibition of cell proliferation, with no evidence of cytotoxicity following a 3-day or 5-day ex-

posure to drug, while charybdotoxin (large-conductance BK_{Ca} inhibitor), iberiotoxin (BK_{Ca} inhibitor), margatoxin (Kv1.3 channel inhibitor), and apamin (small-conductance SK_{Ca} inhibitor) at supra-maximal channel blocking concentrations had no effect on MCF-7 cell proliferation, viability, or cell cycle distribution (Woodfork et al. 1995; Klimatcheva et al. 1999; Wonderlin et al. 1995).

3.2 Knocking Down the Expression of K^+ Channels

Promotion of cancer cell proliferation by K^+ channels critically depends on up-regulation of expression of the encoding genes. As aforementioned, in cancerous cells multiple K^+ channels are overexpressed, resulting in enhanced K^+ channel activities favoring cell growth. This process also opens up an opportunity for interfering with cancer progression by interfering with expression of K^+ channels genes and proteins. While the classical small-molecular-weight pharmaceutical agents currently available have limited channel specificities, the strategy of knocking-down expression of K^+ channels provides a surrogate for the problem. In particular, small molecule nucleic acids or short stretch nucleotides have demonstrated tremendous potential as therapeutic agents.

Decoy Oligodeoxynucleotide (dODN) Technology. The dODN technology involves synthetic double-stranded ODN containing a *cis* element with high affinity for a target transcription factor (TF) but with low affinity for non-target TFs, which can bind the TF after being introduced into target cells and attenuate authentic *cis–trans* interaction, leading to removal of *trans* factors from the endogenous *cis* element with subsequent modulation of gene expression (Gao et al. 2006; Bielinska et al. 1990; Morishita et al. 1995, 1997, 2001). Intriguingly, the transcription of K^+ channel genes characterized thus far appears to be exclusively driven by oncogenes/oncoproteins. We have found that stimulating protein-1 (Sp1) is the transactivator for most of the human K^+ channel genes including HERG (Lin et al. 2007), KCNQ1 (Luo et al. 2007), KCNE1 (Luo et al. 2007), HCN2 (unpublished observation), HCN4 (unpublished observation), Kv4.2 (unpublished observation) and Kv4.3 (unpublished observation). We also identified E2F as the transactivator for EAG (unpublished observation). These findings rationally recommend K^+ channel genes as targets for dODNs. Indeed, we have used Sp1-dODN to successfully down-regulate HERG and KCNQ1 expression (Lin et al. 2007; Luo et al. 2007).

Small Interfering RNA (siRNA) Technology. siRNA, a class of 20–25 nucleotide-long RNA molecules, is used in RNA silencing, a method that allows one to "knock down" expression of genes or more specifically to degrade the target mRNAs, in a sequence-specific fashion (Chakraborty 2007; Kumar

and Clarke 2007; Masiero et al. 2007). siRNAs are designed and synthesized to have complete complementarity to the target mRNAs. Although RNA silencing occurs naturally to protect organisms from aberrant transcription, it is now being exploited to silence genes implicated in diseases and to determine the functions of various genes. This strategy has been employed by several laboratories to successfully knock-down the gene expression of distinct K^+ channels. Its application to Ca^{2+}-activated K^+ channels and HERG has also been reported (Table 2).

Antisense Oligodeoxynucleotide (ODN) Technology. Synthetic antisense ODN fragments (17–30 bp) hybridize to the coding regions of target mRNA transcripts by full base-pairing and prevent the mRNAs from being translated into a protein (Prakash and Bhat 2007; Flaherty et al. 2001; Pawlak et al. 2000). When the genetic sequence of a particular gene is known to be causative of a particular disease, it is possible to synthesize ODN that will bind to the mRNA produced by that gene, effectively turning that gene "off". This technique has been used by Pardo et al. (1999) to successfully downregulate EAG levels and decrease the proliferation of several tumor cell lines (Table 2).

microRNA (miRNA) Mimic Technology. miRNAs are endogenous ~22-nt non-coding RNAs that anneal to inexactly complementary sequences in the 3′UTRs of target mRNAs of protein-coding genes to specify translational repression or/and mRNA cleavage. MiRNAs are an abundant RNA species both in terms of their sheer number in the genome (>1% of the predicted human genes, which regulates ~10% of genes) and in terms of their expression levels (some miRNAs >1000 copies per cell); therefore, miRNAs likely have widespread functions in the cells (Alvarez-Garcia and Miska 2005; Ambros

Table 2 Nucleic acid approaches tested for their ability to inhibit tumor cell proliferation and/or migration

Technology name	Target gene	Tested cells	Refs.
siRNA	SK4 (intermediate-conductance K_{Ca})	Endometrial cancer	Wang et al. 2007
siRNA	SK3 (small-conductance K_{Ca})	Metastasizing mammary cancer cell	Potier et al. 2006
siRNA	*EAG*	Human colonic carcinoma cells	Spitzner et al. 2007
siRNA	*EAG*	Human breast cancer cells	Borowiec et al. 2007
siRNA	*HERG*	Breast cancer cells	Wang et al. 2002
Antisense ODN	*EAG*	Breast cancer cells, carcinoma of the cervix, neuroblastoma, colonic carcinoma	Pardo et al. 1999

2004). Recent evidence has suggested that miRNAs might be viable therapeutic targets for a wide range of human disease (Alvarez-Garcia and Miska 2005; Yang et al. 2007). However, each miRNA has the potential to regulate hundreds or even thousands of mRNAs (Miranda et al. 2006), which renders actions of miRNAs non-gene specific and their applications as therapeutic targets are therefore limited. This consideration prompted us to explore the possibility of developing strategies to render miRNA actions gene-specific. We therefore tested an artificial miRNA designed based on the sequence of the EAG gene, which can presumably mimic the endogenous miRNAs to produce silencing of EAG expression, but in a gene-specific manner (Xiao et al. 2007). We reasoned that two conditions must be satisfied in order to generate such a miRNA mimic: (1) the artificial miRNA must be complementary

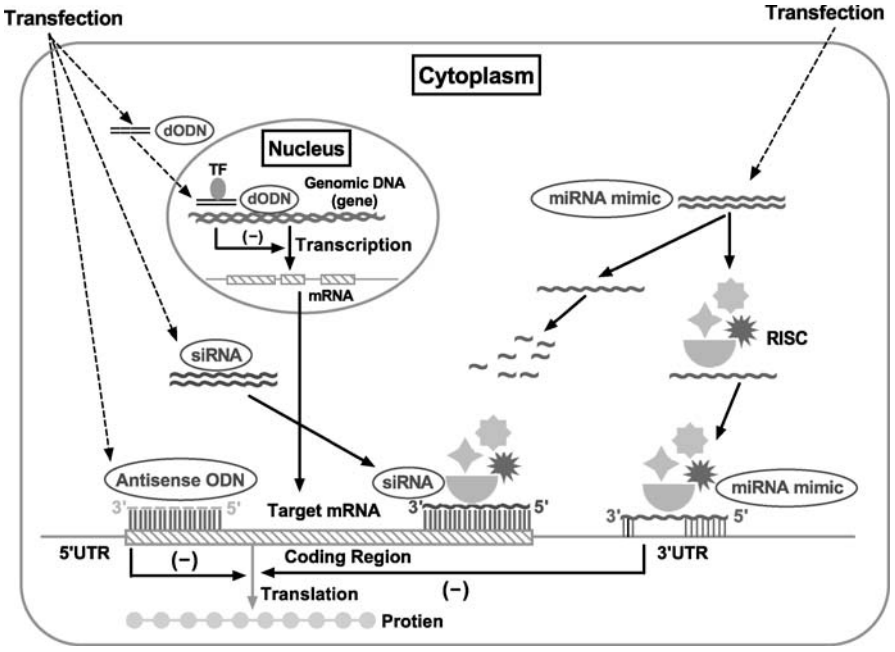


Fig. 3 Schematic illustration of the mechanisms of nucleic acid therapy or gene-silencing techniques. dODN (decoy oligodeoxynucleotides) is delivered into the nucleus by transfection or virus-mediated infection, where it binds transcription factor (TF) and prevents TF from binding the target genomic DNA resulting in transcription inhibition. Antisense ODN, siRNA and miRNA mimic are delivered into the cytoplasm by transfection or virus-mediated infection. Antisense ODN is usually designed to completely base-pair with the coding region of the target mRNA so to block the protein translation process; siRNA can be designed to fully base-pair with any regions of the target mRNA to cause degradation of mRNA; and miRNA mimic is designed to partially base-pair with the region within the 3′UTR (3′-untranslated region) of the target mRNA to cause sessation of translation and probably also degradation of the mRNA. ((–): inhibition)

only to the 3′UTR of the target gene to elicit miRNA action, i.e. repressing EAG at the post-transcriptional level with minimal effects on the mRNA level, and (2) the 3′UTR of the target gene must contain a unique sequence distinct from other genes to elicit gene-specific action (Fig. 3). Following the rules, we first identified a stretch of sequence in the 3′UTR unique to the *EAG* gene that is expectedly long enough for miRNA action. On the basis of the unique sequence we designed a 22-nt miRNA mimic that at the 5′ end has eight nucleotides (nucleotides 2–8), and at the 3′end has seven nucleotides, complementary to the *EAG* sequence. This design is expected to yield the strongest action of a miRNA, according to the work reported by Lewis et al. (2003) and by Brennecke et al. (2005). Our pilot study indeed supported our hypothesis; the miRNA mimic decreased EAG protein level in colon cancer cells and caused significant cell death (unpublished observations).

4 Concluding Remarks

On the basis of our current knowledge, it is conceivable that K^+ channels are viable diagnostic biomarkers and therapeutic targets for human cancers. Nonetheless, it should be emphasized that there is still a long way to go towards obtaining solid proof for this hypothesis. There are many unanswered questions and unsolved problems, and answering these questions will shape the future directions of the research field. Not until the key issues will have been resolved, will the clinical implications of K^+ channels in cancer therapy be a reality.

1. **Specificities of actions of K^+ channel inhibitors.** This concern involves at least four issues. (**a**) Multiple K^+ channels may co-exist in a cell and we do not know in each single cell type which of the K^+ channels plays the major role in cancer progression. Inhibition of all these K^+ channels might produce the best anticancer effect, but such an action may well elicit many unwanted effects. (**b**) A single K^+ channel subtype may express in multiple cell types. An inhibitor of this K^+ channel, when administrated systematically, can affect all of these cells. Therefore, drug delivery to the target site becomes critical in this situation. (**c**) K^+ channels in general have multiple cellular functions, including controlling membrane potential, cardiac repolarization, Ca^{2+} entry and osmolarity, regulating proliferation and apoptosis, etc. Inhibition of K^+ channels can affect all these aspects and induce unwanted effects. For example, K^+ channel blockers, in particular HERG blockers, may be effective against cancer progression, but they can meanwhile cause drug-induced long QT syndrome due to excessive slowing of cardiac repolarization (Fermini and Fossa 2003). (**d**) Most, if not all, of

the K^+ channel blockers currently available are not specific towards a particular K^+ channel. Because of the substantial similarities among different categories of K^+ channels in terms of their protein structures particularly the amino acid sequences lining the pore or the pathway for K^+ ions to pass through the membrane, it is hard, if not impossible, to generate real specific agents. Nevertheless, with respect to cancer therapy, it is unclear whether a drug should target a single particular K^+ channel or co-existing multiple K^+ channels to yield optimal effects. Ideally, the K^+ channels which do not express or express at low abundance in healthy cells but are up-regulated in transformed cells would be the best candidates as a target and a biomarker for cancer diagnosis and treatment. For example, the EAG K^+ channel is normally expressed only in brain. But when cells are transformed to be cancerous, EAG appears in many cells of various histological origins. The HERG K^+ channel has a similar pattern of expression; it expresses in a variety of tumor cells, but is present at a very low level in the healthy cells from the same histological origins.

2. **To verify that the K^+ channels expressed in tumor cell lines are also present in primary cancerous tissues.** To date, a vast majority of published studies concerning K^+ channels in tumor cells are from the experimental data with cell lines. Whether these K^+ channels are also present in cells from the primary cancerous tissues remains yet to be rigorously verified. Although a few reports indeed have demonstrated expression or overexpression or enhanced activities of K^+ channels in real tumors, more convincing data are needed.
3. **To examine the efficacy of K^+ channel blockers and nucleic acids in tumor growth in vivo or in animal models.** Many K^+ channels known to express in tumor cells have been biophysically characterized, but have not yet been explored in terms of their potential pathophysiological function. In particular, studies in animal models to investigate the role of K^+ channels in carcinogenic process and the efficacy of the K^+ channel blockers, that have been documented to inhibit tumor cell growth in vitro, have been surprisingly sparse despite the increasing enthusiasm for a role of K^+ channels in tumorigenesis. Our current lack of adequate knowledge of the exact functions of K^+ channels and tumor-suppressing efficacy of K^+ channel blockers in animal models hinders the applications of K^+ channels as a therapeutic target for cancers.
4. **To delineate the precise mechanisms underlying regulation of tumor cell proliferation by K^+ channels** (Fig. 4). Most of the studies available to date in the literature regarding the regulation of tumor cell proliferation and apoptosis by K^+ currents have been largely observational and superficial. The mechanistic link between K^+ currents and tumor cell proliferation is still missing. Without knowing the precise mechanisms underlying regulation of tumor cell proliferation by K^+ channels, it is hard, if not impossible, to apply our findings to clinical practice.

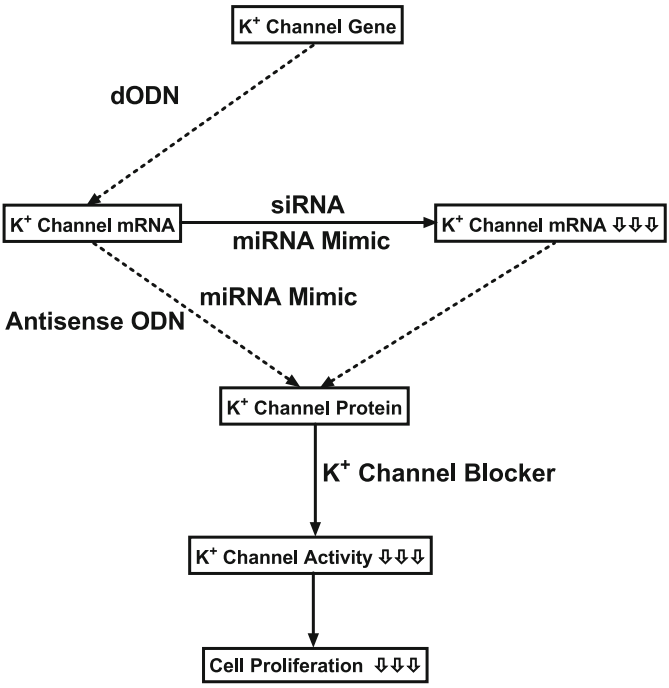


Fig. 4 Schematic illustration of the potential target sites to reduce K^+ channel activity leading to decreases in tumor cell proliferation. ⇓ indicate reduction; *dashed arrows* indicate negative regulation

In summary, great efforts have been made over the past 25 years to understand the role of K^+ channels/currents in tumor cell growth, and, having realized the importance, interest in this field from researchers worldwide is steadily increasing. We have begun to touch on the core issues or the mechanisms by which K^+ channels regulate cancer progression. But we should be aware that our current knowledge in this regard is still rather poor and we are still far away from being able to apply our limited knowledge to the clinical setting.

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Pharmacological Rescue of Mutant CFTR Function for the Treatment of Cystic Fibrosis

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Abstract The vast majority of morbidity and mortality in cystic fibrosis (CF) patients is due to lung disease caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR). CFTR is a PKA-regulated anion channel localized in the apical membrane of bronchial epithelia where it controls salt and fluid regulation to facilitate bacteria clearance. There are over 1000 disease-causing mutations in the gene encoding CFTR and, depending on the type of mutation, the cell surface density and/or functional activity of CFTR in the apical membrane is reduced. A therapeutic strategy for the treatment of lung disease in CF patients is to use pharmacological agents that increase mutant CFTR-mediated anion secretion. High-throughput screening strategies have iden-

tified multiple chemotypes that increase mutant CFTR-mediated anion secretion. These chemotypes can be grouped into two classes based on their mode of action. The first class is known as CFTR correctors because they correct the processing and trafficking of CFTR to increases its cell surface density. The second class is known as CFTR potentiators as they potentiate the amount of anion secretion through CFTR at the cell surface. In vivo analysis of CFTR activity in CF patients indicates that it is correlated with the severity of lung disease and supports the hypothesis that CFTR modulators that restore mutant CFTR activity to >10% of wild-type-CFTR would improve lung function. The use of high-throughput screening and medicinal chemistry optimization to improve the efficacy, potency, and pharmaceutical properties of the multiple potentiator or corrector scaffolds identified to date offers a promising approach for the treatment of CF by directly targeting the root cause of the disease.

Keywords Corrector · DF508-CFTR · Genetic disease · High-throughput screening · Misfolded protein · Potentiator

1 Introduction

Cystic fibrosis (CF) is a genetic disease affecting 1 in 3300 births in the United States [1]. The root cause of CF disease is mutations in the gene encoding the CF transmembrane conductance regulator (CFTR), an epithelial anion channel that is opened by cyclic AMP-dependent protein kinase A (PKA; Fig. 1) [2–6]. Over 1500 mutations in the CFTR gene have been identified in CF patients [7]. Depending on the mutation, there is a decrease in the amount of CFTR at the cell surface, its ability to pass anions, or both [7–11]. The decrease in anion flow through mutant-CFTR impairs salt and water transport across the epithelia lining of multiple organs [12–16]. In the lung, this leads to dehydration of the airway surface, decreased cilia movement, and accumulation of thick sticky mucus that obstructs the airways and prevents the clearance of infection causing bacteria [17]. The chronic bacterial infection and corresponding inflammation cause bronchiectasis and eventually cardiorespiratory failure in most CF patients [18].

Pulmonary treatment strategies are limited to symptomatic treatments and aimed at improving lung function and reducing pulmonary exacerbations by clearing the airways of the thick sticky mucus and reducing infection-causing bacteria (Fig. 1) [19]. To loosen and get rid of the mucus, the majority of CF patients undergo daily chest physiotherapy and administration of inhaled bronchodilators, mucolytics, and hypertonic saline. Antibiotics are used to fight infection-causing bacteria and anti-inflammatory medications are used to decrease the inflammatory response of the body to the bacterial infections. Symptomatic pulmonary disease treatments in development include restoring salt and water transport by targeting ion channels other than CFTR expressed in epithelia, including epithelial Na^+ channels and Ca^+-activated Cl^- channels. Although maintenance of lung function is

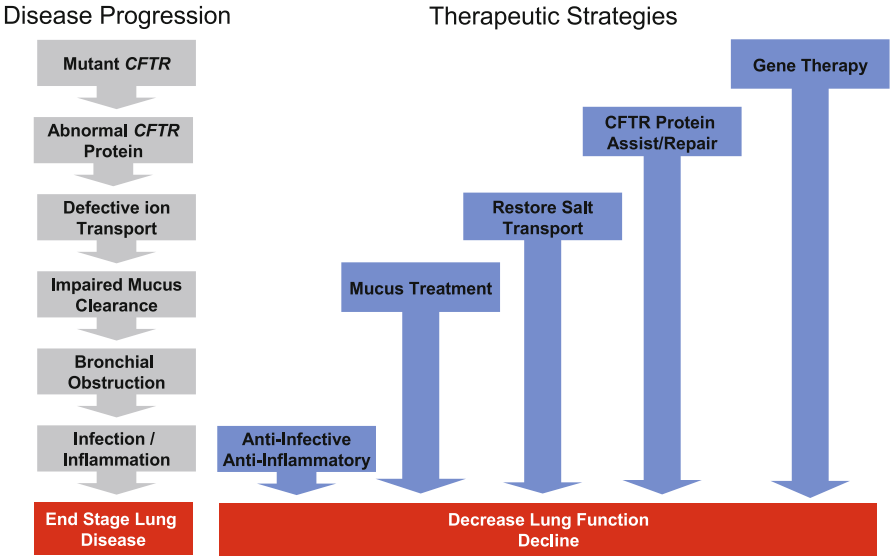


Fig. 1 Therapeutic treatment strategies targeting different points along the CF pathogenesis cascade. Mutations in the gene encoding the epithelial anion channel, CFTR, impair its function leading to a cascade of deleterious events that result in progressive lung disease and other organ failures. Symptomatic treatment strategies are aimed at restoring salt transport, reducing mucus plugging, or preventing recurring infections and inflammation. Treatment strategies aimed at the core defect in CF include the use of pharmacological agents to increase mutant CFTR function or replacement of the defective *CFTR* gene using gene therapy

the primary concern, symptomatic treatments help control many of the other clinical manifestations of CF disease. For example, malabsorption due to pancreatic insufficiency is treated with pancreatic enzyme supplements leading to improved nutritional status.

Despite improvements in the symptomatic treatment of CF disease that have greatly increased the life span of CF patients over the last 25 years, most patients do not survive beyond their mid 30's [18]. In addition, existing treatment strategies require hours of daily treatment and multiple medications that cause significant interruptions to daily life. For these reasons, drug discovery efforts are aimed at identifying pharmacological agents to restore the underlying defect of CF disease, mutant CFTR function.

2 Molecular Consequence of Mutations in the CFTR Gene

CFTR is a member of the ATP-binding cassette (ABC) superfamily and is localized in the apical membrane of epithelial cells [20]. Like other ABC pro-

teins, CFTR is composed of two sets of six membrane spanning domains (TMD1 and TMD2) and two cytoplasmic nucleotide binding domains (NBD1 and NBD2) and is thought to function as a monomer. In addition, it contains a regulatory "R" domain with multiple PKA and protein kinase C (PKC) phosphorylation sites (Fig. 2A; [2, 21]). Following phosphorylation by the cAMP/PKA signaling pathway, CFTR is gated by the binding and hydrolysis of ATP. The open channel passes Cl^- and bicarbonate to regulate fluid transport across epithelia. The amount of anion flow is determined by the number of channels at the cell surface (density), the amount of time the channel remains open (open probability), and its ability to pass anions (single-channel conductance) and is defined by Eq. 1:

$$\text{Total CFTR current} = \text{density} \times \text{open probability} \times \text{conductance} . \quad (1)$$

The number of channels synthesized, processed, and trafficked to the apical membrane and rate of CFTR internalization at the apical membrane determines the cell surface density. Mutations in CFTR that alter one or more of these parameters decrease total CFTR-mediated anion flux and impair epithelial cell function.

Mutations in the *cftr* gene have been grouped into five classes based on their molecular consequences (Table 1) [9, 18, 22]. Class I, II, and V mutations decrease the cell surface density of CFTR due to complete lack of CFTR protein synthesis (Class I), impaired processing and trafficking to the membrane (Class II), or reduced synthesis of otherwise normal protein (Class V). Class III and IV mutations cause CFTR to open less frequently (impaired gating) or reduce its ability to pass Cl^- (altered conductance), but do not decrease the cell surface density of CFTR. In general, the reduction in CFTR-mediated anion secretion is more pronounced for Class I, II, and III mutations compared to Class IV and V mutations. Accordingly, patients with Class IV and V mutations generally have less severe CF disease [18]. Within each class, however, the amount of residual mutant CFTR activity and disease severity can vary and may be influenced by genetic modifiers and environmental factors [1, 18, 23].

Some mutations alter one or more properties of CFTR to impair its function. This is best exemplified by the in-frame deletion of three nucleotides resulting in the loss of phenylalanine at position 508 (ΔF508) in NBD1 (ΔF508-CFTR) [2, 10, 11]. Close to 90% of CF patients carry at least one copy of the ΔF508-CFTR allele and ~55% of these carry two copies of the mutant gene [7]. The phenylalanine deletion is thought to prevent the proper folding of NBD1 and domain–domain assembly of the multi-domain CFTR protein [24, 25]. Because of this, the endoplasmic reticulum (ER) quality control machinery recognizes the protein as defective and targets it for ubiquitin-mediated degradation by the proteasome [26, 27]. Consequently, less than 1% of the total ΔF508-CFTR protein synthesized exits the ER and traffics to the cell surface (Fig. 2B). The small amount of defective CFTR protein

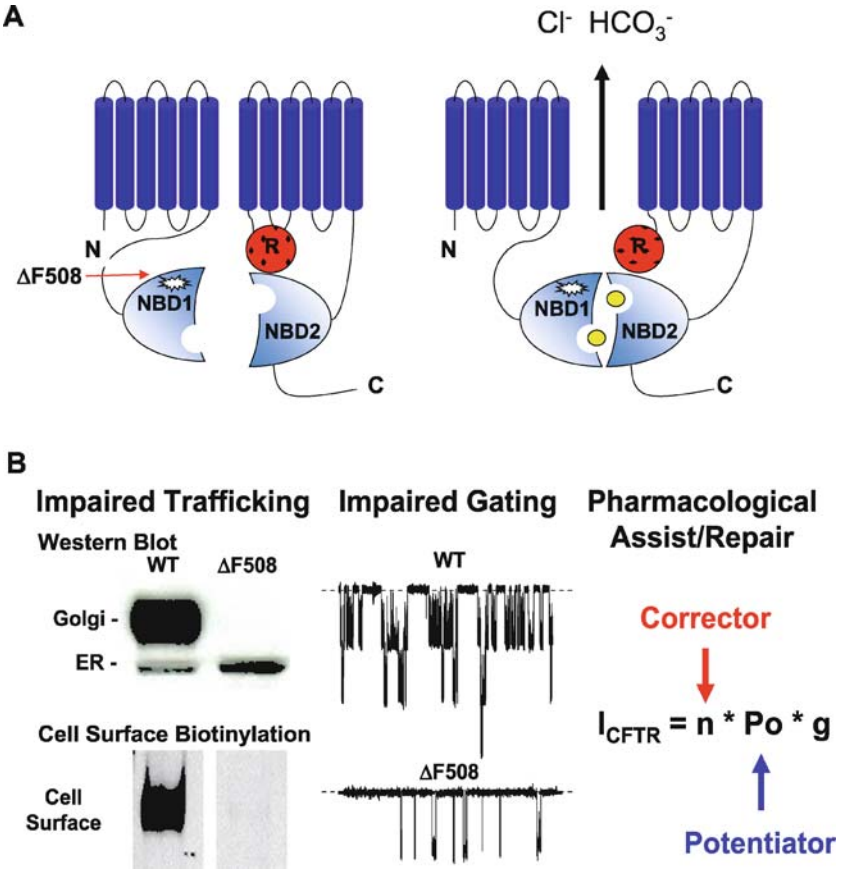
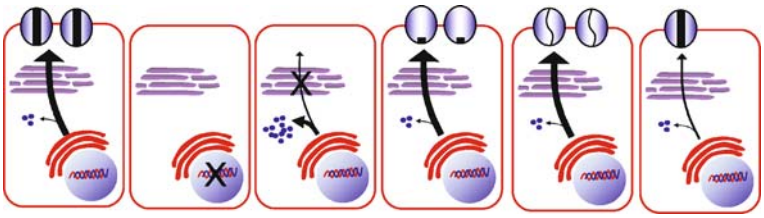


Fig. 2 Pharmacological agents called correctors and potentiators target the core defects caused by mutations in the gene encoding CFTR. **A** Schematic of ΔF508-CFTR in the closed (*left panel*) and open (*right panel*) channel state. The phenylalanine deletion in ΔF508-CFTR is located near the interface between the first transmembrane domain and NBD1 (*star*). **B** *Left panel*, a hallmark of a corrector is its ability to increase the amount of extensively glycosylated (150–170 kDA) CFTR in Western Blot assays. This form is also known as mature CFTR (so-called band C) and is indicative of its passage through the Golgi network from where it is trafficked to the cell surface. Core glycosylated CFTR is located in the ER and is known as immature CFTR (so-called band B). Cell surface biotinylation can be used to directly monitor the cell surface density of CFTR. *Middle panel*, single-channel recording techniques are used to directly monitor CFTR gating activity. A representative excised patch recording of the gating activity for wild-type- and ΔF508-CFTR in the presence of 75 nM PKA and 1 mM ATP on the cytoplasmic side is shown. The closed state is indicated by the *dashed line*. *Right panel*, the total CFTR-mediated current (I_{CFTR}) is determined by the cell surface channel density (n), the open probability of the channel (P_o), and the single-channel conductance (g). A corrector is aimed at increasing n, whereas a potentiator is aimed at increasing P_o. An increase in one or both of these parameters is expected to increase I_{CFTR}

Table 1 CFTR mutant classes and their molecular consequence



Class	Wt	I	II	III	IV	V
Molecular defect		No synthesis	Reduced processing	Reduced gating	Altered conductance	Reduced synthesis
Prevalence (%)		9	55	4	2	2
Type of mutations		Nonsense frameshift	Missense amino acid deletion	Missense amino acid change	Missense amino acid change	Missense amino acid change, alternative spicing
Common genotypes		G542X W1282X	ΔF508 N1303K	G551D	R117H	A445E 2789+5G→A
Desease severity		Severe	Severe	Severe	Moderate–mild	Moderate–mild
Potential therapy		PTC-124	Corrector potentiator	Potentiator	Potentiator	Potentiator

that reaches the cell surface undergoes rapid internalization and lysosomal degradation [28], further reducing the cell surface density of ΔF508-CFTR. ΔF508-CFTR also exhibits reduced gating activity compared to wild-type CFTR (Fig. 2B) [10]. Together, the reduced cell surface density and gating activity markedly reduce CFTR-mediated anion secretion.

3 Pharmacological Approaches to Restore Mutant CFTR Function

Because mutant CFTR is the core defect in CF, it is expected that pharmacological agents designed to increase CFTR function will prevent or slow disease progression (Fig. 1). There are two pharmacological approaches to increase mutant CFTR function. The first is to increase the cell surface density of mutant CFTR. This class of compounds is known as CFTR correctors (Fig. 2B). The second approach is to increase the open probability of mutant CFTR at the cell surface. This class of compounds is known as CFTR potentiators (Fig. 2B). Depending on the molecular consequence of the mutation

and disease severity, potentiators and correctors may be co-administered to maximize clinical efficacy or therapeutic window, if needed.

3.1 CFTR Correctors

CFTR correctors are pharmacological agents that increase the cell surface density of CFTR by correcting the defective protein synthesis, conformation, or cellular processing and trafficking of mutant CFTR. Correctors that promote the proper protein conformation may also rescue other known defects associated with mutant CFTR, including increased internalization and impaired gating activity of channels at the cell surface. Because of the presumed effect on protein folding or trafficking of mutant CFTR, these compounds are also referred to as pharmacological or chemical chaperones. The main target of correctors is the Class II mutation, ΔF508-CFTR, which is the predominate CF-causing mutation. An increase in the maturation and cell surface density of functional ΔF508-CFTR was first observed in response to low-temperature incubation and later by pharmacological and chemical chaperones such as 4-phenylbutyrate (4-PB) and glycerol [29–31]. These early studies provided the rationale for identifying pharmacological agents to increase the cell surface density of functional ΔF508-CFTR.

3.2 CFTR Potentiators

CFTR potentiators act directly on CFTR to potentiate its open probability in the presence of endogenous cAMP agonists. Unlike pharmacological activators of channel function, the activity of CFTR potentiators requires prior channel phosphorylation by the cAMP/PKA-signaling pathway. This maintains normal physiological control over CFTR function while allowing the potentiator to increase its activity when needed. A prerequisite for potentiators to act is that CFTR is expressed at the cell surface. Because of this, potentiators are expected to be most effective against class III and IV mutants, which decrease CFTR gating or conductance but not the cell surface density. Potentiators may also act on other mutant classes that result in low to moderate levels of CFTR in the apical membrane or be co-administered along with CFTR correctors.

4 HTS Assays to Identify Potentiators and Correctors of Mutant CFTR

To facilitate identification and optimization of novel, drug-like correctors and potentiators of ΔF508-CFTR, high-throughput screening (HTS) strate-

gies have been used in both academic and industrial settings [32–34]. These strategies employed the use of fluorescent-probes to monitor CFTR function in cell-based assays.

4.1 Fluorescent-Probes to Monitor CFTR Function

Several fluorescent-based corrector and potentiator HTS assays have been developed to identify chemical starting points from small molecule libraries and track the structure–activity relationship (SAR). These include the use of membrane potential-sensitive fluorescent probes and halide sensors, including genetically targeted fluorescent proteins [35, 36]. These assay formats are amenable to automation and the use of high-density plate formats to enable large-scale screening campaigns for the purposes of hit identification. The high-throughput capabilities, sensitivity, and reproducibility of these assay formats also facilitate SAR evaluation.

Membrane potential-sensitive fluorescent probes offer a sensitive and convenient approach to indirectly monitor anion flux through CFTR in a variety of different cells types, including recombinant cells and primary cell cultures. Changes in membrane potential due to anion flux through CFTR are monitored by measuring the change in fluorescence resonance energy transfer (FRET) between a membrane soluble voltage-sensitive dye [bis-(1,2-dibutylbarbituic acid)] and a plasma membrane bound fluorescent, coumarin-linked phospholipid (CC2-DMPE). The change in membrane potential causes the negatively charged bis-(1,2-dibutylbarbituic acid) to redistribute across the plasma membrane and the amount of energy transfer from the plasma membrane bound phospholipid changes accordingly [35]. The use of FRET allows emission ratio detection that reduces experimental error due to well-to-well or plate-to-plate differences in cell density and dye-loading compared to single intensity indicators.

Another assay approach is to directly monitor CFTR-mediated Cl^- flux using genetically encoded anion-sensitive fluorescent probes, such as yellow fluorescent protein (YFP) [34, 36]. YFP is a mutant form of green fluorescent protein that is rapidly quenched by anion binding at physiological pH. Because YFP quenching is more sensitive to I^- than Cl^-, I^- is often used to increase the signal intensity and minimize the potential impact of anion co-transporters, which are relatively poor transporters of I^-. Unlike fluorescent-based membrane potential-sensitive probes, YFP-based assays are a readout of anion flux and do not require extensive processing and handling prior to monitoring channel activity. Some challenges with the use of YFP-based assays include the generation of cell lines that stably or transiently express YFP and the target of interest at the desired levels, changes in cytosolic pH that alter YFP fluorescence, and YFP quenching due to basal or non-specific anion transport.

4.2
Fluorescent-Based Assay Formats to Identify CFTR Potentiators and Correctors

High-throughput screening assays to identify CFTR potentiators have been developed using FRET- and YFP-based assay formats (Fig. 3A). Cell lines expressing wild-type-, ΔF508-, or G551D-CFTR can be used. When considering which mutant CFTR to use it is important to note that the potency of potentiators can be altered by the location of the defect in CFTR [34]. In the case of ΔF508-CFTR, it is necessary to first incubate the cells at a low temperature (27 °C) for 16 hours to increase the cell surface density of ΔF508-CFTR. Test compounds are added a few seconds prior to activating CFTR with forskolin and monitoring the fluorescent response in a fluorescent plate reader and liquid handler (Fig. 3C). The dynamic range of the assay can be optimized by increasing the driving force for Cl^- efflux by reducing extracellular Cl^-. To reduce baseline drift and maintain an effective assay window, it is important to select cell lines with minimal basal anion flux through unstimulated CFTR, as well as other anion channels or co-transporters. Because activators of the cAMP-signaling pathway can be detected under these assay conditions, these screening hits must be removed using assays designed to identify adenylate cyclase activators or phophodiesterase inhibitors. To confirm that the putative potentiator hits increase the open probability of CFTR, single-channel recording techniques are used (Fig. 3D; left Panel).

A similar HTS assay format can be used to identify correctors of ΔF508-CFTR with the following three exceptions (Fig. 3B). First, the cells are maintained at 37 °C to prevent temperature correction of ΔF508-CFTR. Second, test compounds are added for 16 hours and subsequently removed by repeated washing prior to recording CFTR activity. Third, a CFTR potentiator is added along with forskolin to increase the assay window. Like the potentiator HTS assay, putative corrector hits that activate cAMP signaling must be eliminated. In addition, incomplete washout of the test compound can cause false positives due to potentiator activity. To confirm that the putative corrector hits increase the trafficking or cell surface density of mutant CFTR, Western blot or cell surface biotinylation techniques can be used (Fig. 3D; right panel).

4.3
Limitations of Fluorescent-Based Flux or Membrane Potential Assays

Fluorescent-based flux or membrane potential assays provide superior throughput compared to traditional electrophysiology-based screening technologies. For fluorescent-based membrane potential assays, however, the response to increasing channel activity can be non-linear due to the inherent non-linear relationship between channel activity and membrane potential (Fig. 4A). This prevents an accurate readout of efficacy and reduces the reso-

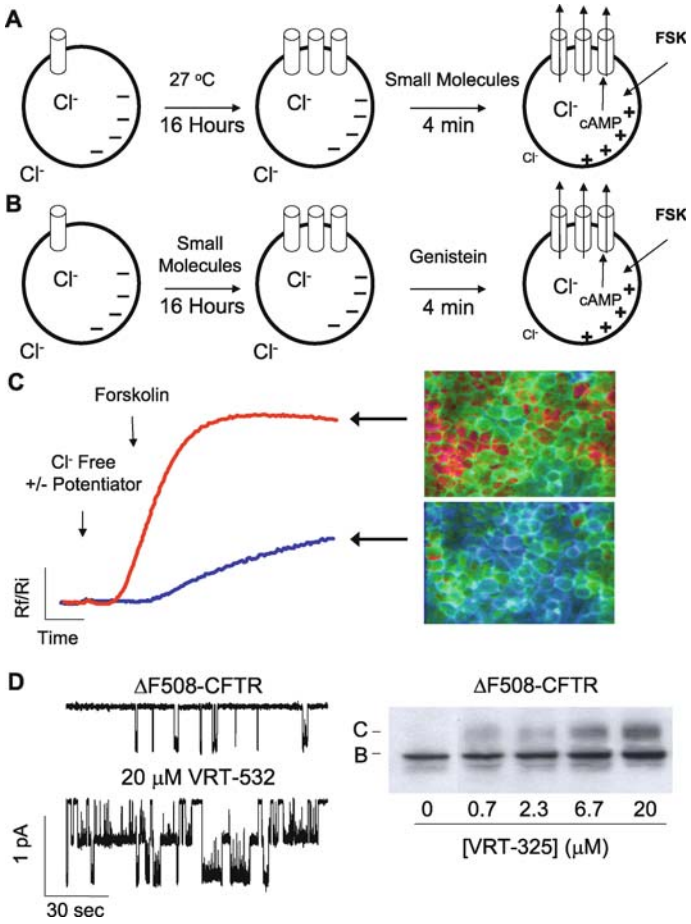


Fig. 3 HTS assay format for identification of CFTR potentiator and correctors. Schematic of the cell-based potentiator (**A**) or corrector (**B**) HTS assay formats based on voltage-sensitive fluorescent probes. For the corrector HTS assay, the cells were incubated at 37 °C in the presence of test compound for 16 hours. The test compound was removed by repeated washing prior to activating CFTR activity with forskolin in the presence of Cl^--free media and genistein. For the potentiator HTS assay, the cells were incubated at 27 °C for 16 hours to temperature correct the cell surface density of ΔF508-CFTR. Test compounds from the screening library were added in place of genistein and were present during the forskolin stimulation. **C** Membrane potential response (R_F/R_I) to forskolin in the presence (*red line*) and absence (*blue line*) of a CFTR potentiator in temperature corrected NIH-3T3 cells. **D** *Left panel*, single-channel activity of ΔF508-CFTR in temperature corrected NIH-3T3 cells before and during the application of the CFTR potentiator, VRT-532. *Right panel*, glycosylation pattern of ΔF508-CFTR in HEK-293 cells treated for 16 hours with 0–20 μM of the CFTR corrector, VRT-325

lution of the SAR (Fig. 4B). The dynamic range for efficacy can be improved by measuring the rate of membrane potential change rather than the absolute magnitude. To do this, the temporal response of the dye must be sufficiently fast to accurately track the membrane potential. For this reason, FRET-based dyes with response times <50 ms are superior to redistribution dyes with much slower kinetics. Alternatively, electrophysiological techniques could be used as ion flux or membrane potential is under voltage- or current-clamp control, providing a more linear readout with respect to CFTR function (Fig. 4A). Although this is an advantage over fluorescent-based assays, as there is greater resolution of compound efficacy (Fig. 4B), throughput is markedly lower. The expression levels of the channel of interest can also impact the apparent potency in fluorescent-based assays due to the non-linear relationship between channel activity and membrane potential [37]. For agonist assays, increasing channel densities will progressively shift the half-maximal response (EC_{50}) to lower compound concentrations. Therefore, control of channel expression is important to accurately report and maintain consistency in the potency of channel agonists.

5
Chemotypes Identified by HTS for CFTR Correctors and Potentiators

5.1
CFTR Correctors

High-throughput screening of chemically diverse and known drug libraries in academic and industrial settings identified a number of small molecule correctors of ΔF508-CFTR (Table 2). The overall hit rate of these screens was relatively low (0.04–0.1%) resulting in few chemical starting points. In addition, many of the hits identified in the optical HTS assays do not confirm in subsequent electrophysiological assays using immortalized or primary epithelia expressing ΔF508-CFTR. This may be due to lower expression of CFTR in native cells or higher sensitivity of the optical assay formats compared to biochemical or electrophysiological assays formats.

Quinazolinones represent one of the most efficacious corrector scaffolds identified to date [32]. Parallel synthesis efforts around the hit, VRT-422 (compound 1), led to minor improvements in potency but significantly increased the efficacy (total amount of Cl^- secretion at any given concentration). The key to the improved efficacy was the replacement of the 3-alkylquinazolinone group with a 4-alkoxyquinazoline moiety. As a result, compounds like VRT-325 (compound 2) were prepared. In the Tic diamide series, compound 3a was identified from a HTS and subsequent analog synthesis resulted in a ~80 fold improvement in potency (compound 3b) with no changes in efficacy [38]. Finally, bisaminomethylbithiazoles, like Corr-4a

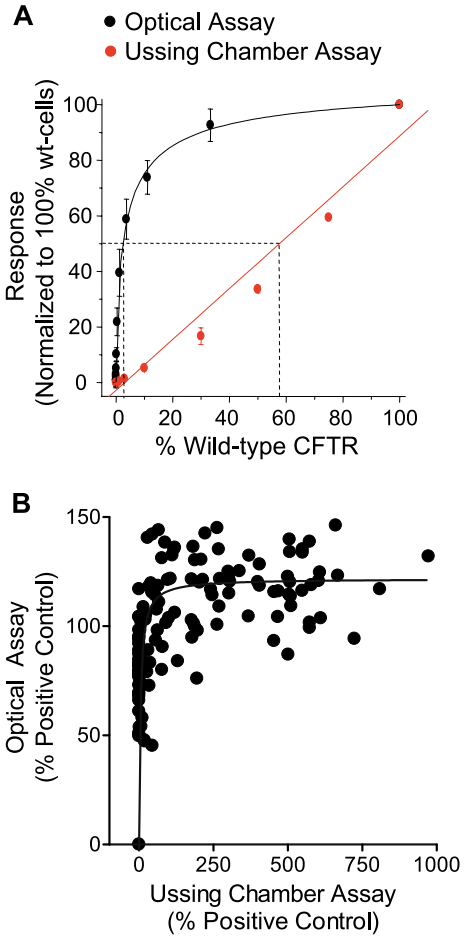
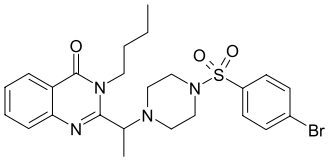
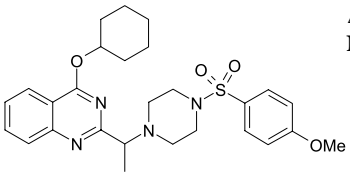
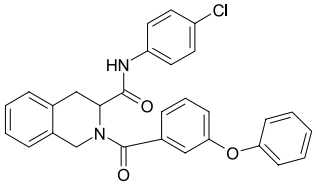
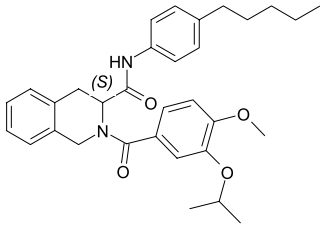
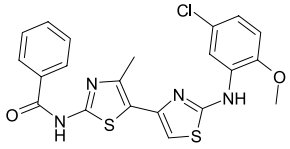


Fig. 4 The fluorescence-based membrane potential HTS optical assay format does not accurately report corrector efficacy. **A** The range in efficacy for the optical and Ussing chamber assay was assessed by monitoring the forskolin response in the presence of increasing amounts of wild-type CFTR expressing cells. Wild-type-CFTR-expressing cells were mixed with ΔF508-CFTR-expressing cells at the indicated proportions (% wild-type CFTR). The forskolin response was monitored in the optical assay (*black circles*) using fluorescent-based probes and in the Ussing chamber assay (*red circles*) under voltage-clamp control. The response in both assays was normalized to the response using 100% wild-type CFTR-expressing cells. In the fluorescent-based assay, the half maximal response was observed at ~3% wild-type CFTR and was nonlinear as the concentration of wild-type CFTR expressing cells was increased. In contrast, the half-maximal response in the Ussing chamber assay was reached at ~60% wild-type CFTR and was linear. **B** Correlation between the optical and Ussing chamber corrector assay formats for the ΔF-508-CFTR-mediated response. The response in each assay format was normalized to the same positive control. The maximum efficacy was limited in the optical assay compared to the Ussing chamber assay. As a result, the Ussing chamber assay allows better resolution of corrector efficacy to drive SAR evaluation

Table 2 Corrector chemotypes

Compound	Structure	Cell line[a]	Assay format	EC_{50} (μM)/ efficacy
VRT-422 (1)		ΔF508-CFTR-NIH-3T3	FRET-based	~4 μM/ ~47% 27 °C
VRT-325 (2)		ΔF508-CFTR-NIH-3T3	FRET-based	~3 μM/ ~74% 27 °C
Corr-3a (3a)		ΔF508-CFTR-FRT	YFP-based	~3 μM/ ~108% 27 °C
Corr-3b (3b)		ΔF508-CFTR-FRT	YFP-based	~12 μM/ ~142% 27 °C
Corr-4a (4)		ΔF508-CFTR-FRT	YFP-based	~3 μM/ ~175% 27 °C

[a] The potency of potentiators was assessed using wild-type-, G551D-, or ΔF508-CFTR expressed in fisher rat thyroid (FRT) cells or mouse fibroblast (NIH-3T3) cells

(compound 4), are potent (<1 μM) and efficacious correctors of ΔF508-CFTR cell surface density and function [33].

PTC124 was identified after solid-phase combinatorial optimization of a screening hit from an 800 000 compound screening library [39]. Because the compound induces ribosomal read-through of premature termination codons

it can be potentially used to correct the cell surface density of Class I CFTR mutations such as G542X and W1282X. PTC124 is currently in Phase 2 clinical trials.

5.2 CFTR Potentiators

High-throughput screening of diverse chemical libraries, analogs of known potentiator scaffolds, and known drugs led to the identification of multiple chemical scaffolds that potentiate CFTR. These include; flavones and isoflavones, benzo[*c*]quinoliziniums, tetrahydrobenzothiophenes, phenylglycines, sulfamoyl-4-oxoquinoline-3-carboxamides, pyrazoles, 1,4-dihydropyridines, and 6-phenypyrrolo[2,3-*b*]pyrazines (Table 3). The greater diversity and number of potentiator hits is reflective of the higher hit rate (0.2–0.6%) from HTS compared to that observed for CFTR correctors. The potency of some potentiator scaffolds is influenced by the class of the CFTR mutation, suggesting that the binding site(s) may be affected by the location of the specific CFTR defect or the conformational structure of the protein. Consequently, the SAR trends may be genotype specific. Lead optimization of a number of potentiator scaffolds has led to improved potency against CFTR and revealed SAR trends in each scaffold.

Flavones and isoflavones-type CFTR potentiators, such as genistein (compound 5) and apigenin (compound 6), potentiate wild-type- and mutant-CFTR in multiple different cell types (Table 3). Several publications describe lead optimization efforts using flavone-like compounds as starting points. Springsteel et al. identified UCCF-029 (compound 7) as a CFTR potentiator with improved potency compared to genistein [40]. Systematic modifications of compound 7 led to UCCF-339 (compound 8), a potent flavonoid potentiator of wild-type-CFTR. This effort revealed the following SAR trends; (1) benzanulation at the 7,8-positions of the flavone ring significantly improved wild-type-CFTR activity and (2) the pyridyl B-ring was less influential for activity. These analogs, however, were inactive against G551D-CFTR, the most common gating mutation known to cause CF.

Benzo[*c*]quinolizinium derivatives, like MPB-07 (compound 9), are reported to be potent potentiators of CFTR [41]. The effort started with the discovery that MPB-07 (compound 9) at concentrations >100 μM potentiated wild-type-CFTR, but not G551D-CFTR. SAR evaluation revealed that the hydroxyl at position 6 and the ammonium salt and a chlorine atom at positions 7 or 10 are essential for CFTR potentiation. In addition, addition of an alkyl chain at the 5 position, as in MPB-104 (compound 10), improved the potency ~100 fold compared to MPB-07. These modifications also resulted in activity against G551D-CFTR, which was not observed for MPB-07.

Evaluation of the SAR trends for tetrahydrobenzothiophene derivatives, like compound 11, revealed that a variety of aromatic amides were tolerated

Table 3 Potentiator chemotypes

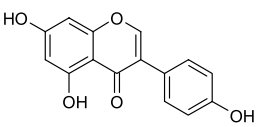
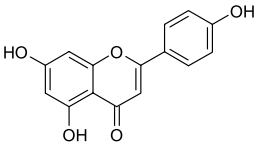
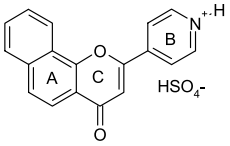
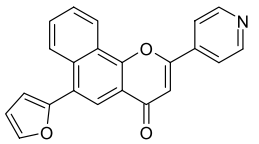
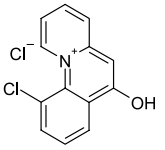
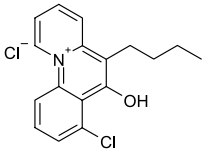
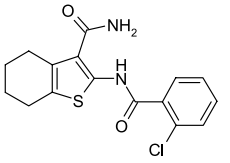
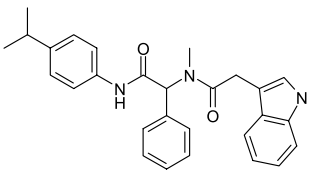
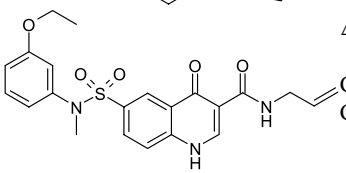
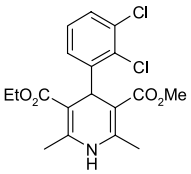
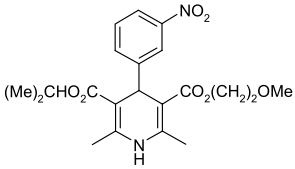
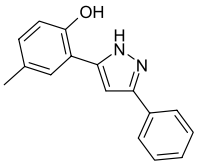
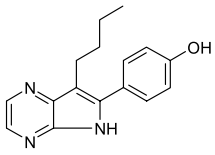
Compound	Structure	Cell line [a,b]	Assay format	Activity reported
Genistein (**5**)		ΔF508-CFTR-FRT	YFP-based	EC_{50} = 24 μM
				EC_{50} = 19 μM
		ΔF508-CFTR-NIH-3T3	FRET-based	EC_{50} ~ 10–20 μM [c]
				EC_{50} ~ 50–150 μM [c]
		ΔF508-CFTR-FRT	Short-circuit	EC_{50} ~ 10 μM [c]
		G551D-CFTR-FRT	Short-circuit	
		Wild-type-CFTR-FRT	Short-circuit	
Apigenin (**6**)		Wild-type-CFTR-Calu-3	Short-circuit	EC_{50} = 9 μM
UC_{CF}-029 (**7**)		Wild-type-CFTR-FRT	YFP-based	EC50 = 10 μM
				Inactive
		G551D-CFTR-FRT	YFP-based	
UC_{CF}-339 (**8**)		Wild-type-CFTR-FRT	YFP-based	EC_{50} = 1.7 μM
MPB-07 (**9**)		Wild-type-CFTR-CHO	^{125}I efflux	EC_{50} = 141 μM
				Inactive
		G551D-CFTR-CHO	^{125}I efflux	
MPB-104 (**10**)		Wild-type-CFTR-CHO	^{125}I efflux	EC_{50} = 1.7 μM
				EC_{50} = 0.75 μM
		G551D-CFTR-CHO	^{125}I efflux	
ΔF508act-02 (**11**)		ΔF508-FRT	YFP-based	EC_{50} = 0.18 μM
		G551D- FRT	Short-circuit	Not active at 10 μM
		Wild-type-FRT	Short-circuit	EC_{50} = 1.3 μM

Table 3 (continued)

Compound	Structure	Cell line [a,b]	Assay format	Activity reported
PG-01 (12)		ΔF508-FRT	YFP-based	EC_{50} = 0.3 μM
			Short-circuit	EC_{50} = 0.3 μM
		G551D-FRT	Short-circuit	EC_{50} = 1.0 μM
		G1349D-FRT	Short-circuit	EC_{50} = 0.04 μM
SF-01 (13)		ΔF508-FRT	YFP-based	EC_{50} = 0.3 μM
			Short-circuit	EC_{50} = 0.1 μM
		G551D-FRT	Short-circuit	Inactive
		G1349D-FRT	Short-circuit	Inactive
Felodipine (14)		ΔF508-CFTR-FRT	YFP-based	EC_{50} = 0.9 μM
				EC_{50} = 23 μM
		G551D-CFTR-FRT	YFP-based	
Nimodipine (15)		ΔF508-CFTR-FRT	YFP-based	EC_{50} = 0.7 μM
				EC_{50} = 36 μM
		G551D-CFTR-FRT	YFP-based	
VRT-532 (16)		ΔF508-CFTR-NIH-3T3	FRET-based	EC_{50} = 2.4 μM
				EC_{50} = 3.8 μM
				EC_{50} = 20 μM
		ΔF508-CFTR-FRT	Short-circuit	
		G551D-CFTR-FRT	Short-circuit	
RP107 (17)		ΔF508-CFTR-CF15	^{125}I efflux	EC_{50} = 0.111 μM
				EC_{50} = 0.001 μM
		G551D-CFTR-CHO	^{125}I efflux	EC_{50} = 0.152 μM
		Wild-type-CFTR-CHO	^{125}I efflux	

[a] In most cases, the activity of potentiators on ΔF508-CFTR was assessed using cells incubated at low-temperatures to increase the cell surface density of ΔF508-CFTR. [b] The potency of potentiators was assessed using wild-type-, G551D-, or ΔF508-CFTR expressed in fisher rat thyroid (FRT) cells, Chinese hamster ovary (CHO) cells, human airway epithelial (CF15) cells, a human airway cell line of adenocarcinoma origin (Calu-3), or mouse fibroblast (NIH-3T3) cells. [c] Data obtained from multiple publications

at the 2-position, while the 4,5-fused tetrahydro-benzothiophene with a fused 6- or 7-membered aliphatic ring and the unsubstituted carboxamide in position 3 were critical for activity [42]. Like other compounds, compound 11 did not potentiate G551D-CFTR.

Phenylglycines, such as compound 12, are potent potentiators of G1349D-, G551D, ΔF508-CFTR [43]. SAR trends indicated that preferred moieties were a *para* alkylphenyl group (Me, iPr) at R1 and a hydrogen group at R2. Cell-attached patch-clamp experiments in FRT cells indicated that the addition of compound 12 restored the defective gating of the mutant CFTR to wild-type-CFTR levels.

Another structural class of CFTR potentiators identified by HTS is the sulfamoyl-4-oxoquinoline-3-carboxamides (compound 13; [43, 44]). Compound 13 (SF-01) has been shown to selectively potentiate the gating of ΔF508-CFTR and is ~500 fold more potent than genistein. However, it was less effective than genistein at potentiating wild-type-CFTR and did not potentiate G551D- or G1349D-CFTR. SAR analysis revealed that the sulfonamide at position 6 and the primary amide at position 3 are critical for activity. In addition, substituted alkyl, aryl, and benzyl amines are preferred on the amide and sulfonamide groups.

Dihydropyridine analogs are widely used as hypertensive agents through their ability to block the L-type calcium channel [45]. Screening of drug libraries identified dihydropyridine analogs, like felodipine (compound 14) and nimodipine (compound 15), that appear to act directly on CFTR to potentiate its activity with an improved potency compared to genistein. Felodipine and nimodipine were more active against ΔF508-CFTR and wild-type-CFTR compared to G551D-CFTR.

High-throughput screening of compound libraries identified 2,5-diaryl pyrazoles derivatives like VRT-532 (compound 16) as CFTR potentiators with an EC_{50} against ΔF508-CFTR of 2–4 μM [32]. Single-channel patch-clamp experiments were used to study the effects of VRT-532 on single channels expressed in a small patch of membrane excised from the cell. Because the second messenger pathways are no longer present under these conditions, it was necessary to add PKA and ATP to the cytoplasmic surface of the channel to activate ΔF508-CFTR. Under these conditions, VRT-532 restored the gating of the ΔF508-CFTR to wild-type-CFTR levels by increasing the open burst duration. Also, VRT-532 does not inhibit phosphodiesterases or activate adenylate cyclase to increase cAMP signaling. Taken together, these results indicate that VRT-532 acts directly on the channel to restore the defective gating of ΔF508-CFTR. VRT-532 also potentiated the defective gating of G551D-CFTR but with ~5 fold lower affinity (EC_{50}= 20 μM).

6-Phenyl pyrrolo[2,3-*b*]pyrazine derivatives, previously described as CDK and GSK-3 inhibitors, also potentiate CFTR [46]. The potentiator activity appears to be modulated by substituents in positions 2 and 3 of the ring. At position 2, the best potency was obtained with a 4-hydroxyphenyl group

(4-OHPh>4-ClPh≫4-OMePh). Linear alkyl chains were evaluated in position 3 and the best activity was obtained with a butyl. This led to the identification of RP107 (compound 17), which is a submicromolar potentiator of ΔF508-, G551D-, and wild-type-CFTR expressed in recombinant cells.

6
Mechanism of CFTR Potentiator and Corrector Action

6.1
Mechanism of Action for CFTR Correctors

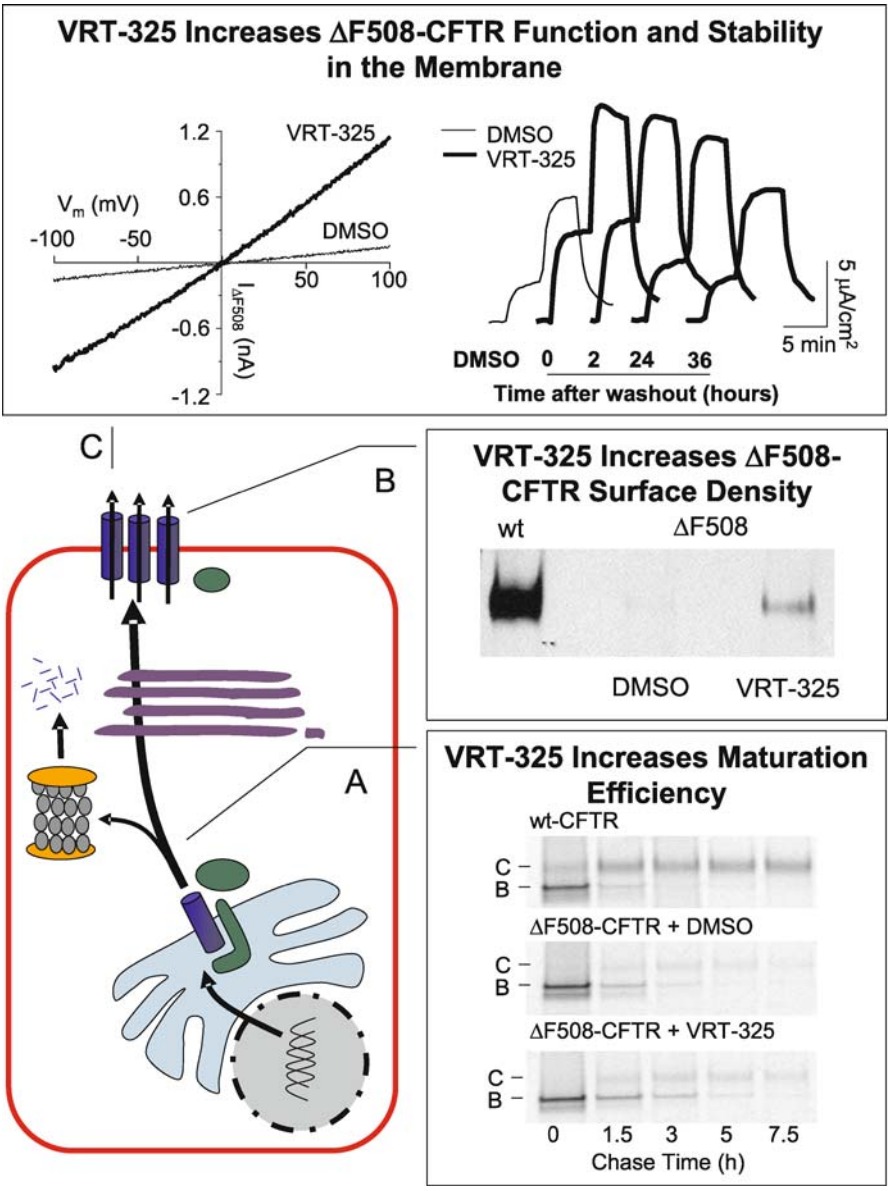
Because phenotypic HTS strategies have been used to identify CFTR correctors, elucidation of the mechanism of action and identification of the molecular target requires extensive biological characterization. These efforts could potentially benefit medicinal chemistry optimization by allowing development of target or structure-based screens to more directly assess the SAR. In addition, understanding the molecular target or pathway could provide insight into the potential for adverse effects in vivo.

The mechanistic data on the quinazolinone corrector, VRT-325, suggest a model whereby the compounds act primarily or initially in the ER to promote the proper folding and ER export of ΔF508-CFTR (Fig. 5) [32]. This is supported by the improved packing and orientation of the transmembrane domains [47], increase in maturation efficiency, and lack of effects on CFTR transcription or proteasome-mediated degradation [32]. Once ΔF508-CFTR exits the ER, it undergoes further glycosylation in the Golgi and is transported to the cell surface plasma membrane. In addition to facilitating ΔF508-CFTR maturation and trafficking to the membrane, VRT-325 in-

Fig. 5 VRT-325 restores the cell surface density and function of ΔF508-CFTR. **A** Pulse-chase analysis indicates that VRT-325 increases the maturation efficiency of ΔF508-CFTR. This leads to an increase in the cell surface density of ΔF508-CFTR as indicated by cell surface biotinylation analysis (**B**). In addition to increasing the trafficking and cell surface density of ΔF508-CFTR, VRT-325 increases its residence time at the cell surface (**C**; *right panel*). The residence time of ΔF508-CFTR at the cell surface was determined by first incubating epithelial cell cultures for 96 h with 6.7 μM VRT-325. CFTR activity was then monitored from 10 min to 72 h after compound removal. Representative forskolin- and potentiator-stimulated I_{SC} in uncorrected (DMSO) and 0, 2, 24, and 36 h after removal of 6.7 μM VRT-325 are shown. Forskolin was added first to activate CFTR followed by addition of VRT-532 to potentiate CFTR. The increase in ΔF508-CFTR trafficking to the membrane and residence time at the cell surface increases the total CFTR-mediated Cl^- current (**C**; *left panel*). The increase in total CFTR-mediated Cl^- current was measured using whole-cell voltage-clamp recording conditions in NIH3T3 cells expressing ΔF508-CFTR following pretreatment with DMSO (*thin line*) or 6.7 μM VRT-325 (*thick line*) for 16-hours ▶

creases the amount of time ΔF508-CFTR remains at the cell surface. Together, the increase in trafficking and cell surface stability increase the steady-state cell surface density of functional CFTR leading to enhanced Cl^- secretion.

The increased maturation efficiency and cell surface density of ΔF508-CFTR in the presence of VRT-325 and other correctors scaffolds represents



~10% or less of that observed for wild-type CFTR [32]. This suggests that the correctors act on a small fraction of the available ER pool of ΔF508-CFTR to promote its exit from the ER. The ubiquitin proteasome pathway rapidly degrades the remaining ΔF508-CFTR protein. The low efficacy does not appear to be due to lack of available ΔF508-CFTR protein, as the effect of VRT-325 is additive to other known CFTR correctors [48]. This also suggests that different chemical classes act on distinct molecular targets or pathways to correct ΔF508-CFTR. Although medicinal chemistry optimization can moderately increase the efficacy of correctors, it is not known if correction to wild-type-CFTR levels is possible using pharmacological modulation of a single mechanism or target.

6.2 Potential Molecular Targets for CFTR Correctors

There are a number of potential molecular targets for CFTR correctors that could promote the normal folding and/or maturation of mutant-CFTR, including increasing the synthesis of mutant protein, direct binding to the channel to stabilize the native protein conformation, modulating molecular chaperones involved in the folding or degradation of the protein, inhibiting the ubiquitin proteasome degradation pathway, and decreasing CFTR turnover at the membrane. The intention here is not to provide an exhaustive list of the potential molecular targets that CFTR correctors could act upon to increase the cell surface density of CFTR, but to highlight a couple of well-studied examples. For a more complete review of the molecular targets involved in the processing, trafficking, and degradation of CFTR see [49–52].

Early approaches were aimed at increasing ΔF508-CFTR expression using butyrate and sodium 4-phenylbutyrate (4-PB), a histone deacetylase inhibitor approved for use in patients with urea cycle disorders [30]. It was believed that increasing ΔF508-CFTR expression would allow more ΔF508-CFTR to escape the ER quality control pathway and traffic to the cell surface. Although low milimolar concentrations of 4-PB failed to increase ΔF508-CFTR transcription, the maturation and cell surface density of ΔF508-CFTR was increased. Subsequent studies demonstrated that 4-PB down-regulates the 70-kDa heat shock cognate protein (Hsc70) [53]. Hsc70 with the aid of its co-chaperone, Hdj-2 (Hsp 40), form a transient complex with NBD1 to facilitate domain assembly of CFTR [54, 55]. The association of Hsc70 with ΔF508-CFTR has a longer half-life than that of the wild-type CFTR-Hsc70 complex, suggesting that Hsc70 retains ΔF508-CFTR in the ER and targets it for degradation by the ubiquitin-proteasome pathway [56]. Down-regulation of Hsc70 by 4-PB is believed to reduce ΔF508-CFTR-Hsc70 complex formation, allowing ΔF508-CFTR to exit the ER and traffic to the membrane. Deoxyspergualine, a spermidinyl, α-hydroxyglycyl, 7-guanidinoheptanoly peptidomimetic that binds Hsc70 to prevent its interaction with cellular proteins, also in-

creases maturation of ΔF508-CFTR in expression systems [57]. These results indicate that pharmacological modulation of interactions between CFTR and molecular chaperones, like Hsc70, can increase ΔF508-CFTR trafficking to the apical membrane. In addition to Hsc70, multidimensional protein identification technology identified 30 chaperones and co-chaperones for CFTR, many of which preferentially bind to ΔF508-CFTR [49]. Which of these chaperones and co-chaperones of CFTR can be targeted by small molecules to increase ΔF508-CFTR maturation remains to be determined.

Another potential therapeutic strategy to correct ΔF508-CFTR is to prevent the degradation of the misfolded protein by the ubiquitin proteasome degradation pathway. Degradation of proteins by this pathway occurs in multiple steps. First, the protein is recognized as misfolded by quality control checkpoints in the ER. Second, the misfolded protein is conjugated with multi-ubiquitin molecules, which tag the molecule for degradation. Third, the ubiquitin-tagged protein is transferred to the cytoplasmic 26 S proteasome where it is unfolded and degraded. The proteasome inhibitor Velcade™, which is approved for use in patients with refractory multiple myeloma, increases ΔF508-CFTR maturation in some cells [58]. Small molecules that modulate molecular chaperones involved in the recognition of misfolded proteins (Derlin-1, Hsc70-Chip) or their ubiquitination, deubiquitination (Usp4), or transport to the proteasome (p97/valosin-containing protein) may also rescue ΔF508-CFTR [59, 60].

A more direct and potentially selective strategy to correct ΔF508-CFTR is to identify small molecules that bind to CFTR to facilitate its folding and trafficking to the membrane. Ligand binding to misfolded proteins, such as ΔTyr490-P-glycoprotein [61], D148A-Vasopressin V1a receptor [62], and G601S-human ether-a-go-go (hERG) K^+ channels [63] is believed to stabilize a more native protein conformation to facilitate ER export. Ligand binding to CFTR may also modulate its interaction with other proteins involved in the folding or degradation of mutant CFTR. For example, benzo(c)quinolizinium drugs are believed to selectively bind to the cytoplasmic domains of ΔF508-CFTR to mask a proteolytic cleavage site involved in its degradation [64].

6.3
Potential Sites of Action for CFTR Potentiators Identified by HTS

The opening and closing of the CFTR channel is believed to be regulated by cycles of ATP binding and hydrolysis at two sites located in the interface between NBD1 and NBD2 [65]. The ATP binding site 1 is formed by the Walker A and B motifs of NBD1 and the LSGGQ motif of NBD2 and exhibits slow turnover. The ATP binding site 2 is formed by the Walker A and B motifs of NBD2 and the LSGGQ motif of NBD1 and is catalytically more active than site 1. ATP binding causes the dimerization of the NBD1–NBD2 to open the channel, whereas ATP hydrolysis closes the channel [65]. Accord-

ingly, mutations in the CFTR *gene* that alter the ATP binding sites profoundly decrease channel gating. These include the class III CF-causing mutations in which glycine is changed to aspartic acid at codon 551 (G551D) located in the LSGGQ motif of NBD1 and G1349D, which affects the equivalent residue in NBD2 [66–68]. In addition to altering the gating activity of CFTR, these mutations decrease the binding affinity of CFTR potentiators ([34, 68]; Table 3). This led to the hypothesis that CFTR potentiators bind at the interface between the NBD1 and NBD2 to stabilize the dimer, thereby increasing channel opening. This is supported by homology model building studies of the NBD1–NBD2 dimer that suggests that there is at least one binding pocket located at the dimer interface [67].

In addition to increasing channel gating, most potentiators identified to date decrease channel gating at high concentrations [32, 69]. This results in the characteristic "bell-shaped" curve of CFTR potentiators. The inhibition of CFTR gating by potentiators is believed to be due to a low-affinity binding site in the NBD2 domain, as mutations in this domain prevent channel inhibition. Some potentiators may also bind with low affinity in the pore of the CFTR channel to block anion permeation. The presence of two binding sites suggests that medicinal chemistry optimization may improve the high affinity binding site without affecting the low affinity site to maximize the window between the EC_{50} and IC_{50}. Whether the affinity of potentiators to the two bindings sites can be independently optimized remains to be demonstrated.

7
How Much Repair of CFTR Function is Necessary?

A fundamental question in CF drug discovery is how much mutant CFTR repair is required to ameliorate severe lung disease. The clinical spectrum of CFTR-related diseases includes classical CF characterized by severe or mild lung disease and the clinical presentation of obstructive azoospermia secondary to congenital bilateral absence of the vas deferens (CBAVD) or chronic pancreatitis in the absence of lung disease. Several key indicators associated with the severity of lung disease have been identified. These include the patient genotype, the age at diagnosis, the age at death or transplant, and the incidence of pancreatic, sweat gland, intestinal, liver, and reproductive dysfunction (Table 4). In addition, treatment strategies, genetic modifiers, and environment can impact pulmonary disease severity. The aim of a corrector therapy is to increase ΔF508-CFTR activity enough to decrease the rate of lung function decline in patients with severe lung disease to levels observed in patients with mild to no lung disease.

To estimate how much mutant-CFTR-function must be increased to ameliorate severe lung disease, mutant-CFTR-mediated Cl^- secretion was measured in subjects with different clinical phenotypes [70–86]. These include

CF-subjects with mild or severe lung disease and CBAVD subjects with one or two CFTR mutations (Fig. 6). To measure mutant CFTR function in vivo, nasal potential difference (NPD) recording techniques are used. This in vivo electrophysiology assay measures ion transport across the nasal membrane of human subjects. CFTR activity is isolated by addition of Cl^- free media and β-adrenergic agonists to increase cAMP signaling. The non-CF subjects respond with a robust increase in wild-type CFTR activity. In contrast, the response in subjects with no, mild and severe lung disease was $68 \pm 9\%$ (44–88%), $9 \pm 6\%$ (0–33%), and <1% of that observed for wild-type CFTR, respectively (Fig. 6; Table 4).

Another approach to determine the amount of CFTR activity that could be beneficial is to measure the amount of wild-type CFTR mRNA in patients with missense mutations and mutations in introns that regulate mRNA splicing (Table 5) [87–93]. In these patients, a small amount of wild-type CFTR mRNA is produced. Patients with 4–10% wild-type CFTR mRNA typically exhibit a more moderate decline in lung function, whereas patients with >30% wild-type CFTR mRNA have mild to no lung disease.

Taken together, these studies suggest that a pharmacological agent producing ~10% wild-type CFTR activity may decrease the rate of lung function decline in severe patients to levels observed in patients with a more moderate decline in lung function, which may result in decreased morbidity and mortality.

8 Use of Primary Disease Bronchial Epithelia to Characterize CFTR Modulators

To estimate the potential clinical efficacy of CFTR modulators and bridge the gap between monitoring CFTR activity in engineered cell lines and in vivo, cultures of differentiated human bronchial epithelial (HBE) cells isolated from the bronchi of CF patients have been used [32]. Primary cultures of HBE, grown as monolayers and in the presence of an air–liquid interface on permeable supports, differentiate into polarized, ciliated cells that transport ions and fluid. Many characteristic features associated with lung disease in vivo are observed in differentiated HBE cultures. For example, HBE isolated from CF subjects homozygous for ΔF508-CFTR exhibit reduced CFTR-mediated Cl^- flux, impaired fluid regulation, and reduced cilia beating compared to non-CF HBE [17, 32].

To monitor CFTR activity in HBE cultures, Ussing chamber recording techniques are used (Fig. 7). Like the nasal potential difference recording technique used in vivo, the Ussing chamber technique is an extracellular recording of the membrane potential or short circuit current (I_{SC}) due to ion flux through channels and transporters. To isolate CFTR activity, a basolateral to apical Cl^- gradient is established and amiloride is added to block the ep-

Table 4 Spectrum of CF disease severity and key indicators

Severity of lung disease	None	Mild	Severe
Genotypes (ΔF508/X)	R117H-7T	A455E 3849+10kbC→T 3272-26A→G 2789+5G→A	ΔF508 G551D 1811+1.6kbA→G G542X N1303K
Age at diagnosis	Adulthood	Adolescence	Infant
Age at death/transplant; mean (range)	> 50	38 (28–48)	24 (8–40)
Pancreatic insufficient	0%[a]	50%	100%
Sweat [Cl^-] mmol/L mean; (range)	54 (22–100)	81 (28–116)	103 (65–158)
Other clinical presentations	CBAVD Pancreatitis Sinusitis	CBAVD	CBAVD Meconium Ileus Diabetes, Cirrhosis
Lung FXN (FEV) decline/year; mean (range)	ND[b]	– 0.9% (0.8–1.1)	– 3% (2.3–3.6)
% wild type-CFTR activity	68 (44–90)	9 (0–33)	< 1 (0–15)
surface density (% wild type-CFTR mRNA)	33	7 (4–11)	4
% Patient population	1–2[c]	10–20[d]	80–90

[a] Congenital bilateral absence of the vas deferens

[b] Although a longitudinal assessment of FEV_1 in CBAVD patients has not been done, these patients typically have normal FEV_1 scores. However, occasional mild respiratory disorders have been reported in some patients. These patients have one or two CFTR mutations and typically have normal sweat Cl^- levels and FEV_1 scores

[c] Accounts for 1–2% of male infertility

[d] Based on the patient population with mild mutations, pancreatic sufficiency, and residual CFTR activity

ithelial Na^+ channel (ENaC) induced currents. In the presence of amiloride, forskolin or β-agonists are added to the apical surface to stimulate cAMP signaling and activate CFTR. To evaluate the potential clinical efficacy of the CFTR modulators, the isolated CFTR current in CF-HBE can be compared to that in non-CF HBE endogenously expressing wild-type CFTR (% wild-type CFTR). This evaluation of in vitro activities in airway epithelia isolated from CF subjects is important because of the lack of clinically predictive animal models of CF lung disease.

In non-CF HBE, forskolin stimulated a large, biphasic increase in Cl^- secretion, whereas the response in ΔF508-HBE was monophasic and <5% of the wild-type response. The forskolin response in non-CF and ΔF508-HBE is blocked by CFTR inhibitors but not the Ca^{2+}-activated Cl^- channel blocker, DIDS, indicating that the response is due to CFTR activity in the apical membrane. Residual ΔF508-CFTR activity in the apical membrane of CF airway

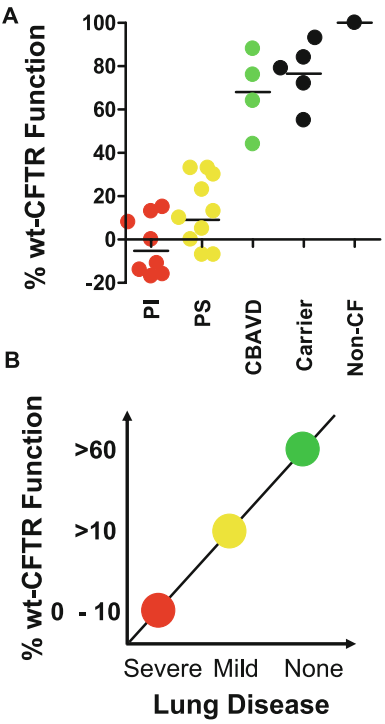


Fig. 6 Disease severity in CF patients is determined by the amount of mutant CFTR activity. **A** In vivo data were pooled from multiple published studies using NPD techniques to measure CFTR-mediated Cl^- secretion in subjects with pancreatic insufficiency (PI), pancreatic sufficiency (PS), or congenital bilateral absence of the vas deferens (CBAVD) and obligate heterozygotes (carriers). CFTR-mediated Cl^- secretion in each study was normalized to the non-CF control subject and expressed as % wild-type (wt)-CFTR. **B** The severity of lung disease in each group was determined from the longitudinal decline in FEV_1 and classified as severe (*red*; ~3%/year), mild (*yellow*; ~1%/year), or no (*green*) lung disease

epithelia has been recorded in primary cultures of airway epithelia [32]. Other studies, however, failed to demonstrate the presence of ΔF508-CFTR on the apical membrane of cultured HBE [94]. The fraction of the ΔF508-homozygous patient population with residual CFTR activity and a method to identify this population is an important issue to resolve from a clinical perspective since it is possible that such patients could benefit from a potentiator therapy.

Sustained treatment of ΔF508-HBE with a number of different corrector scaffolds increases ΔF508-CFTR-mediated Cl^- secretion (Fig. 7). The most effective correctors known to date include VRT-325 (compound 2: Table 2) and Cor-4a (compound 4; Table 2), which increase the total ΔF508-CFTR-mediated Cl^- secretion at low micromolar concentrations to ~10% of that observed in wild-type HBE. At significantly higher concentrations (1.5 mM),

Table 5 Severity of lung disease and predicted CFTR activity in different patient genotypes

Genotype[a]	Channel density	Channel gating	Channel conductance	Wild-type CFTR activity	Severity of lung disease
wt-CFTR	1	1	1	1	None
ΔF508/wt-CFTR	0.5	1	1	0.5	
5T	0.3	1	1	0.3	Mild
5T-TG12-M470V	0.11	1	1	0.11	Moderate
A455E	0.1	1	1	0.10	
3849+10kbC→T	0.08	1	1	0.08	
3272-26A→G	0.05	1	1	0.05	
2789+5G→A	0.04	1	1	0.04	
1811+1.6A→G	0.01	1	1	0.01	Severe
G551D	1	0.025	1	0.025	
ΔF508/ΔF508	0.01	0.25	1	0.0025	

[a] The wild-type-, ΔF508/wild-type-, G551D- and ΔF508-genotypes were added for comparison. All other genotypes are missense or splice mutations. All values for wild-type CFTR are normalized to 1

4-PB caused a marginal increase in ΔF508-CFTR activity in HBE that did not reach the 10% wild-type-CFTR activity threshold. Other known correctors identified using recombinant cell systems failed to increase ΔF508-CFTR-mediated Cl^- secretion in HBE. A further increase in the ΔF508-CFTR mediated current is observed following addition of a CFTR potentiator, suggesting that a dual therapeutic approach may improve clinical efficacy.

9 Conclusions and Perspective

Much progress has been made in the last decade in the identification and characterization of various classes of CFTR modulators. Despite this progress, to the best of our knowledge there are only few CFTR modulators that have been studied clinically (see above). The most advanced compounds are the potentiator VX-770 and the CFTR corrector PTC124, both of which are in Phase II clinical studies in 2007. The limited clinical progress may in part be due to the fact that relatively little medicinal chemical optimization of the various modulator hits has been undertaken or reported. Thus, many of the potentiators and correctors described here may not have drug-like properties, limiting application in the clinic. Despite the fact that various potentiator and corrector chemotypes have been described, the identification of a more general potentiator or corrector pharmacophore has proven to be hard. In addition, the binding modes or even the molecular targets for CFTR modu-

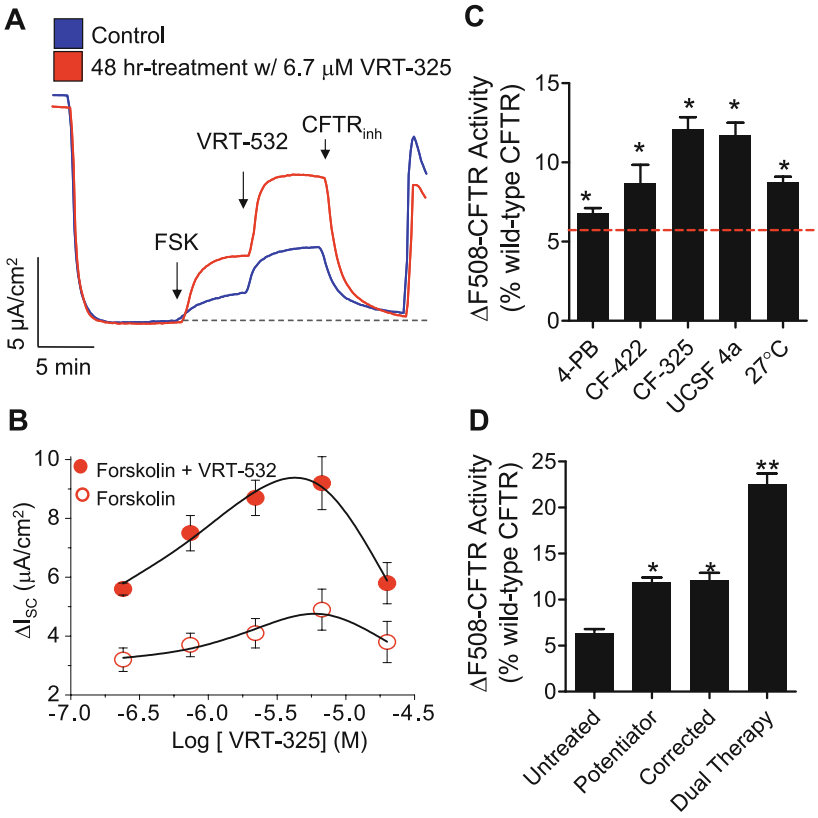


Fig. 7 Rescue of ΔF508-CFTR in HBE isolated from ΔF508-homozygous CF patients. **A** Representative short circuit current in ΔF508-HBE pre-treated for 48-hours with DMSO- (*blue line*) or 6.7 μM VRT-325 (*red line*). **B** Dose response to VRT-325 in FSK-stimulated ΔF508-HBE in the presence (*filled circles*) and absence (*open circles*) of the CFTR potentiator, VRT-532 ($n = 5$). **C** Maximum response to 10 μM FSK in ΔF508-HBE pre-treated with 1500 μM 4-PBA-, 6.7 μM VRT-422- (compound 1), 6.7 μM VRT-325- (compound 2), or 1 μM Corr-4a (compound 4), as well as 27 °C-treated ΔF508-HBE. The peak response to forskolin was normalized to that in wild-type-HBE isolated from non-CF subjects (% wild-type CFTR). The *dashed line* indicates the level of residual (un-treated) ΔF508-CFTR activity in ΔF508-HBE. **D** Cumulative data for uncorrected and VRT-325-corrected ΔF508-HBE with and without addition of the CFTR potentiator, VRT-532. *Single asterisk* = $p<0.05$ compared to un-treated controls; *double asterisk* = $p<0.01$ compared to un-treated controls

lators are unknown or speculative. Finally, we note that even the elucidation of the X-ray structure of NBD1 has had only a limited impact on CFTR modulator design so far, although it has stimulated modeling and mutagenesis studies. Despite these complexities, it may be expected that more drug-like optimized CFTR modulators will become available, in particular when clinical studies reveal a clinical benefit to CF patients.

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On the Process of Finding Novel and Selective Sodium Channel Blockers for the Treatment of Diseases

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Abstract Blockers of voltage-gated sodium channels have several therapeutic uses, including use as anticonvulsants, antiarrhythmics, and analgesics. However, voltage-gated sodium channels are challenging drug targets, and most of the clinically used drugs were found before their sodium channel blocking mechanism was known. Sodium channels are a family of ten homologous subtypes, and family members are expressed differentially throughout the nervous system and in cardiac and skeletal muscle tissue. They exist in closed, open, and inactivated conformational states, and selective interactions with one or more of these states differentiates therapeutically useful drugs from neurotoxins. Therefore, assays used in drug discovery need to be sensitive to these mechanisms of action

and preferably able to distinguish between drug interactions with different conformational states. Electrophysiological assays are ideally suited for this task, and the recent development of automated electrophysiology instrumentation affords medium throughput. Higher capacity assays amenable to studying sodium channel pharmacology include ligand binding, flux, and fluorescent assays.

Keywords Analgesics · Antiarrhythmics · Anticonvulsants · Local anesthetics · Sodium channels

Abbreviations

BPBTS	*N*-{[2′-(Aminosulfonyl)biphenyl-4-yl]methyl}-*N*′-(2,2′-bithien-5-ylmethyl)succinamide
BTX	Batrachotoxinin A 20-α-benzoate
CHO	Chinese hamster ovary
HEK	Human embryonic kidney
Na_V	Voltage-dependent sodium channel
STX	Saxitoxin
TTX	Tetrodotoxin

1 Introduction

Blockers of voltage-gated sodium channels are used clinically as anticonvulsants, antiarrhythmics, and local anesthetics [1], and more recently to treat a number of chronic pain conditions [2]. The therapeutic efficacy of these agents is based on the critical role that voltage-gated sodium (Na_V1) channels play during the initial rising phase of action potentials in nearly all excitable cells. The contribution of sodium channel conductances to action potential firing was first recognized by Hodgkin and Huxley, more than 30 years before the cloning of the first member of the Na_V1 channel family, by voltage clamp recordings from squid giant axons [3]. The critical importance of this role of Na_V1 channels is further illustrated by the effects of the highly potent neurotoxins tetrodotoxin (TTX) and saxitoxin (STX), which are among the deadliest poisons known.

Despite the essential role of Na_V1 channels in normal cell signaling, a number of clinically beneficial sodium channel blockers exist. Most were discovered empirically before they were known to block Na_V1 channels. A key feature of these drugs is their channel blocking mechanism: by blocking channels in a voltage- and use-dependent manner, they spare normal conduction in healthy tissues. Advances in molecular biology and assay technologies now enable a target-driven approach to the development of novel Na_V1 channel blockers.

2
Sodium Channels

2.1
Gating

Early voltage clamp experiments from squid giant axons revealed two important properties of sodium currents essential for their role in shaping action potentials: voltage-dependent activation and inactivation [3]. Long before any knowledge of the molecular or structural features of sodium channels, Hodgkin and Huxley described these properties in terms of two gates that control sodium flux across the plasma membrane: the m gate that controls activation and the h gate that controls inactivation. Since the cloning and expression of Na_V1 channels, it is known that opening and closing of the m and h gates correspond to a number of conformational states that Na_V1 channels can adopt. Based on the findings from voltage clamp experiments, these conformational states are classified as resting, open, and inactivated (Fig. 1). Distribution of channels across these conformational states is controlled by membrane voltage (for a comprehensive review see [4]). At hyperpolarized (negative) membrane potentials, most channels reside in resting states, from which they are ready to open as soon as the membrane becomes depolarized. At more depolarized (positive) membrane potentials, channels occupy inactivated states and remain non-conducting until membrane hyperpolar-

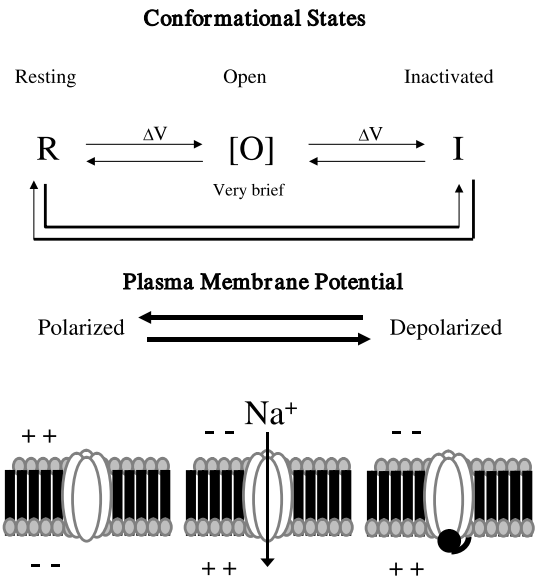

Fig. 1 Gating scheme of voltage-gated sodium channels

ization causes them to recover from inactivation and return to the resting state. The open state of Na_V1 channels is short-lived (typically 1–2 ms), so that, at any given membrane potential, the large majority of sodium channels exist in an equilibrium between resting and inactivated states. Detailed studies of the voltage- and time-dependence of inactivation reveals the existence of at least two inactivated states: a fast inactivated state that channels enter and exit on the millisecond time scale, and a slow inactivated state that may persist for several seconds after the cell is repolarized [5, 6]. We further understand that channels may enter the inactivated state directly from the resting state.

A small overlap in the voltage dependence of activation and inactivation leads to a persistent sodium current, also referred to as a window current. This current is very small compared to the peak current and may go unnoticed in typical whole cell current recordings. However, this current may play an important role in amplifying subthreshold depolarizations and thus in controlling excitability [7].

The neurotoxins TTX and STX bind with similar affinity to all conformational states. Most other small molecule sodium channel blockers appear to interact preferentially with the open state, an inactivated state, or a combination of open and inactivated states. Since channel residence in these states is controlled by membrane voltage, state-dependent sodium channel blockers show both voltage- and use-dependence, i.e., their potency increases with more depolarized holding potentials and higher frequency stimulation [8–10].

2.2 Structure–Function

Na_V1 channels have been purified from mammalian brain and skeletal muscle [11–13]. In these tissues, sodium channels are a complex of a large α-subunit (~260 kDa) and one or two smaller β-subunits (30–40 kDa). The α-subunit contains the pore that sodium ions pass through and, when expressed alone, forms functional channels that display sodium selectivity, voltage-dependent activation and rapid inactivation. Nine distinct α-subunits ($Na_V1.1$–$Na_V1.9$) have been identified, cloned, and functionally expressed (Table 1). Homology between these Na_V1 subtypes is high (>50% amino acid identity) within the membrane-spanning domains and extracellular loops. A tenth, more distantly related, subunit, Na_x, has been identified but not yet functionally expressed. All sodium channel modulators known to date interact with the α-subunit.

Four Na_V1 β-subunits have been cloned and functionally expressed (β1–β4) [14–17]. All contain a single membrane-spanning domain and are highly glycosylated. At least some β-subunits also appear to function as cell adhesion molecules [18]. The β2- and β4-subunits form disulfide bridges

Table 1 Properties of Na_V1 subtypes. Reproduced with permission from [113]

Subtype	Former name	Gene	V_h activation [a] (mV)	V_h inactivation [a] (mV)	Primary location
$Na_V1.1$	Brain type I	*SCN1A*	–33	–72	CNS, PNS
$Na_V1.2$	Brain type II	*SCN2A*	–24	–53	CNS
$Na_V1.3$	Brain type III	*SCN3A*	–23	–69	Mostly embryonic
$Na_V1.4$	SkM1/μ1	*SCN4A*	–18	–71	Skeletal muscle
$Na_V1.5$	SkM2/H1	*SCN5A*	–48	–92	Cardiac muscle
$Na_V1.6$	NaCh6/PN4	*SCN8A*	–29	–72	CNS, PNS
$Na_V1.7$	hNE-Na/PN1	*SCN9A*	–19	–68	PNS
$Na_V1.8$	SNS/PN3	*SCN10A*	3 [b]	–63 [b]	PNS
			6 [c]	–38 [c]	
$Na_V1.9$	NaN/SNS2/PN5	*SCN11A*	–32 [d]	–43 [d]	PNS
			–61 [e]	–99 [e]	

[a] Recombinant α-subunit expressed alone in mammalian cell line
[b] Recombinant α-subunit expressed in CHO cells
[c] Recombinant α- and β1-subunits expressed in HEK cells
[d] Current in DRG neurons from $Na_V1.8$ null mutant mice with Cl^- as intracellular anion
[e] Current in DRG neurons from $Na_V1.8$ null mutant mice with F^- as intracellular anion

with the α-subunit, whereas the β1- and β3-subunits associate non-covalently. All four β-subunits are expressed in the CNS and in the peripheral nervous system, and significant levels of β1 and β4 have been reported in heart and skeletal muscle. When co-expressed with a Na_V1 α-subunit, β-subunits can modulate the amplitude, voltage dependence, and kinetics of the currents. Other auxiliary subunits, such as annexin II light chain, have been reported [19]. In addition, sodium channels may interact with contactin, the extracellular matrix protein tenascin, the cytoskeletal protein ankyrin [20–22], and some of these interactions may involve β-subunits.

The Na_V1 α-subunits contain four homologous domains (I–IV) (Fig. 2). Similar to voltage-gated potassium and calcium channels, each domain has six membrane-spanning segments (S1–S6) and a pore loop, which forms part of the permeation pathway and is located between S5 and S6. The fourth membrane-spanning segment (S4) of each domain contains a number of positively charged amino acids, spaced approximately one helical turn apart, that form part of the voltage sensor. Movement of these charged residues within the membrane electric field ultimately causes the conformational changes that open and close the channel. Accordingly, charge-neutralizing mutations affect the voltage dependence of channel activation.

The intracellular loop connecting domains III and IV plays a critical role in fast inactivation. Indeed, mutation of a conserved IFM motif to QQQ results in channels with little or no fast inactivation [23], which is restored by

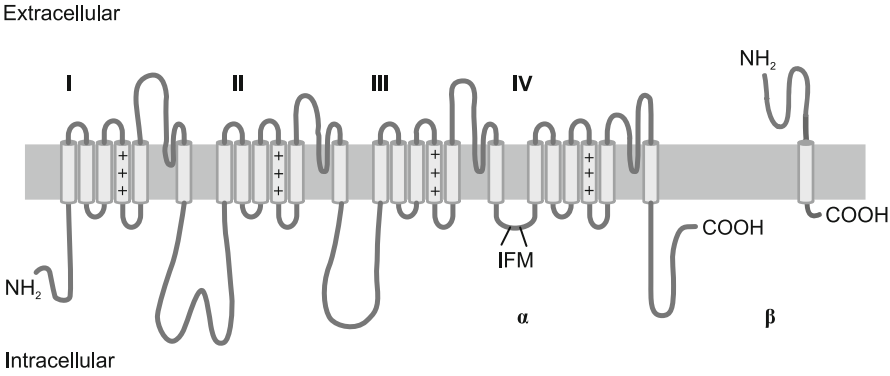

Fig. 2 Structural domains of $Na_V1.2$ subunits. Reproduced with permission from [113]

application of a small peptide corresponding to part of the domain III–IV linker.

2.3 Channelopathies

The important physiological roles of Na_V1 channels are highlighted by the number of inherited disorders, referred to as channelopathies, that have been linked to mutations in Na_V1 subunits. $Na_Vl.1$ (SCN1A) is expressed in both the central and peripheral nervous system and mutations are associated with several forms of epilepsy, including generalized epilepsy with febrile seizures plus types 1 and 2 (GEFS+l, GEFS+2) and severe myoclonic epilepsy of infancy (SMEI) [24, 25]. $Na_V1.2$ (SCN2A) is predominantly expressed in the central nervous system, and mutations in $Na_V1.2$ are associated with forms of early childhood epilepsy [26]. $Na_V1.3$ is mostly expressed during embryonic development, but can be detected in adult central and peripheral nervous system. No human channelopathies are known for $Na_V1.3$ (SCN3A). $Na_V1.4$ (SCN4A) is expressed exclusively in skeletal muscle and mutations have been linked to paramyotonia congenital, hyperkalemic periodic paralysis, and hypokalemic periodic paralysis [27, 28]. The $Na_V1.5$ subtype (SCN5A) is almost exclusively expressed in cardiac muscle and mutations in this channel are the cause of at least some forms of long QT syndrome (LQT3), Brugada's syndrome, and isolated cardiac conduction disease [29]. No human channelopathies are known for $Na_V1.6$ (SCN8A), but, in mice, mutations in SCN8A are associated with motor endplate disease (*med* locus). Null mutations cause loss of skeletal muscle innervation, followed by paralysis and death [30, 31]. A less severe loss-of-function mutation is associated with the jolting phenotype, characterized by cerebellar ataxia [32]. $Na_V1.7$ (SCN9A) is preferentially expressed in peripheral sensory and sympathetic systems. In humans, complete loss of $Na_V1.7$ function has been linked to a congenital inability to sense

pain without apparent deficits in other sensory functions [33]. Conversely, mutations in $Na_V1.7$ that increase channel activity are associated with two inherited pain syndromes: familial erythromelalgia and paroxysmal extreme pain disorder [34, 35]. Both syndromes are characterized by bouts of severe burning pain that affect predominantly the extremities, in the case of familial erythromelalgia, and the rectum, genitalia, and face in the case of paroxysmal extreme pain disorder. In mice, deletion of SCN9A causes pups to die within a few days after birth. However, mice with a nociceptor-specific deletion of SCN9A are viable but show reduced mechanical and thermal pain thresholds and reduced inflammatory pain responses [36]. $Na_V1.8$ (SCN10A) and $Na_V1.9$ (SCN11A) are expressed primarily in the peripheral nervous system. There are no known channelopathies associated with these subtypes, but altered expression of $Na_V1.8$ has been observed in patients suffering from multiple sclerosis [37].

2.4 Modulation

Most or all Na_V1 channel subtypes are subject to modulation through second messenger pathways. Phosphorylation of neuronal and cardiac sodium channels via PKA- and/or PKC-dependent pathways can alter current amplitudes, kinetics, and voltage dependence [38, 39]. Interestingly, phosphorylation of homologous residues can have divergent effects on biophysical properties of different sodium channel subtypes [40]. The physiological consequences of sodium channel phosphorylation have been studied in a number of tissues. For example, in the hippocampus, cholinergic input inhibits sodium currents via PKC phosphorylation, leading to a shift from bursting to tonic firing [41]. In sensory neurons, phosphorylation of $Na_V1.8$ channels appears to be involved in the modulation of TTX-resistant currents and in the sensitization by prostaglandins [42, 43].

In addition to their effects on second messenger systems, G-proteins may modulate sodium channels by direct binding of G_$\beta\gamma$ subunits to the Na_V1 α-subunit. In the case of recombinant $Na_V1.2$ channels, this modulation results in a dramatic increase in persistent sodium current [44].

3 Sodium Channel Blockers

Sodium channels are associated with a rich pharmacology, including the potent neurotoxins TTX and STX, as well as numerous clinically used therapeutic agents. TTX and STX bind with similar affinity to all conformational states and prevent sodium permeation by physically occluding the pore [45, 46]. Most other non-peptide sodium channel blockers appear to interact preferen-

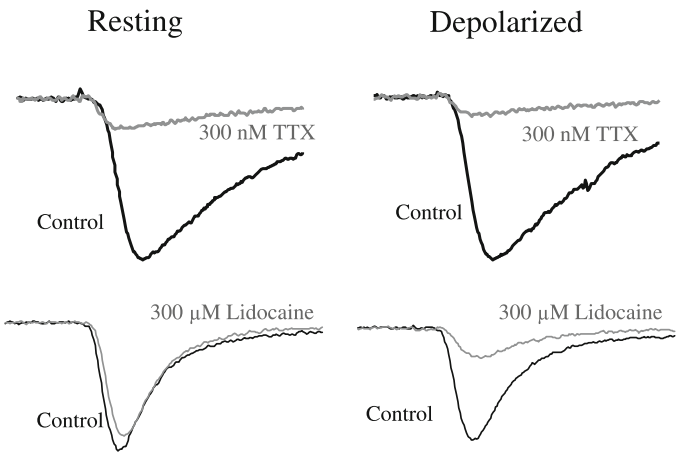

Fig. 3 Block of $Na_V1.7$ channels by TTX and lidocaine. Block by TTX is equally potent at hyperpolarized (resting) and depolarized holding potentials, whereas block by lidocaine becomes more potent at depolarized holding potentials

tially with the open state, an inactivated state, or a combination of open and inactivated states (Fig. 3).

Since channel residence in the different conformational states is controlled by membrane voltage, state-dependent sodium channel blockers show voltage- and use-dependence, i.e., their potency increases with more depolarized holding potentials and with repetitive stimulation [8–10]. State-dependent sodium channel blockers are used clinically as local anesthetics and to treat epileptic seizures and certain arrhythmias.

3.1 Therapeutic Agents

3.1.1 Local Anesthetics

Local anesthetics have been used in clinical practice for well over a century. Experimenting with cocaine, Carl Koller and Sigmund Freud first discovered the local anesthetic properties of this class of drugs, which produce anesthesia by blocking conduction in peripheral nerves. This type of conduction block is not generally considered functionally selective, although motor neurons appear somewhat less sensitive to block than sensory neurons [47]. Most local anesthetics contain a benzene moiety connected by a linker of varying length to a tertiary or quaternary amine. Many local anesthetic are also used as antiarrhythmics (Fig. 4).

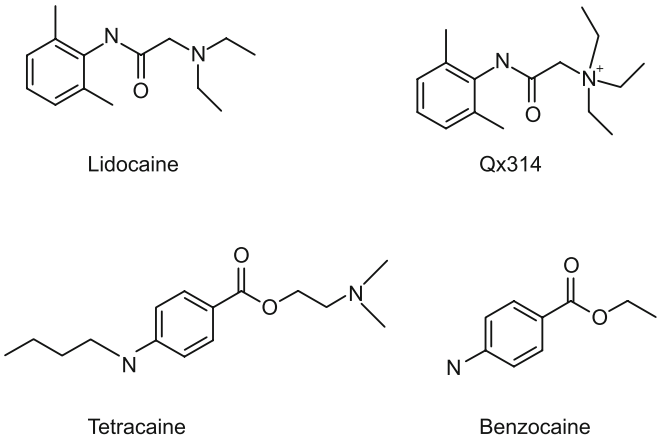

Fig. 4 Structures of common local anesthetics

3.1.2 Anticonvulsants

Epilepsy affects ~0.5% of the world's population and can have a multitude of underlying etiologies, including several mutations in CNS sodium channels. Sodium channel mutations linked to human epileptic syndromes typically shift activation to more hyperpolarized potentials, slow inactivation kinetics, accelerate recovery from inactivation, and/or increase the persistent current [48]. Seemingly paradoxically, some mutations appear to result in non-functional channels [48–50].

Early anticonvulsants, such as phenytoin and carbamazepine, were developed based on their efficacy in animal models (Fig. 5). Indeed, phenytoin and carbamazepine were recognized for their potential to suppress electroshock-induced seizures in rodents decades before they were known to block Na_V1 channels. Originally developed as an anticonvulsant for its ability to reduce folate concentrations, lamotrigine was also shown to block Na_V1 channels. Since epilepsy is associated with increased neuronal excitability and abnormal action potential firing, it is not surprising that sodium channels may be involved in epileptogenesis and that sodium channel blockers may be efficacious in suppressing seizures. Conversely, several proconvulsant agents, such as veratridine and pyrethroids, increase sodium currents.

Epileptic seizures are characterized by high-frequency bursts of action potentials that occur synchronized throughout neuronal networks. This type of epileptiform repetitive firing can be induced by the removal of extracellular magnesium. Use-dependent sodium channel blockers such as lamotrigine block the epileptiform activity at concentrations well below those required to block normal conduction [51]. Since the firing associated with epileptic seizures occurs with high frequency, the use-dependent properties of anticon-

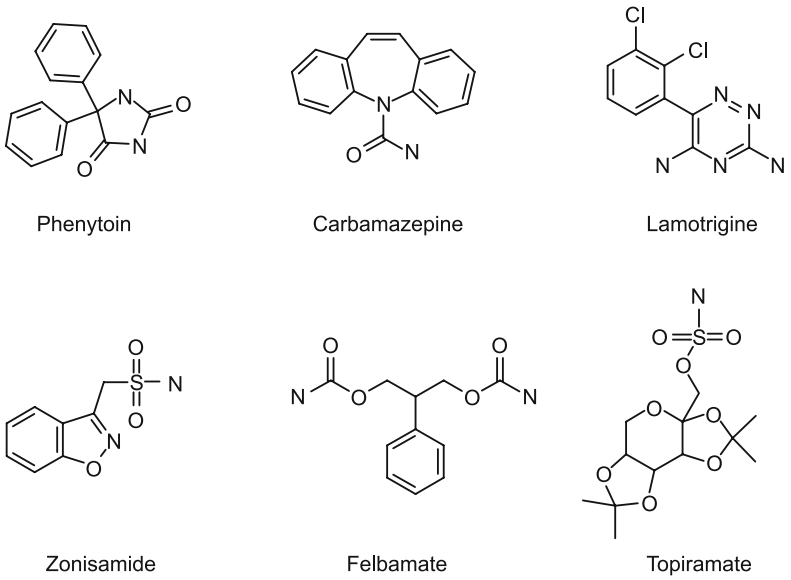

Fig. 5 Structures of common anticonvulsants

vulsant drugs are thought to contribute to this functional selectivity for block of the pathological, epileptiform firing. In addition, the characteristic high-frequency bursts may be evoked by a sustained depolarization at the epileptic focus, favoring block by voltage-dependent drugs. High affinity of anticonvulsant drugs for channels in the open state may also contribute to functional selectivity by favoring block of persistent sodium current. Indeed, several anticonvulsant drugs have been reported to selectively inhibit the persistent current [52–55].

In addition to phenytoin, carbamazepine, and lamotrigine, metabolically optimized analogs of these drugs, such as fosphenytoin and oxcarbazepine, show clinical promise. Other anticonvulsants that block sodium channels, among several mechanisms of action, include zonisamide, felbamate, topiramate, and valproate (Fig. 5).

3.1.3 Antiarrhythmics

The efficacy of sodium channel blockers as antiarrhythmics was first discovered when local anesthetics were used during cardiac surgery, although their sodium channel blocking mechanism was not known at that time. Antiarrhythmic drugs have been classified according to their mechanism of action, with class I drugs acting through sodium channel blockade. Class I antiarrhythmics include lidocaine, mexiletine, procainamide, quinidine, flecainide,

and disopyramide. They are further subdivided into classes IA, IB, and IC based on their effects on conduction velocity and refractory period [56]. Class IC drugs, including flecainide and propafenone, have the greatest effect on conduction velocity and refractory period and are the most efficacious antiarrhythmics; however, in the Cardiac Arrhythmia Suppression Trial (CAST), a large multicenter clinical trial, class IC drugs were associated with proarrhythmic effects and increased mortality [57].

3.1.4 Analgesics

Local application of concentrated sodium channel blockers can provide complete pain relief through nerve conduction block (local anesthetics). This approach to pain relief is limited to a few applications involving short-term treatment of acute pain, since sodium channels are also vital to conduction in the heart, CNS, skeletal muscle, and non-nociceptive sensory neurons. However, some types of chronic pain signaling appear to be sensitive to sodium channel blockers at concentrations that do not cause conduction block. In particular, neuropathic pain, defined as "chronic pain resulting from a primary lesion or dysfunction of the peripheral nervous system" by the International Association for the Study of Pain (IASP) [58], is thought to originate from aberrant signaling in the nervous system and can be ameliorated by sodium channel blockers.

The local anesthetic lidocaine provides significant relief of neuropathic pain in the clinic when administered systemically at subanesthetic doses. In several placebo-controlled studies, intravenous infusions of lidocaine significantly reduced neuropathic pain at plasma concentrations of 1.5–5 $\mu g\,mL^{-1}$ [59–61]. Lightheadedness is a common side effect at therapeutic doses, whereas cardiac depression is associated with plasma concentrations of 20–25 $\mu g\,mL^{-1}$. A transdermal formulation of lidocaine is approved in the United States for the treatment of postherpetic neuralgia [62]. Orally available state-dependent Na_V1 blockers have shown efficacy in the clinic when appropriate plasma concentrations are achieved.

Several sodium channel subtypes are preferentially expressed in pain-sensing neurons of the peripheral nervous system and may be targets for novel analgesic agents. In the absence of subtype-selectivity, state-dependent sodium channel blockers, which favor binding in rapidly firing or partially depolarized tissues, may have efficacy while preserving sodium channel-dependent conduction. Neuropathic pain should be sensitive to this mechanism of block since it is thought to arise from injury-induced areas of depolarizations.

State-dependent sodium channel blockers may also be efficacious in the treatment of chronic inflammatory pain conditions since inflammatory mediators, such as prostaglandins and serotonin, increase sodium channel ex-

pression and modulate sodium currents in sensory neurons. Knock-down of several sodium channel subtypes in rodents reduces inflammatory pain behavior.

3.1.5 Other Therapeutic Uses

Several other therapeutic effects of sodium channel blockers have been suggested. Most of these stem from clinical activities of approved anticonvulsants and antiarrhythmics with sodium channel blocking activity. Beneficial effects of sodium channel blockers for the treatment of bipolar disease are suggested by clinical data with lamotrigine [63–67], phenytoin [68], topiramate [69], and carbamazepine [70, 71]. In addition, clinical studies with lidocaine suggest efficacy in the treatment of tinnitus [72] and, as an inhaled formulation, in the suppression of cough [73, 74].

Multiple sclerosis is associated with changes in sodium channel expression, which may contribute to remission of symptoms but can also contribute to axon degeneration. In patients with multiple sclerosis, peripheral axons become demyelinated, leading to loss of normal saltatory action potential conduction at the Nodes of Ranvier [75]. At least early on during disease progression, many patients experience periods of remission. Increased sodium channel expression in demyelinated axons has been demonstrated in immunocytochemical and binding studies and may contribute to improved action potential conduction during these remissive periods [76]. However, during progressive multiple sclerosis, axonal degeneration contributes to permanent neurological deficits, and sodium influx through Na_V1 channels may play a role in the degeneration process. Indeed, the sodium channel blockers phenytoin and flecainide have proven beneficial in rodent models of multiple sclerosis [77, 78].

3.2 Pharmacophore Models

In general, pharmacophore models can be derived by characterizing the binding site on the receptor protein, by comparing the structures of known ligands, or by computational docking analysis, which is essentially a combination of the first two approaches.

The Na_V1 α-subunit is a large protein, and several receptor sites for natural product neurotoxins have been identified by photoaffinity labeling and site-directed mutagenesis [79]. The small hydrophilic guanidines TTX and STX and the μ-conotoxin peptides bind at the extracellular mouth of the conduction pathway and are thought to physically occlude the pore. Binding of these toxins shows little state- or use-dependence. A number of α scorpion, sea anemone, and spider toxins also bind to an extracellular site, but act as

channel activators by inhibiting inactivation. In contrast, several structurally diverse lipid-soluble neurotoxins, such as veratridine, batrachotoxin, and the pyrethroid insecticides, bind to a receptor site within the inner vestibule of the channel lined by the S5 and S6 helices. These relatively small molecules are also considered channel agonists since they cause a hyperpolarizing shift in the voltage dependence of activation and a variable inhibition of inactivation [80]. Binding of the lipid-soluble neurotoxins is highly state-dependent: veratridine and batrachotoxin modify channels in the open state [80, 81], whereas the pyrethroid insecticides act primarily on resting channels [82].

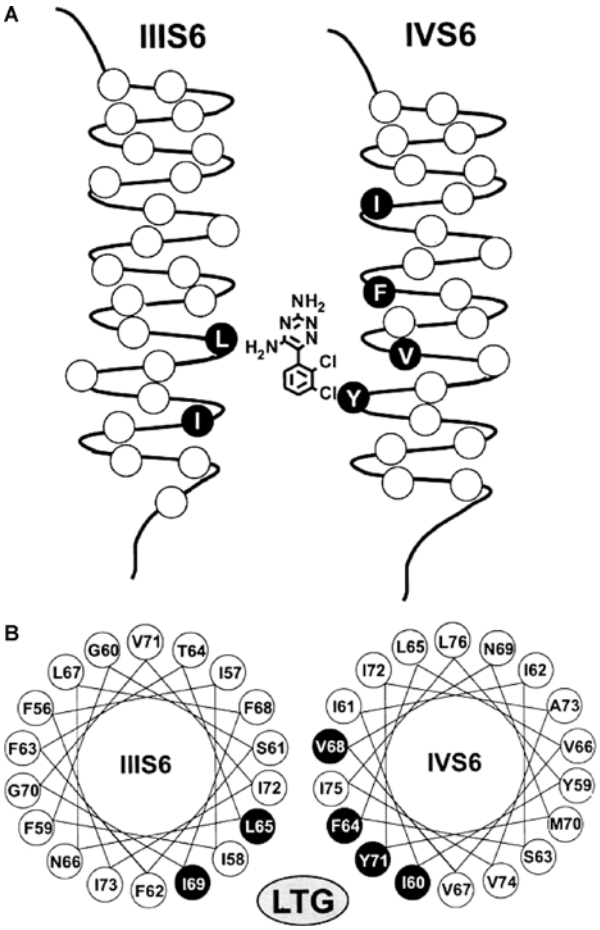

Fig. 6 Lamotrigine binding to transmembrane segments IIIS6 and IVS6 of the rat $Na_V1.2$ channel. **A** Side view of the proposed location of the lamotrigine binding site within the pore. **B** α-Helical representation showing the axial position of mutations causing reduction in affinity of lamotrigine (*LTG*) for inactivated Na^+ channels. Reproduced with permission from [87]

Detailed electrophysiological and site-directed mutagenesis studies suggest that the state-dependent sodium channel blockers used clinically as local anesthetics, anticonvulsants, and antiarrhythmics bind to a common site within the inner vestibule of the sodium channel [83]. This common receptor site involves aromatic residues in transmembrane segment S6 of domain IV [84–87], which may interact with the aromatic rings found in most sodium channel blockers (Fig. 6). Interestingly, the phenylalanine residue (F1764 in $Na_V1.2$) that is part of this receptor site also plays a role in binding of the lipid-soluble neurotoxins. Both types of sodium channel modulators are state-dependent, and it is possible that they differ predominantly in their interactions with the inactivation gate.

Comparative analysis of known sodium channel blockers has resulted in several somewhat distinct models [88–91]. These models suggest that local anesthetic and anticonvulsant sodium channel blockers share a pharmacophore consisting of a hydrophobic group (typically an aryl ring) separated by 5–6 Å from a hydrogen bond acceptor-donor group.

4 Design of Novel Sodium Channel Blockers

The development of clinically successful sodium channel blockers should involve improving on the tolerability of existing drugs while retaining therapeutic efficacy. One approach to limiting undesirable side effects is to target specific Na_V1 subtypes. Considerable evidence now links $Na_V1.7$ and $Na_V1.8$ to pain signaling [33, 92, 93]. Patients suffering from multiple sclerosis may benefit from $Na_V1.6$-selective blockers [94], and, for anticonvulsants, arguments can be made for selectively blocking $Na_V1.2$ and/or $Na_V1.6$ channels [95]. Recently, the first subtype-selective small molecule sodium channel blocker was reported [96]. This compound, A-803467, was at least 100-fold selective for the human $Na_V1.8$ subtype over $Na_V1.2$, $Na_V1.3$, $Na_V1.5$, and $Na_V1.7$, and showed antinociceptive activity in several rat models of inflammatory and neuropathic pain.

While the binding site for A-803467 is at present unknown, sodium channel blockers currently used in the clinic largely interact with the S5 and S6 pore lining segments. These segments are highly conserved among different channel subtypes, and, consistent with this finding, these drugs interact with similar potency with all channel subtypes tested. Targeting compounds that bind to the extracellular domains of the channel and either block the pore or modulate gating may hold greater promise for subtype selectivity. While technically challenging, it may also be possible to target channel domains involved in protein–protein interactions responsible for the control of channel distribution/trafficking.

Even in the absence of molecular subtype selectivity, it has been possible to specifically target sodium channels in a given conformational state while preserving sodium channel-dependent conduction. Using this approach has resulted in sodium channel blockers that display functional selectivity and an acceptable therapeutic index for the treatment of epilepsy (e.g., lamotrigine, phenytoin, and carbamazepine) and certain cardiac arrhythmias (e.g., lidocaine, tocainide, and mexiletine). The therapeutic index of these compounds is primarily a consequence of their use-dependent and state-dependent properties arising from higher binding affinity for open and inactivated states than for channels in resting closed states [10, 95]. This mechanism of block favors binding in rapidly firing or partially depolarized tissues.

5 Assay Technologies

The ideal ion channel assay provides a linear, accurate measure of channel activity under conditions that predict the desired therapeutic effects. Sodium channels undergo large voltage-dependent conformational changes as they transition between resting, open, and inactivated states. Clinically used sodium channel blockers preferentially bind to open and/or inactivated states, and thus selectively inhibit channels in rapidly firing or partially depolarized tissues. A sodium channel assay therefore needs to be sensitive to this mechanism of action to be therapeutically relevant. A number of assay technologies exist that are applicable to sodium channel blockers and that vary greatly in throughput, cost, and information content. These assays typically require stable heterologous expression of the recombinant channel of interest in an immortalized mammalian cell line, in order to study this channel in isolation, without the potentially confounding influences of other channel types, and to provide an easily renewable source of target material.

5.1 Recombinant Expression

For most of the TTX-sensitive sodium channel subtypes, stable expression in human embryonic kidney (HEK) or Chinese hamster ovary (CHO) cells is readily achieved using standard protocols. In contrast, $Na_V1.8$ and $Na_V1.9$ have been notoriously difficult to express heterologously, and some features of the recombinant channels differ from their native counterparts [97].

Rat $Na_V1.8$ channels have been stably expressed in the neuroblastoma cell line ND7-23 [98] and in F11 cells [99], both cell lines of neuronal origin. However, ND7-23 and F11 cells both express endogenous TTX-sensitive sodium channels that have to be blocked to study the pharmacological properties of $Na_V1.8$ channels. Human $Na_V1.8$ channels have been stably expressed in

CHO cells, using an inducible expression system [100], and in HEK cells [97]. While the recombinant channels show the expected pharmacology, some of the biophysical properties differ from native TTX-resistant currents, suggesting the existence of auxiliary subunits. In *Xenopus* oocytes and in mammalian cells, expression of $rNa_V1.8$ was increased substantially by co-expression with $Na_V\beta1$ and $Na_V\beta3$, respectively [98, 101].

To date, a single report of a small persistent current in transiently transfected HEK cells is the only evidence for the functional expression of $Na_V1.9$ in mammalian expression systems [102].

5.2 Electrophysiology

Electrophysiology has been the gold standard method for studying ion channels and can provide very detailed and quantitative information on ion channel function and pharmacology. One of the unique advantages of electrophysiological/voltage clamp recordings is the ability to control and manipulate membrane potential. Since residence of sodium channels in specific conformational states is dependent on membrane potential, voltage clamp recordings can provide detailed information about the ability of test compounds to interact with these conformational states of the channel. However, traditional electrophysiology is extremely time-consuming, limiting its use in drug discovery to the detailed characterization of a few compounds identified in higher throughput assays.

Recently, higher throughput automated electrophysiological instruments have been developed. Most of these instruments use a planar substrate with holes (Fig. 7). Cells that seal to the substrate around a hole may be voltage clamped by rupturing or perforating the section of plasma membrane covering the hole, and thus allow electrical access to the cell's cytoplasm. Currently, the IonWorks HT and IonWorks Quattro are the highest throughput devices available. On these instruments, increased throughput is achieved by use of a planar substrate in combination with synchronous operation (simultaneous recording from 48 wells), allowing for a simplified hardware and software design.The IonWorks HT uses a disposable 384-well PatchPlate and a common ground chamber. Typically, cells form medium resistance seals, and whole cell access is obtained by perfusion with a membrane-perforating agent. The IonWorks Quattro uses a modified PatchPlate with 64 holes per well, thus recording the average current in up to 64 cells in each well.

The PatchXpress, the QPatch (Sophion Biosciences) and the Patchliner (Nanion Technologies) also use planar substrates, but recording from individual cells occurs in asynchronous fashion, limiting the throughput of these instruments. Commercially available instruments use 16-well or 48-well disposable cartridges. High-resistance seals between the cells and the substrate

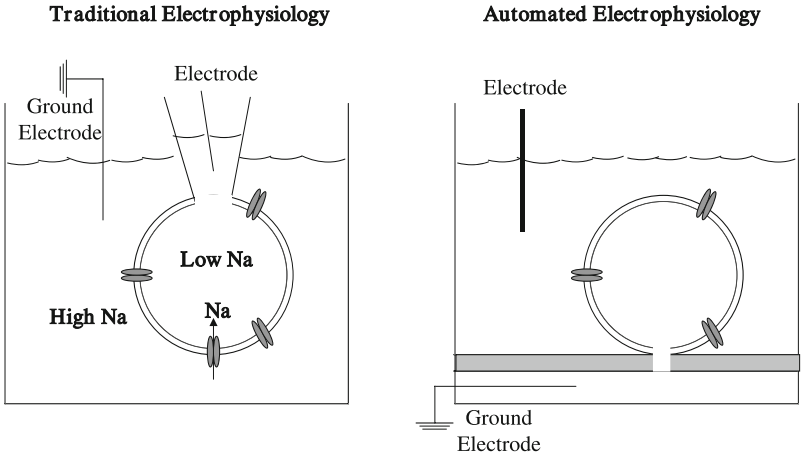

Fig. 7 Schematic showing the recording configuration during traditional and automated whole cell voltage clamp recordings

enable voltage clamp recordings similar in quality to those obtained by traditional manual electrophysiology.

Sodium channels activate and inactivate rapidly, and capacitive transients can significantly obscure the peak current if not compensated properly. Lack of capacitance and series resistance compensation, combined with relatively low seal resistance and moderate sampling frequency (10 Hz maximum) limits the quality of sodium current recordings on the IonWorks instruments. However, due to the reasonably low capacitance of the seal chip and the relatively small and very stable series resistance of the perforated patch configuration, it is still possible to obtain information on sodium channel pharmacology. On the lower throughput platforms, seal resistance, capacitance, series resistance, and non-specific leak currents can be monitored and compensated, resulting in high quality recordings.

5.3 Flux Assays

A medium throughput approach to evaluating sodium channel activity is the measurement of sodium flux across cell membranes [103]. In these experiments, a tracer that permeates the channel and is easily quantifiable is used to analyze sodium influx. Traditionally, radioactive tracers such as $^{22}Na^{+}$ or [^{14}C]guanidinium have been used. Alternatively, Li^{+} can be used as a tracer and analyzed by atomic absorption spectrometry. Sodium flux assays can be used to test approximately 10^5 compounds per year. They offer a robust readout of channel activity, but lack voltage control and temporal resolution. To examine sodium channel blockade by measuring sodium flux,

sodium channels have to be opened. This may be achieved by depolarizing cells through the addition of potassium in cells where potassium controls resting membrane potential, or by adding a sodium channel agonist such as veratridine.

5.4 Binding Assays

Ligand binding assays using radiolabeled or fluorescent-labeled ligands are similar to flux assays with regard to throughput; however, they are non-functional assays and rely on the test compounds binding to the same site or to a site allosterically coupled to the labeled ligand binding site. To date, the commercially available radioligands for sodium channels are TTX, STX, and batrachotoxinin A 20-α-benzoate (BTX-B). As mentioned previously, TTX and STX have very low affinity for $Na_V1.8$ and $Na_V1.9$ channels. Both are external pore blockers that bind independently of the channel's conformational state and do not compete with traditional state-dependent blockers. In contrast, the BTX-B binding site is allosterically coupled to other drug binding sites on the channel, but requires co-application of a scorpion toxin for efficient [^{3}H]BTX-B binding [104].

Recently, specific binding to sodium channels was demonstrated for the state-dependent sodium channel blocker [^{3}H]BPBTS [105]. BPBTS is a potent blocker of all sodium channel subtypes tested, including native TTX-resistant channels expressed in mouse sensory neurons. Binding of [^{3}H]BPBTS is inhibited by the local anesthetics tetracaine and lidocaine at concentrations known to interact with channels in the open and/or inactivated state.

5.5 Fluorescent Assays

Fluorescent dyes that detect changes in membrane potential [106, 107] can provide a robust measure of ion channel activity. If the ion channel of interest conducts current at the resting membrane potential, and thus contributes to setting the resting membrane potential, changes in the concentration of the permeant ion result in a fluorescent signal. This signal can then be blocked with the appropriate pharmacology. Sodium channels conduct little standing current at any given membrane potential, and therefore sodium channels require activation in order to affect the membrane potential. In a fluorescent whole cell assay, sodium channel activation can be achieved by the addition of pharmacological agonists [108, 109] or by electrical field stimulation [110, 111].

While fluorescent assays afford the highest throughput among ion channel assays and do not require specialized instrumentation, they suffer from some limitations, including potential fluorescent interference by the test compound

and, in the case of membrane potential-dependent assays, a highly non-linear dependence of the signal on fractional channel block.

Several functional sodium channel assays that use membrane potential sensing dyes and pharmacological activators have been described [99, 106, 108, 112]. In addition, there are recent reports of fluorescent sodium channel assays using changes in an electric field applied across cells to activate sodium channels [110, 111], thus avoiding potential interactions of the test compound with the channel activator.

6 Summary

Blockers of voltage-gated sodium channels are commonly used as local anesthetics and anticonvulsants, whereas their use as antiarrhythmics is limited due to potential proarrhythmic activity. More recently, sodium channel blockers have generated considerable interest as analgesics. A great deal is known about the molecular biology, biophysical properties, and expression patterns of the ten subtypes that constitute the family of voltage-gated sodium channels, and much progress has been made in developing assay technologies suitable for sodium channel drug discovery. It is expected that progress in these areas will soon lead to the development of novel therapies and to the first blockers selective for individual Na_V1 subtypes.

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Kv1.5 Potassium Channel Inhibitors for the Treatment and Prevention of Atrial Fibrillation

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Abstract The Kv1.5 channel subunit is the putative molecular correlate of the ultrarapidly activating delayed rectifier potassium current, I_{Kur}, detected in human atrial but not ventricular myocytes. Inhibition of Kv1.5 has been widely recognized as a potential therapeutic strategy for the treatment of atrial fibrillation (AF), a major clinical concern, especially in aging populations. This review centers on recent Kv1.5 drug discovery and development, in the context of action potential prolonging antiarrhythmics for the treatment of AF. Collectively, most novel agents are nonselective for Kv1.5. Preclinical along with very limited clinical efficacy data appear to validate Kv1.5 as an effective target to increase atrial refractoriness and to convert fibrillating atria to sinus rhythm. Functional characterization of Kv1.5-specific structural domains and novel assays can help discover better Kv1.5 drugs. Definite proof of concept for Kv1.5 inhibition as an effective antiarrhythmic strategy awaits agents with greater Kv1.5 selectivity and a more detailed characterization of I_{Kur} in human atria and the consequence of its inhibition.

Keywords Antiarrhythmic · Atrial fibrillation · I_{Kur} potassium current · Kv1.5 channel

1 Introduction

This review focuses on the recent discovery and development of chemical entities targeting the Kv1.5 potassium channel subunit for the treatment of atrial arrhythmias. This strategy is based on the expression of the ultrarapidly activating delayed rectifier potassium current, I_{Kur}, in atria but not ventricles of humans, and on the molecular characterization of the Kv1.5 channel subunit as a structural correlate of I_{Kur} [1–6]. Although no perfectly selective Kv1.5

channel inhibitors have been described in the literature, a potential advantage of such an inhibitor over existing pharmacological agents to treat atrial fibrillation (AF) has been proposed [7] and the effect of I_{Kur} inhibition on atrial action potential has been modeled [8, 9]. Here, we present preclinical and limited clinical pharmacological evidence indicating that agents, which are more or less selective for Kv1.5, prolong atrial action potential, increase atrial refractoriness, and terminate and convert AF to sinus rhythm. We discuss structural determinants of Kv1.5 selectivity, as well as the methods and technologies being used to discover and optimize Kv1.5 inhibitors. An important unanswered question is the exact identity of the target. While human Kv1.5 homotetrameric channels appear to recapitulate most of the properties of the native I_{Kur} channel, additional complexity has been reported. Related considerations are whether desirable therapeutic effects can be ascribed specifically to Kv1.5 inhibition, and whether Kv1.5/I_{Kur} itself is a target in the remodeling processes leading to AF.

Atrial flutter and AF are the most common sustained cardiac arrhythmias, and are likely to increase in prevalence with the aging of the population [10, 11]. Briefly, in AF, the upper cardiac chambers (atria) develop sustained, very rapid (400–600 per minute), and irregular firing. In atrial flutter, the firing is slower and more regular. Even with conduction filtered by the atrio-ventricular node, these waves of electrical activity reach the ventricle at an abnormally high rate ($\sim$ 150 per minute). The maintenance of such a rapid rate, left untreated, is likely to lead to heart failure over time. In addition, during sustained AF ineffective atrial contraction results in inadequate ventricular filling and provides conditions that favor clotting [12]. It is forecast that by 2050, AF will be present in approximately 2% of the general population and in a far higher proportion of elderly patients [13]. Therefore, while AF is rarely lethal, it has serious health consequences and societal impact for affected individuals and for medical care delivery systems, particularly in the areas of stroke, quality of life, morbidity, and mortality [14].

AF develops when ectopic or reentrant excitation encounters fibrillation-prone atrial tissue [15]. Typical triggers include rapidly firing ectopic foci in the pulmonary veins [16], sometimes connected with abnormal activity of the autonomic nervous system [17]. The most common mechanism of AF is complex atrial reentry, including single- and multiple-circuit reentry [12, 18]. Underlying the progressive nature of the disease are structural and electrical remodeling processes. Structural remodeling is connected with activation of the renin–angiotensin system and inflammation. Electrical remodeling is connected with decreases in major atrial ionic currents and concomitant changes in atrial action potential configuration and refractoriness [15, 19–21].

AF is quite heterogeneous in its clinical presentation [19], sometimes associated with structural heart disease, and other times without any associated medical conditions. Recently, an international consensus has been reached re-

garding the definitions used to described AF clinical presentations [22]. Thus, AF is defined as *initial* (or first detected) versus *recurrent*. In turn, recurrent AF can be defined as *paroxysmal* (episodes terminate spontaneously within 2–7 days), *persistent* (intervention is needed to terminate), and *established* or *permanent* (episodes of AF cannot be cardioverted or reversion to AF is observed within 24 h of cardioversion). Throughout the published literature, the terms *chronic* and *paroxysmal* have been used but are variably defined; the consensus attempts to facilitate study comparisons. A related consideration addressed for consensus was the possible curable cause. In this regard, the document reflected a prevalent view: AF can occur in the setting of acute cardiovascular diseases, and in these instances it rarely represents the major problem; in most cases (approximately 30%), it can occur in the absence of cardiac disease (*lone* AF) or any disease (*idiopathic* AF).

This assessment of asymptomatic AF and its clinical prognosis and management has been recently questioned, in light of a clinical study on the progression of AF, from an initial diagnosis of lone AF, to understand the rate and predictors of progression [23, 24]. The study followed patients meeting these criteria at the time of initial diagnosis: < 60 years of age, a diagnosis of lone AF, and classification along the paroxysmal to permanent AF spectrum. The study reported several surprising findings: less than 3% of the population diagnosed with AF during a span of 30 years qualified for inclusion in the study; the overall survival of this cohort was statistically comparable to the population at large; finally, age-related co-morbidities were found to significantly modulate the progression and complications of AF. Despite acknowledged limitations, the study presents a picture of AF as a heterogeneous disorder with progression and complications modulated by co-morbidities. Different treatment strategies would be warranted for AF manifested primarily as an electrophysiological phenomenon, versus the final common pathway of a vascular inflammatory process [24].

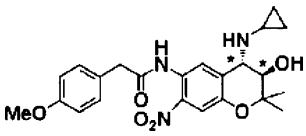
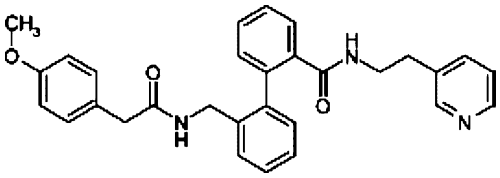
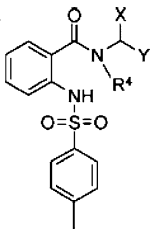
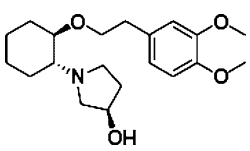
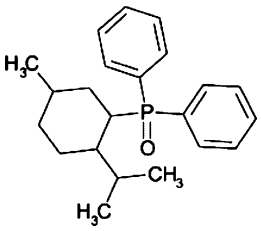
In this evolving clinical context, antiarrhythmic drugs are commonly used for the treatment of AF, to restore sinus rhythm, to facilitate electrical cardioversion, to prevent AF recurrence, and to control ventricular rate [25]. The discovery and development of novel antiarrhythmic agents for the treatment of AF can be best understood in the framework of a recent clinical debate comparing rhythm control versus rate control. Recently, AFFIRM, a large-scale randomized placebo-controlled clinical trial, compared the strategies of rhythm and rate control for the treatment of AF, with all-cause mortality as an end point. The rhythm control group included antiarrhythmic agents with different and varied mechanisms of action: amiodarone, disopyramide, flecainide, moricizine, procainimide, propafenone, quinidine, sotalol, and combinations of these drugs. The rate control group included beta-blockers, calcium-channel blockers (verapamil and diltiazem), digoxin, and combinations of these drugs. No survival advantage was found between either strategy on an "intention-to-treat" basis [26]. Other prospective studies, smaller in

scope and with different end points, have come to similar conclusions [27]. In spite of these findings, a more recent "on treatment" analysis of the AFFIRM data associates the presence of sinus rhythm with reduced mortality [28]. Furthermore, AFFIRM substudies have indicated differences in the advantage and risk of specific antiarrhythmic agents. For example, a comparison among amiodarone, sotalol (both considered to be class III or action potential prolonging agents), and class I agents (sodium current-blocking) concluded that amiodarone provides significantly more advantage and less risk than sotalol or class I agents [29]. A separate systematic meta-analysis of several randomized trials comparing antiarrhythmic drugs came to similar conclusions [30]. Amiodarone, generally proven to be the most effective antiarrhythmic agent for atrial and ventricular arrhythmias, is unique and very nonselective in that it prolongs action potential duration and refractoriness of all cardiac fibers and acts through blockade of sodium channels, β-receptors, the L-type calcium channel, and several potassium channels. Although it rarely causes the lethal ventricular arrhythmia torsade de pointes, its use is limited by serious noncardiovascular end-organ toxicities [19, 25]. Thus, the preeminence of amiodarone in the clinical setting underscores the need for better class III antiarrhythmics. Most clinically available class III agents block at least one of the major cardiac repolarizing K^+ currents, I_{Kr} or I_{Ks}, which are expressed in both human atria and ventricles. Because these agents prolong ventricular refractoriness and the electrocardiographic QT interval, they pose a risk for ventricular proarrhythmia, which limits their safety and utility for treatment of atrial arrhythmias [12, 13, 19, 25, 31, 32].

2 Novel Kv1.5 Channel Inhibitors

Efforts to develop safer and more selective class III atrial antiarrhythmics targeting the ultrarapidly activating delayed rectifier K^+ current, I_{Kur}, and human Kv1.5 (hKv1.5), the cloned channel pore-forming subunit underlying I_{Kur}, have been systematically reviewed, including information disclosed only in patent applications [33–36]. Here, we include new structural series, or new demonstrations of biological activity with some of the structures previously reviewed. Tables 1 and 1 show salient examples of the discovery and development of Kv1.5 channel inhibitors for AF: benzopyrans NIP-141 and NIP-142, by Nissan [37–40]; bisaryls, such as AVE0118 (and the related compound, AVE1231), and anthranilic acid amides, by Sanofi-Aventis [41–43]; the phenethoxycyclohexane vernakalant (RSD1235), and related 2-aminoalkyl ethers by Cardiome [44–46]; phosphine oxides, such as DPO-1 [47–49], diisopropylamides [50], and isoquinolinones [51, 52], by Merck; tetrahydroindolone-derived semicarbazones, and tetrazole derivatives by Procter and Gamble [53, 54]; aryl sulfonamide indanes, by Icagen and

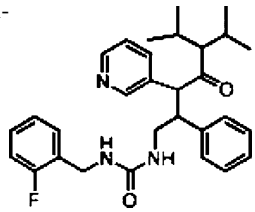
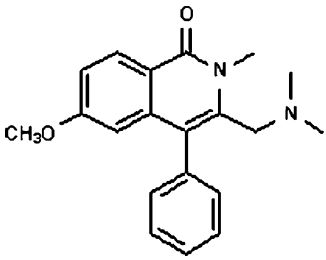
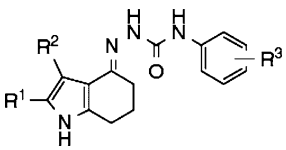
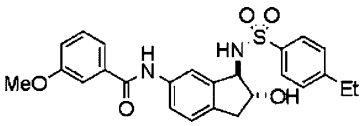
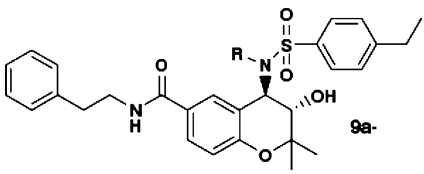
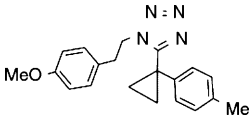
Table 1 Novel Kv1.5 channel blockers

Compound designation	Structure	Kv1.5 IC_{50} (μM)	hERG/I_{Kr} IC_{50} (μM)	Refs.
NIP-142 (free amine)		4.75	N.A.	[39] [37]
NIP-141 (hydrochloride)		5.3	≫ 5	
AVE0118		1.1	10	[42]
Anthranilic acid amide 3i ($R^4 = CH_2Ph$, X = H, Y = 3-pyridyl)		0.7	≫ 10	[43]
RSD1235		13	21	[44]
DPO-1		0.16	≫ 3	[47]

Bristol–Myers Squibb [55]; and benzopyran sulfonamides, by Bristol–Myers Squibb [56].

Historically, the strategies pursued in the discovery of Kv1.5 inhibitor lead compounds have varied: chemistry utilizing potassium channel modulator "privileged structures" as cores or scaffolds, as previously reviewed [34]; rescaffolding of disclosed chemical series [41, 54, 56]; and pharmacophore

Table 1 Novel Kv1.5 channel blockers (cont.)

Compound designation	Structure	Kv1.5 IC_{50} (μM)	hERG/I_{Kr} IC_{50} (μM)	Refs.
1-[3-(Diisopropyl-carbamoyl)-2-phenyl-3-(pyridin-3-yl)propyl]-3-(2-fluoro-benzyl)urea		0.15	N.A.	[50]
ISQ-1		0.11	5.3	[51, 52]
Tetrahydro-indolone-derived semi-carbazone 8i (R^1 = Me, R^2 = Et, R^3 = H)		0.13	21	[53]
Aryl sulfonamide indane (1R, 2R)		0.033	> 10	[55]
Amide benzopyran analog 9a (R = *n*-Bu)		0.057	> 10	[56]
Tetrazole derivative 2f		0.33	16 (48-fold)	[54]

modeling followed by 3D similarity determinations to identify potential leads [43]. In addition, leads belonging to truly novel chemical series have been increasingly mined from compound collections and characterized with the recent availability of high-content, high-throughput screens in the last decade. A separate strategy that has been pursued is the discovery of amiodarone analogs, such as dronedarone and SSR149774C [57, 58]. This strategy is not concerned with a particular mechanism for providing atrial-specific action potential prolongation and increased refractoriness, but rather with preserving the apparent benefits of mixed ion channel activity that amiodarone provides, while aiming to minimize its adverse effects.

Significant effort has been focused on bicyclic scaffolds, such as chromanes and indanes, but only recently published research has attempted to establish the role of conformational rigidity in these series, utilizing bicyclic substitutions, or substitution with acyclic derivatives. On the one hand, results from this type of chemistry on an indane scaffold suggested that conformational constraint is not required for Kv1.5 activity [55]. On the other hand, during the study of tetrahydroindolone-derived semicarbazones (see 8i in Table 1), it was observed that replacement of the tetrahydroindolone core with other heteroaromatic cores provided inactive compounds [53].

Some of the other scaffolds used to synthetize Kv1.5 channel inhibitors are prototypes of vicinally substituted heterocycles, such as the tetrazole 2f shown in Table 1. This series was developed to circumvent a metabolic liability of thiazolidinone derivatives previously identified as potent Kv1.5 blockers [54]. Some chemistry efforts have been directed toward novel structures that provide practical advantages in addition to Kv1.5 potency. This is the case of Kv1.5 inhibitors based on a diisopropyl amide scaffold [50].

Structure–activity relations within chemical series have helped establish whether specific scaffolds and substitutions are an integral part of the Kv1.5 pharmacophore, or simply help display it. Frequently, property changes unrelated to Kv1.5 activity, such as unacceptable pharmacokinetic profiles, have prevented a thorough understanding of a particular pharmacophore. Other times, detailed pharmacophore characterization is compromised by a need to preserve or improve desirable properties in addition to Kv1.5 activity, such as oral bioavailability [43] or *P*-glycoprotein susceptibility, a predictor of reduced brain exposure [50].

Despite the large variety of molecules recently described as desirable Kv1.5 inhibitors, there has been limited success in predicting ligand-based Kv1.5 pharmacophores. Recently, a pharmacophore model was derived from several members of the bisaryl series (see Table 1) and another series. The model consists of three hydrophobic centers in a triangular arrangement, and it was used to identify anthranilic amides as novel Kv1.5 inhibitors [43]. A similar paucity of published data exists with regard to pharmacophores based on the Kv1.5 channel structure [59].

All these novel agents inhibit the Kv1.5 channel, but without absolute selectivity; however, some are more selective than others. RSD1235 and related 2-aminoalkyl ethers are at best mixed ion channel inhibitors, blocking a number of ionic currents, including I_{Kur}, the transient outward potassium current (I_{to}), the rapidly activating delayed rectifier potassium current, I_{Kr} (hERG), and the cardiac sodium current, all with comparable potencies [44–46]. Molecular Kv1.5 selectivity issues aside (see Sect. 3 below), atrial-specific in vivo efficacy by many of these agents has been shown in a number of preclinical AF models. DPO-1 and ISQ-1 have shown a selective increase in atrial versus ventricular effective refractory period, and afford AF termination in acute cardiac electrophysiological studies in anesthetized dogs [49, 52]. Similar effects on atrial refractory period have been shown for DPO-1 in African green monkeys [48].

Representative members of the biphenyl class described above, namely AVE0118, AVE1231, S9947, and S20951, produce dose-dependent increases in atrial effective refractory period in anesthetized pigs [40, 60]. AVE0118 and AVE1231 have shown similar efficacy in a goat model of persistent AF [40, 61, 62]. Semicarbazone 8i and tetrazole 2f produce selective prolongation of atrial effective refractory period in anesthetized mini-pigs [53, 54]. RSD1235 has demonstrated efficacy increasing atrial refractoriness, reducing paroxysmal AF and cardioverting AF to sinus rhythm in a goat model, while having no effects on ECG or hemodynamic parameters in telemetered conscious dogs [45].

Among the chemical structures reviewed here, only RSD1235 has been reported to show clinical efficacy. It rapidly converts recent-onset AF in $\sim$ 50% of patients in clinical phase II and III studies, as recently reviewed [45]. In addition to the disclosed chemical series, other agents have been reported to impart preclinical AF efficacy by effects on Kv1.5. AZD7009 displays mixed ion channel activity, similar to RSD1235 [63], while XEN-D0101 is presumably highly selective for I_{Kur} [12]. Both drugs have progressed to clinical trials, but AZD7009 apparently has been withdrawn due to extra-cardiac side effects [12].

3 Kv1.5 Selectivity

The agents discussed here display varying degrees of selectivity for Kv1.5 over other ion channels. Some of them, such as AVE0118, have been found to block I_{to} and the acetylcholine activated potassium current, I_{KAch}, with potency similar to that of Kv1.5. RDS1235 and AZD7009 appear to derive benefit from an additional block of cardiac sodium channels. One frequently disclosed indicator of selectivity, because of the concern about life-threatening proarrhythmic effects, is the effect of drugs on the hERG channel, the struc-

tural correlate of I_{Kr} and a primary determinant of ventricular proarrhythmic effects. Tables 1 and 1 summarize IC_{50} values of Kv1.5 and hERG for the lead compounds in the chemical series of interest.

In this discussion of Kv1.5 selectivity, it is worth mentioning that a large variety of compounds, outside of discovery efforts explicitly focusing on Kv1.5 as a therapeutic target, have been found to inhibit Kv1.5. Not only a large number of antiarrhythmic agents, but also many compounds developed for therapeutic classes other than ion channels have been reported to inhibit the channel, in many instances at their therapeutic concentrations [34].

This observation is not entirely surprising, since most compounds inhibiting ion channels appear to do so by blocking the inner cavity of the channel pore. There is some degree of structural conservation among ion channels, and a high degree of structural similarity among potassium channel families, particularly around the pore-forming region. The crystallographic characterization of prototypical potassium channel pores [64–66], in conjunction with primary sequence comparisons around the pore region, has served to highlight commonalities and specific differences among cloned potassium channels.

Figure 1 compares the amino acid sequence of several voltage-dependent potassium channel alpha subunits across the putative pore region. This sequence alignment underscores the challenges not only for strategies seeking absolute Kv1.5 specificity, but also for the feasibility of attaining atrial versus ventricular selectivity. Little sequence divergence exists among members of the KCNA (Kv1.x) family; more when comparing relevant members of the KCNB (Kv2.1), KCNC (Kv3.1), KCND (Kv4.3), KCNQ (KvLQT1), and KCNH (hERG) families. The major contributors to ventricular repolarization are the rapidly and slowly activating delayed rectifier potassium currents, I_{Kr} and I_{Ks}, with alpha subunits encoded, respectively, by KCNH2 and KCNQ1 (hERG and KvLQT1, Fig. 1). As the sequence alignment indicates, there is structural conservation of the selectivity filter, underlying a commonly shared mechanism of selective ion conduction in K^+ channels, and variation of the inner pore structure, related to conformational changes that open and close the pore. Notable examples of functionally connected divergence are the outer vestibular turret and the S6 helix.

Figure 2 highlights structural landmarks of the Kv1.5 pore. It associates the amino acid sequence of Kv1.5 with important domains of the pore-forming region (top), and shows a schematic cross-sectional representation of the Kv1.5 channel pore (bottom), derived from homology to the crystal structure of prototypical potassium channels [64–66]. In assessing the design of highly selective Kv1.5 blockers, our review of Kv1.5 structural information and drug–channel interactions is focused on domains of the channel pore that appear to be directly "blockable" targets. In addition, we review limited, yet intriguing, evidence of functional allosteric inhibition in the Kv1.5 channel that may prove relevant to the discovery of Kv1.5-selective drugs [67, 68].

```
                                                                  Outer helix/S5
hKv1.5      MSLAILRVIRLVRVFRIFKLSRHSKGLQILGKTLQASMRELGLLIFFLFIGVILFSSAVYFAE
hKv1.1      TSLAILRVIRLVRVFRIFKLSRHSKGLQILGQTLKASMRELGLLIFFLFIGVILFSSAVYFAE
hKv1.2      MSLAILRVIRLVRVFRIFKLSRHSKGLQILGQTLKASMRELGLLIFFLFIGVILFSSAVYFAE
hKv1.3      MSLAILRVIRLVRVFRIFKLSRHSKGLQILGQTLKASMRELGLLIFFLFIGVILFSSAVYFAE
hKv1.4      MSFAILRIIRLVRVFRIFKLSRHSKGLQILGHTLRASMRELGLLIFFLFIGVILFSSAVYFAE
hKv1.6      MSLAILRVIRLVRVFRIFKLSRHSKGLQILGKTLQASMRELGLLIFFLFIGVILFSSAVYFAE
hKv2.1      NVRRVVQIFRIMRILRILKLARHSTGLQSLGFTLRRSYNELGLLILFLAMGIMIFSSLVFFAE
hKv3.1      DVLGFLRVVRFVRILRIFKLTRHFVGLRVLGHTLRASTNEFLLLIIFLALGVLIFATMIYYAE
hKv4.3      ---GAFVTLRVFRVFRIFKFSRHSQGLRILGYTLKSCASELGFLLFSLTMAIIIFATVMFYAE
hKvLQT1     FATSAIRGIRFLQILRMLHVDRQGGTWRLLGSVVFIHRQELITTLYIGFLGLIFSSYFVYLAE
hERG        GSEELIGLLKTARLLRLVRVARKLDRYSEYGAAVLFLLMCTFALIAHWLACIWYAIGNMEQPH
                  .  .:  :::*:.:. *:       * .:          :      :      :  ..

                       Turret              Pore helix
hKv1.5      AD----------------------NQGTHFSSIPDAFWWAVVTMTTVGYGDMRPIT
hKv1.1      AE----------------------EAESHFSSIPDAFWWAVVSMTTVGYGDMYPVT
hKv1.2      AD----------------------ERESQFPSIPDAFWWAVVSMTTVGYGDMVPTT
hKv1.3      AD----------------------DPTSGFSSIPDAFWWAVVTMTTVGYGDMHPVT
hKv1.4      AD----------------------EPTTHFQSIPDAFWWAVVTMTTVGYGDMKPIT
hKv1.6      AD----------------------DDDSLFPSIPDAFWWAVVTMTTVGYGDMYPMT
hKv2.1      KD----------------------EDDTKFKSIPASFWWATITMTTVGYGDIYPKT
hKv3.1      RIGAQPNDPSA-------------SEHTHFKNIPIGFWWAVVTMTTLGYGDMYPQT
hKv4.3      KG----------------------SSASKFTSIPASFWYTIVTMTTLGYGDMVPKT
hKvLQT1     KDAVNE------------------SGRVEFGSYADALWWGVVTVTTIGYGDKVPQT
hERG        MDSRIGWLHNLGDQIGKPYNSSGLGGPSIKDKYVTALYFTFSSLTSVGFGNVSPNT
                                          .   .:::   ::*::*:*:  * *

                  Inner helix/S6
hKv1.5      VGGKIVGSLCAIAGVLTIALPVPVIVSNFNYFYHRETDHEEPAVLKEEQGTQSQ------------
hKv1.1      IGGKIVGSLCAIAGVLTIALPVPVIVSNFNYFYHRETEGEEQAQLLHVSSP---------------
hKv1.2      IGGKIVGSLCAIAGVLTIALPVPVIVSNFNYFYHRETEGEEQAQYLQVTSC---------------
hKv1.3      IGGKIVGSLCAIAGVLTIALPVPVIVSNFNYFYHRETEGEEQSQYMHVGSCQ--------------
hKv1.4      VGGKIVGVLCAIAGVLTIALPVPVIVSNFNYFYHRETENEEQTQLTQNAVSCPY------------
hKv1.6      VGGKIVGSLCAIAGVLTIALPVPVIVSNFNYFYHRETEQEEQGQYTHVTCGQP-------------
hKv2.1      LLGKIVGGLCCIAGVLVIALPIPIIVNNFSEFYKEQKRQEKAIKRREALERAKRNGSIVSMNMKDA
hKv3.1      WSGMLVGALCALAGVLTIAMPVPVIVNNFGMYYSLAMAKQKLPKKKKKHIPRP-------------
hKv4.3      IAGKIFGSICSLSGVLVIALPVPVIVSNFSRIYHQNQRADKRRAQKKARLARIR------------
hKvLQT1     WVGKTIASCFSVFAISFFALPAGILGSGFALKVQQKQRQKHFNRQIPAAASLIQTAWRCYAAEN--
hERG        NSEKIFSICVMLIGSLMYASIFGNVSAIIQRLYSGTARYHTQMLRVREFIRFHQIPNPLRQRLEEY
                  ..    : .    *     :   :          .
```

Fig. 1 Structural considerations about drug binding in the Kv1.5 channel pore. Amino acid sequence comparison of alpha subunit sequences from human Kv1.1–1.6 and Kv2.1, Kv11.1 (hERG), Kv7.1 (KvLQT1). Symbols indicate complete amino acid conservation (*), conservative substitution among all aligned sequences (:), and critical substitution in a generally conserved position (.)

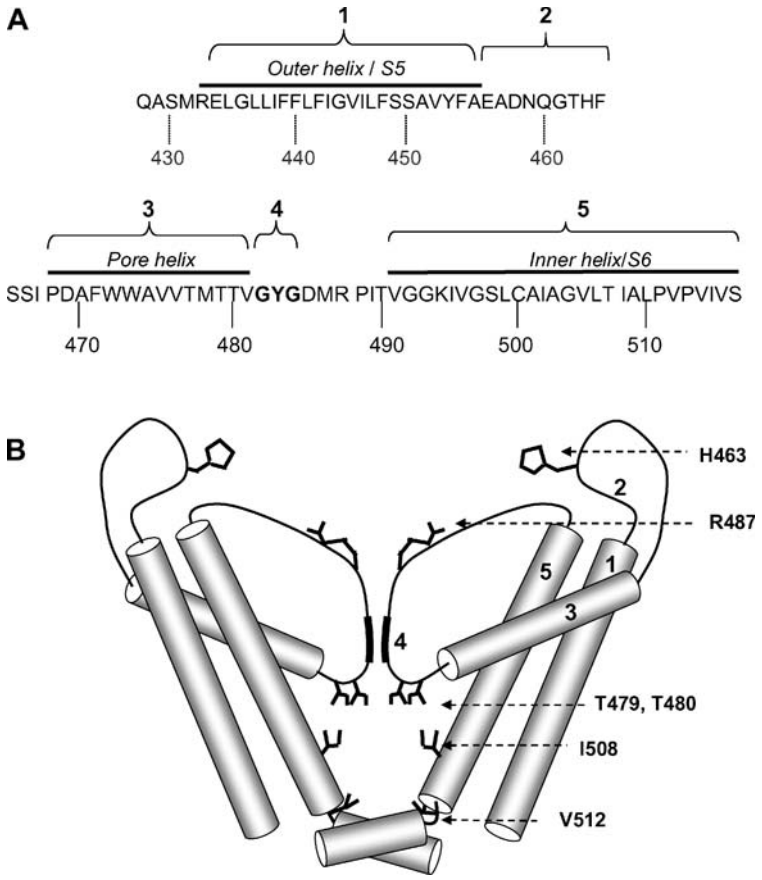


Fig. 2 Schematic representation of Kv1.5-selective features in the putative pore region. **a** Amino acid sequence of the human Kv1.5 alpha subunit around the pore region, as predicted by homology to the KcsA bacterial potassium channel, along with predicted structural landmarks. **b** Diagrammatic spatial representation of the Kv1.5 pore, deduced by comparison with elucidated crystal structures for other potassium channels [37]. A cross section of the channel pore revealing domains of two Kv1.5 alpha subunits in their predicted spatial orientation is shown. The side chains of nonpolar amino acids I508 and V512, and polar amino acids T479 and T480, are depicted as important determinants of drug block within the context of structural landmarks of the Kv1.5 channel pore: *1* the pore helix, N-terminal component of the pore loop; *2* the GFG signature sequence/selectivity filter in the nonhelical portion of the pore loop; *3* R487 in the outer vestibular loop; *4* the S6 or inner helix

The elucidation of structural pharmacophores in the Kv1.5 channel pore has not been nearly as extensive as analogous efforts conducted on hERG [69]. Briefly, in these types of studies, a drug of interest is used to probe a battery of single amino acid substituted channels with properties similar to wild type channels, to "scan" a structural domain and identify de-

terminants of drug binding. For the hERG channel, this strategy has been used to help elucidate the molecular basis of adverse cardiac effects, identifying determinants for the binding of high affinity methanesulfonanilides [69], and recognizing that these determinants may not be the sole contributors to the binding of lower affinity agents [70, 71]. A similar strategy was recently used to identify structural determinants for the binding of an anthranilic acid amide to the Kv1.5 potassium channel pore [72]. Residues T-479 and T-480 in the pore loop, just upstream of the GYG signature sequence, and residues V505, Ile-508, and Val-512, on the inner helix/S6 segment facing the inner cavity, were identified as important drug-binding elements. In the context of the more detailed characterization of drug binding residues in the hERG potassium channel pore, it is important to note that Ile-508 and Val-512 occupy positions equivalent to Tyr-652 and Phe-656, key residues for the binding of methanesulfonanilides as high affinity ligands to the hERG channel [69]. The importance of V505, I508, V512, and several other S5 and S6 helix residues shared by Kv1.x channels has also been independently confirmed during the characterization of structural determinants of high affinity Kv1.3 binding by the immunosuppressant correolide [73, 74]. Figure 2 highlights the critical role of these specific amino acid residues in the Kv1.5 pore.

Other features depicted in Fig. 2 are: the S6 helix interrupted by a proline–valine–proline motif, yielding a pore cavity comparable to that of other Kv potassium channels, except for the hERG channel family, where the P-*x*-P "kink" is completely absent; the GYG signature sequence characteristic of the potassium selective filter; and finally, a characteristic arginine side chain, several residues downstream (R487), and a histidine side chain (H463) implicated in Kv1.5 slow inactivation. It is important to mention R487, since it is predicted to reside on the outer vestibule of the channel pore at a position analogous to Y379 in Kv1.1. As shown in Fig. 1, this particular residue is substituted among various Kv alpha subunits. This residue, corresponding to Shaker T449 and Kv1.1 Y379, has been extensively studied as a structural determinant of external tetraethylammonium selectivity among potassium channels. It has also been shown that R487 substitutions in Kv1.5 confer TEA sensitivity [75]. Mechanistically, π–cation interactions involving Y379 have been proposed to mediate an observed decrease in TEA sensitivity with the stoichiometric substitution of these residues in the tetrameric channel [76–78]. Recently, this specific mechanism has been challenged, but the importance of the local environment in the outer vestibule has not [79]. Furthermore, the involvement of this Kv channel region in permeation and gating [80, 81], and sensitivity to potent peptide toxins [82, 83], suggests that it may be a structural pharmacophore of interest, particularly to select a homotetrameric channel of choice over other homotetramers or heterotetramers.

Ironically, molecular tools like TEA and charybdotoxin, utilized to characterize this region of Kv channels, bind with low affinity to Kv1.5 homotetramers, and the Kv1.5-specific domains in the outer vestibule cannot be probed with these traditional reagents. Recent studies, however, seem to validate the outer vestibule of Kv1.5 as a pharmacological target. One of these studies focused on the slow, P/C type inactivation process, a process that is similar to P and C type inactivation processes as studied in other Kv channels, but with subtle differences [75, 81]. The substituted cysteine accessibility method was used to characterize the turret of the Kv1.5 outer vestibule and, in particular, the importance of protonatable residue H463 (see Fig. 2). Prior evidence had already pointed to the importance of H463 in Kv1.5 P/C inactivation, by interactions with R487 that can only be described as complex, allosteric, and not simply electrostatic [84–86]. In this study, cysteine-substituted channels, themselves not too different from wild type channels, could be covalently bound to cysteine-modifying reagents, and the current through modified channels was irreversibly inhibited due to effects on P/C inactivation. The modifying agents used (positively or negatively charged methanethiosulfonates) suggested that the effect was not related to charge, and that it was probably not the result of direct plugging of the pore [67]. Thus, Kv1.5 signature side chains in the outer vestibule of the pore and in the turret that forms a rim around it are amenable to effective pharmacological intervention. A separate study points to the feasibility of discovering novel, small organic molecules targeting the domain spanning R487, albeit in other Kv channels. In this study, novel potassium channel inhibitors were discovered in a high-throughput rescue screen in yeast [87]. One of these, 48F10, was a novel agent with a norbornyl group on a catechol ring, and no charge or amines in its chemical structure. Additional characterization identified the molecule as a neuroprotective Kv2.1 channel inhibitor targeting the channel's outer vestibular domain. One of the approaches used to demonstrate that 48F10 acted at a site overlapping the site of external TEA block was a comparison of the inhibitory potency of 48F10 on Kv1.5 channels rendered TEA sensitive by tyrosine substitution ($IC_{50} = 6\ \mu M$) versus wild type Kv1.5 channels ($IC_{50} > 50\ \mu M$). Subsequent studies suggested that the specific binding to the outer vestibule of Kv2.1 was imparted not by the catechol moiety, a previously described low affinity blocker of potassium channels, but by the novel norbornyl group [88]. It might prove difficult to encounter a truly high affinity, low molecular weight, organic ligand for the Kv1.5-specific outer vestibule environment. In this regard, it is worth noting that this domain has already been recognized as a protein-derived pharmacophore, although not pursued for virtual screening efforts [59].

Structural domains in Kv1.5 targeted by posttranslational modifications have been widely studied, primarily in heterologous systems, sometimes as prototypes, in the context of fundamental Kv channel posttranslational regulation [89]. Attention to these studies can be of interest for Kv1.5 drug

discovery, especially if the domains in question are Kv1.5-specific and pharmacologically amenable. In this context, recent evidence of allosteric effects on Kv1.5 inactivation by small ubiquitin-like modifier (SUMO) proteins is worth mentioning. After identifying a Kv1.5-specific SUMO binding motif and a second one shared with Kv1.1 and Kv1.2, investigators demonstrated that loss of SUMOylation by motif disruption shifted the voltage dependence of steady-state inactivation [68]. Besides Kv1.5 specificity and suitability for pharmacological intervention, a related consideration is whether modulatory processes described for Kv1.5 in heterologous systems are relevant to native I_{Kur}. While there are many published studies and reviews about Kv1.5 phosphorylation or phosphorylation-independent kinase interactions [90–97], glycosylation [98], ubiquitination [99], and interaction with beta or other regulatory subunits [100–105], information rooted on I_{Kur} in myocytes [106–108] is much more limited.

Whether directly "blockable" or "allosteric", common structural motifs with sequence specificity appear to be emerging in the study of Kv channels. An intriguing notion is the possibility of identifying specific high affinity ligands for Kv1.5 or other Kv channels by starting with information systematically derived with probing partially selective agents on two or more of their preferred target channels, and using techniques based on site-directed mutagenesis like those reviewed above. A recent example of Kv1.5 structure-based pharmacophore elucidation was the identification of novel leads, starting from potassium channel crystallographic data of the bacterial KcsA channel pore and homology modeling of the Kv1.5 pore domain [59]. These studies led to the modeling of a Kv1.5 protein-derived pharmacophore, the use of this pharmacophore in virtual queries, and the identification of novel chemical leads complementary to the determination of ligand-based pharmacophores.

Although most of the novel agents described in this review affect a variety of other cardiac K^+ channels, including Kv4.3/I_{to}, I_{KAch}, and even I_{Kr} and I_{Ks} [37, 42], in some cases with affinities comparable to their affinity for Kv1.5, a certain degree of atrial-selective efficacy has been demonstrated for many of them. This collective finding has shifted the discussion to achieving AF therapeutic efficacy by other means: affinity for several targets concentrated in atria [45, 61], affinity for particular states of the Kv1.5 channel maximizing efficacy during AF [47, 48], and even strategies aimed at minimizing adverse ventricular effects and maximizing efficacious atrial effects, such as the combination of an antiarrhythmic drug with a calcium-channel blocker, and the reduction of the duration of antiarrhythmic drug therapy targeted to periods of symptomatic or likely AF recurrence [109]. Regrettably, in this context, the concept or principle of Kv1.5 as a therapeutic target for AF remains in search of definitive proof.

4
Tools for Discovery and Lead Optimization of Kv1.5 Inhibitors

The first step to drug discovery typically starts with high-throughput screening (HTS) of millions of small chemical molecules to identify compounds that interact with the target. From here, active compounds graduate to lead optimization where they are developed to become druglike molecules. Ion channel assays that are compatible with the throughput requirements of HTS and/or lead optimization screening have become available only in the past 10 years [110]. The throughput and cost of existing assays has governed their position in screening. Here, we describe the utility and limitations of existing techniques used for Kv1.5 channel drug discovery.

The gold standard for measuring the activity of small molecules on ion channels in vitro is patch-clamp electrophysiology. Traditional patch-clamp electrophysiology incorporates the use of a glass micropipette electrode, microfabricated from glass capillary tubes, for controlling the membrane potential whilst measuring ionic current flow through ion channels expressed in the cell membrane. Via patch clamp, the activity of ion channels can be measured directly and in real time. Traditional patch-clamp electrophysiology is utilized for the most detailed mechanistic studies of Kv1.5 antagonists. Features such as state dependence, use dependence, and on/off rates of dissociation can be measured via the use of traditional patch-clamp electrophysiology [47]. These properties can be used to differentiate, and perhaps improve upon, small molecule Kv1.5 antagonists for the treatment of AF. Due to the high quality and content of the data generated by this method, there has been a longstanding need to automate and improve the speed of patch-clamp electrophysiology. In its traditional configuration, the patch clamp has a very low throughput of 10–20 recordings per person per day. To meet the need for increased throughput, several automated patch-clamp instruments have been developed and are now commercially available.

Automated patch-clamp electrophysiology became possible with the invention of the planar patch-clamp electrode. The traditional single micropipette electrode was replaced with a planar substrate with an array of microapertures. Several instruments are now commercially available that utilize this technology. Throughput varies from a single cell (Nanion, Port-a-Patch), 16 cells (Molecular Devices, PatchXpress; Nanion, Patchliner), or 48 cells at a time (Sophion, QPatch) up to 384 cells per experiment (Molecular Devices, IonWorks). All of these platforms have been utilized for evaluating Kv1.5 pharmacology. Data quality of the recordings on the Port-a-Patch, PatchXpress, and QPatch is comparable to that observed in traditional patch-clamp electrophysiology. Additionally, asynchronous operation and integrated fluidics allow for application of multiple compounds or concentrations, thereby allowing the investigation of dose–response relations with these instruments. These systems provide a 2–30-fold increase in through-

put over traditional patch-clamp electrophysiology, making them suitable for lead optimization/medicinal chemistry support [111–115]. The IonWorks Quattro is currently the highest throughput automated patch-clamp system. IonWorks utilizes single hole (one cell per well) or population patch (up to 64 cells per well) plates and provides up to a 384-fold increase in throughput over traditional patch-clamp electrophysiology. Though less flexible than the other platforms, IonWorks is well suited for the screening of focused libraries ($\sim$ 10 000 compounds) and lead optimization [116–119]. The implementation of automated patch-clamp electrophysiology for lead optimization has eliminated the bottleneck in the development of novel, small molecule Kv1.5 antagonists. The first wave of automated patch-clamp electrophysiology instrumentation has provided invaluable tools for ion channel screening; the next stage of evolution for this technology should provide improved capabilities and increased throughput.

Voltage-sensing dyes are used to track changes in membrane potential. These dyes can be used to measure the activity of Kv1.5 indirectly. The major advantage of Kv1.5 voltage-sensing dye assay is that it can be miniaturized to a 384- or 1536-well format and thus be utilized for primary HTS. The two voltage-sensing dye assays that have been utilized for HTS are the Molecular Devices membrane-potential dye kit and the fluorescence resonance energy transfer (FRET) based voltage sensing dyes. The Molecular Devices membrane-potential dye kit utilizes an oxonol derivative voltage-sensing dye which is negatively charged and associates with the outside layer of a hyperpolarized cell membrane. When the cell membrane is hyperpolarized, the dye emits a low fluorescence signal upon excitation. When the cell membrane is depolarized (i.e., the inner layer of the cell membrane becomes more positively charged) the dye moves to the inner layer of the cell membrane and the fluorescence intensity of emission of this dye increases. For cells expressing Kv1.5, depolarization of the cell membrane can be produced by increasing the concentration of extracellular K^+. This assay is ideally suited to HTS in a homogeneous format. Disadvantages of using the dye kit include high false positive hit rate, low temporal resolution of the dye, and the fact that the activity of the channel is measured indirectly [120–123]. Ratiometric, FRET based, voltage-sensing dye assays for Kv1.5 incorporate the use of a pair of dyes to monitor changes in membrane potential. The FRET donor is a coumarin dye linked to a phospholipid that inserts into the outer leaflet of the cell membrane and the FRET acceptor is an oxonol derivative. The oxonol derivative behaves identically to the Molecular Devices membrane-potential dye, moving from the outer to the inner membrane upon membrane depolarization. The coumarin is insensitive to membrane potential and produces FRET to excite the oxonol derivative when associated with the outer cell membrane. The ratiometric measurement of change in membrane potential helps to reduce assay artifacts. Disadvantages of the FRET assay include low temporal resolution of the dyes, nonhomogeneous assay format (multi-

ple wash steps required), and the fact that the activity of the channel is being measured indirectly. Despite this, several leads have been identified from primary HTS campaigns which have utilized the membrane-potential dyes as the primary readout [120, 121, 124, 125].

Two types of ion flux assays have been developed for Kv1.5 channel screening: radioactive ion flux assay and atomic absorbance spectrometry (AAS). Ion flux assays incorporate the use of a tracer ion to track channel activity. Radioactive flux assays for Kv1.5 utilize $^{86}Rb^{+}$ as a radiotracer to track the flow of ions moving into or out of a cell expressing functional K^{+} channels. AAS ion flux assays for Kv1.5 utilize a nonradioactive ion tracer (Rb^{+}). The signal-to-noise ratio of this assay is robust (six- to tenfold) and these assays are well suited for lead optimization and focused library screening. Disadvantages of using radiotracers are that these assays are not homogeneous and have a low temporal resolution [126–129].

5 Unresolved Questions

An important topic of consideration is the exact identity of the human I_{Kur} current and its counterpart in animal species used to model and validate it as an efficacious target for AF. Pharmacological and biochemical evidence indicate close identity between the human I_{Kur} current and current from heterologously expressed Kv1.5 channel subunits; notable findings are experimental reduction of I_{Kur} but not I_{to} with Kv1.5-specific antisense oligonucleotides, similar sensitivity to 4-aminopyridine, and biophysical signatures, among others [1, 3, 4]. These findings, however, do not preclude complexity such as heterotetramer formation with coexpressed alpha subunits that would not dramatically alter pharmacological properties, e.g., Kv1.2, or with beta subunits, e.g., Kvβ1.2. Quantitative and semiquantitative characterizations of Kv1.x and Kvβ subunit transcripts from human atria and other cardiac tissues, especially the most recent and systematic evaluations, say little about beta subunits, but are consistent with the notion that Kv1.5 alpha subunits are expressed at higher levels in atria than in ventricle, and that Kv1.5 homotetramers essentially constitute I_{Kur} [130–134]. In general, protein data are more limited, but support the notion. Recent Western blot analysis of human atria versus ventricle suggests protein expression mirrors transcription [131]. One outstanding concern is the reported localization of Kv1.5 protein in atrial and also in ventricular myocytes, using immunocytochemistry. Imperfect quantitation and fluorescence signal saturation could explain the apparent discrepancy [135]. It should be understood, in any event, that atrial-selective Kv1.5 expression in the human heart means fold differences in mRNA and protein levels, and not absolute absence in the ventricle.

An anticipated concern with Kv1.5 as atrial-specific target is its prominent expression in vascular smooth muscle, in macrophages, and at some level in the central nervous system. In this regard, it is instructive to compare studies of I_{Kur} in human atria with studies of native potassium currents in vascular smooth muscles [136–138] or macrophages [139], where Kv1.5 has been found to play a major physiological role, but not as a homotetramer. In these instances, biophysical observations suggested heteromerization early on, and biochemical characterization confirmed it. Since most evidence to date validates the idea of I_{Kur} channels as Kv1.5 homotetramers, compounds able to distinguish Kv1.5 homomers from heteromers would appear to be advantageous.

A separate complexity, observed in murine, canine, and human Kv1.5 expressed in atria, is reports of alternatively amino-terminal spliced variants imparting functional differences on inactivation properties [140, 141]. This would not appear to pose a major problem for existing drug discovery strategies, targeting primarily the Kv1.5 pore. Other issues connected with the identity of I_{Kur} are potential species differences. This is a concern for preclinical strategy and validation of AF model systems. The role of Kv1.5 versus Kv3.1 channel subunits in the dog has been controversial. Kv3.1, and not Kv1.5, was proposed to be a structural determinant of dog I_{Kur}, based on pharmacological, electrophysiological, and biochemical characterization [142, 143]. Recently, contradictory evidence suggested that Kv1.5 contributes to the canine atrial I_{Kur} current. Despite biochemical absence of Kv3.1 in this study, there was indication of some heterogeneity in I_{Kur} current sensitivity to external TEA [144]. Since Kv3.1 is known to be TEA sensitive and known not to form heterotetramers with Kv1.5 [47], the coexpression of two distinct ultrarapid currents in dog atria cannot be excluded.

Investigators have argued that a reasonable level of mixed ion channel activity does not prevent atrial-selective, efficacious action toward sinus rhythm maintenance and restoration, but actually enhances it [42, 45]. In fact, an outstanding question is whether Kv1.5 selectivity, as opposed to an atrial-selective profile, is truly beneficial in all AF therapeutic settings. It has been argued, for example, based on action potential prediction considerations [9] and on studies in human atrial trabeculae [145], that blocking of I_{Kur} may affect the early repolarizing phase of the action potential and have proarrhythmic consequences in normal atria, while providing benefit in remodeled atria [12]. Another paradoxical finding regarding Kv1.5 and AF is recent evidence from a study of familial AF, suggesting that Kv1.5 loss of function is associated with AF. A nonsense mutation yielding a truncated Kv1.5 subunit was identified in a familial case of AF, during an evaluation of patients diagnosed with idiopathic AF, but not in 540 unrelated control individuals. The effect of the mutation was a dominant-negative loss of function that could be rescued by translational read-through of the stop codon [146]. Separate reports have shown reduced Kv1.5 protein expression in atria from chronic

(persistent) and paroxysmal AF patients [147, 148]. These findings contrast with previously described and reviewed familial AF gain-of-function mutations in KCNQ1 (KvLQT1) and KCNE2 (minK related protein), and with the prevailing notion linking AF with reduced refractoriness and atrial action potential shortening [149].

In conclusion, there is a clear need for a better understanding of AF, in all its manifestations, as well as for proof of concept molecules with better Kv1.5 selectivity, to address in a more definitive manner questions of clinical efficacy and safety with this type of agent.

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