Cancer Drug Discovery and Development

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Handbook of Anticancer Pharmacokinetics and Pharmacodynamics

Second Edition

Humana Press

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Preface

The *Handbook of Anticancer Pharmacokinetics and Pharmacodynamics* was designed with the objective of having a single reference on clinical pharmacology to serve as a guide to drug development with a focus on cancer therapy. The first edition of the handbook was organized to closely follow a logical flow of events of the drug development plan from identification of cancer-specific targets to preclinical testing to clinical trial design and all phases of clinical trials.

In this thoroughly updated and expanded second edition, we embarked on an even more comprehensive approach to adapt to the ever-changing drug development landscape, highlighting the recent changes involved in shifting the paradigm of the process over the last decade. The outline and objective of the handbook remain focused on a roadmap for moving an agent toward NDA submission. We have incorporated in this revised second edition new material on phase 0 trials in oncology, organ dysfunction trials, drug formulations, and their impact on anticancer drug pharmacokinetics/pharmacokinetics including strategies to improve drug delivery, pharmacogenomics and cancer therapy, high-throughput platforms in drug metabolism and transport pharmacogenetics, imaging in drug development, and nanotechnology in cancer. Together these chapters provide for a comprehensive overview of anticancer drug development.

Advances in understanding the molecular basis of cancer are critical for oncology drug researchers to translate molecular targets into new therapies. As the search for cancer-specific targets continues over the next decade, advances in drug discovery and development efforts are underway, and a practical guide detailing the underlying principles of these processes will be invaluable to cancer researchers. We hope to achieve this with an indispensable reference that should be of interest to both the clinical pharmacologist and the pharmaceutical scientist.

We would like to thank all of the authors for their thoughtful and thorough contributions. Our task of compiling this book was made easy by their high-quality efforts. We continue to be conscious of our patients who keep us focused on the goal of finding treatments and cures for all types of cancers.

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Molecular Targets

Christina M. Annunziata and Phillip A. Dennis

Abstract The optimal targeting of cancer requires not only the selection of the target but also the identification of the patients whose cancer depends on the targeted pathway. The objective of this chapter is to give an overview of molecular targets in cancer therapeutics. Targets have been categorized as either established or novel types. Established targets include those against which most currently licensed anticancer drugs were developed and include DNA, microtubules, and nuclear hormone receptors. Novel targets are those under current preclinical and clinical investigation. The section on novel targets emphasizes the relationships of the novel targets to the biological traits of cancer.

Keywords Drug targets • Signaling pathways • Cancer treatment

1 Introduction

Cancer is a major cause of mortality throughout the world. There are an estimated 10.9 million new cases and 6.7 million deaths from cancer worldwide [1]. Cancer cells differ from normal cells by the following hallmark traits [2]: (a) ability to proliferate due to self-sufficiency in growth signals, (b) insensitivity to growth inhibitory signals, (c) evasion of apoptosis and senescence, (d) limitless replication potential, (e) sustained angiogenesis, and (f) potential to invade tissue and metastasize. Although each of these traits may be targeted for drug development, two additional areas are important: chemoprevention and modulation of resistance (Fig. 1).

Over 100 anticancer drugs have been approved by the US Food and Drug Administration (FDA) since the use of mustine to treat a patient with acute lymphoblastic leukemia in 1943 [3]. Although the development of new anticancer drugs has lead to cure of some patients with rare cancers such as childhood leukemia and testicular cancer, conventional drug development has provided only incremental improvements in survival for the majority of cancers.

Traditionally, anticancer drug development has focused on DNA as a target, based on the fact that a high turnover of nucleic acids in cancer cells during DNA replication and cellular proliferation will provide a therapeutic margin. The molecular biology revolution, epitomized with the sequencing of

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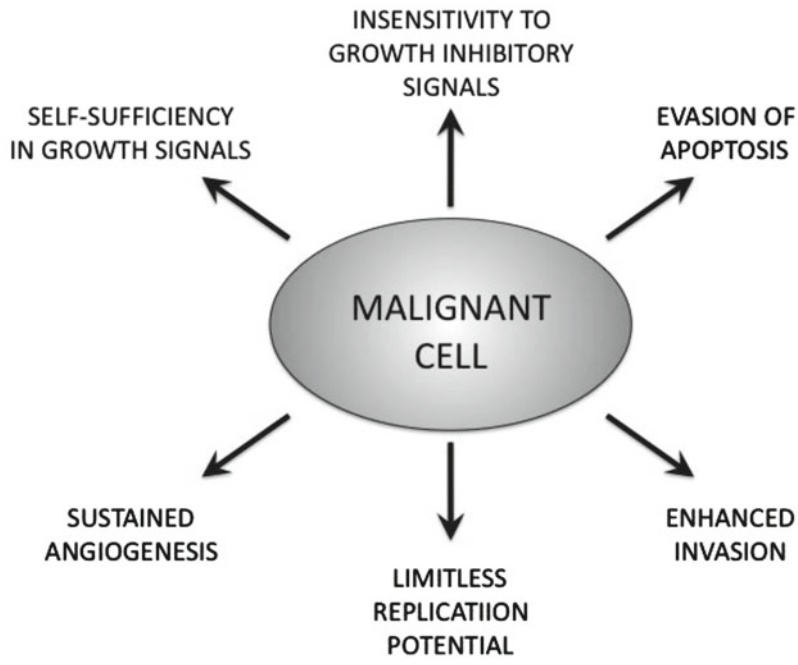

Fig. 1 Characteristic hallmarks of a malignant cell are targeted for anticancer drug development

the human genome and individual cancer genomes, has increased our understanding of cancer at a basic level. More recent efforts in drug development build on this improved understanding of the molecular basis of cancer and use this information to identify and validate new targets for rational drug development [4].

Criteria for target identification and validation include:

- Evidence of pathological deregulation, including mutation leading to constitutive activity of an oncogene or loss of a critical tumor suppressor gene.
- Creation of a malignant phenotype by mutation or increasing or decreasing expression of proposed target.
- Reversal of a malignant process by correcting the genetic abnormality by gene knockout, RNA interference, or transfection.
- Evidence of adverse clinical outcome correlating with target deregulation.

The emergence of high-throughput "-omics" screening and discovery methods will add to the already large number of targets that are a focus for new anticancer drug development [5]. Once a target has been validated, it can be channeled into the drug discovery process that includes screening, lead identification, lead optimization, preclinical toxicology, and clinical trials [6]. Better understanding of the biology and molecular pathology of cancer, coupled to improvements in innovative technologies, is crucial to every step of the drug development process. High-throughput screening, combinatorial chemistry, and the input of structural biology play important roles in lead identification and optimization.

Previously, pharmacokinetic, efficacy and toxicology profiles were the most important criteria as to whether a compound would be a viable candidate for clinical development. With identification and validation of new molecular targets, rational drug design now provides greater opportunity for improving the therapeutic indices of new drugs. Moreover, pharmacokinetic–pharmacodynamic relationships are playing an increasingly important role in the development and use of new

anticancer drugs. A thorough understanding of a drug's pharmacokinetics and pharmacodynamics is dependent upon detailed knowledge of the drug's mechanism and an understanding of the molecular targets on which they act.

Cancer chemotherapy has reached a fascinating stage, in which new molecular therapeutics are being tested individually and in combination with traditional cancer drugs such as the hormonal and cytotoxic agents. It is hoped that agents targeted to the molecular pathology of cancer may minimize the use and maximize the benefit of cytotoxic drugs. A future in which patients will be prescribed personalized mechanism-based anticancer drugs targeted to their individual molecular and genomic profiles is becoming a reality.

The optimal targeting of cancer requires not only the selection of the target but also the identification of the patients whose cancer depends on the targeted pathway. Concurrent biomarker development to identify predictors of response will therefore become essential to the approval process for these new agents and could further refine the use of approved agents [7].

The objective of this chapter is to give an overview of molecular targets in cancer therapeutics. Selected examples and a detailed listing of literature references are included. Targets have been categorized as either established or novel types. Established targets include those against which most currently licensed anticancer drugs were developed and include DNA, microtubules, and nuclear hormone receptors. Novel targets are those under current preclinical and clinical investigation. They include the products of oncogenes and the genes responsible for the multistep transformation of normal cells into cancer cells, such as receptors and receptor tyrosine kinases, as well as new approaches to established targets, such as microtubules and DNA repair. The section on novel targets emphasizes the relationships of the novel targets to the biological traits of cancer as described earlier in this section.

2 Established Molecular Targets

2.1 DNA

DNA is one the most successfully exploited targets for anticancer drug development. The use of mustine to treat leukemia in 1943 predated the discovery of the double helical structure of DNA by Watson and Crick in 1953. However, some of the early attempts at rational drug design led to highly effective drugs such as 5-flurouracil (5-FU). With further understanding of the structure and function of DNA and molecules that regulate it, there may be new targets within and around DNA that can be further exploited for anticancer drug development (Fig. 2).

2.1.1 Nucleotides

The bases of DNA—adenine, guanine, cytosine, and thymine—are heterocyclic rings that make up the genetic code. Adenine and guanine are purines, while cytosine and thymine are pyrimidines. Adenine normally interacts non-covalently with thymine via two hydrogen bonds, whereas guanine forms three hydrogen bonds with cytosine. Methylating agents (e.g., temozolomide) add a methyl group to the O6 position of guanine bases, thus causing mis-pairing of guanine to thymine. Alkylating agents such as melphalan have an active moiety that bind directly to the DNA bases, particularly guanine. Nitrogen mustards are chlorethylating agents that permanently alkylate the N7 position of guanine residues, thus causing interstrand cross-links in the DNA and preventing proper replication of the DNA [8, 9]. Platinum compounds react with the N7 position of guanine to form both monofunctional and bifunctional DNA adducts. Although covalent adduct formation is the mechanism of cytotoxicity

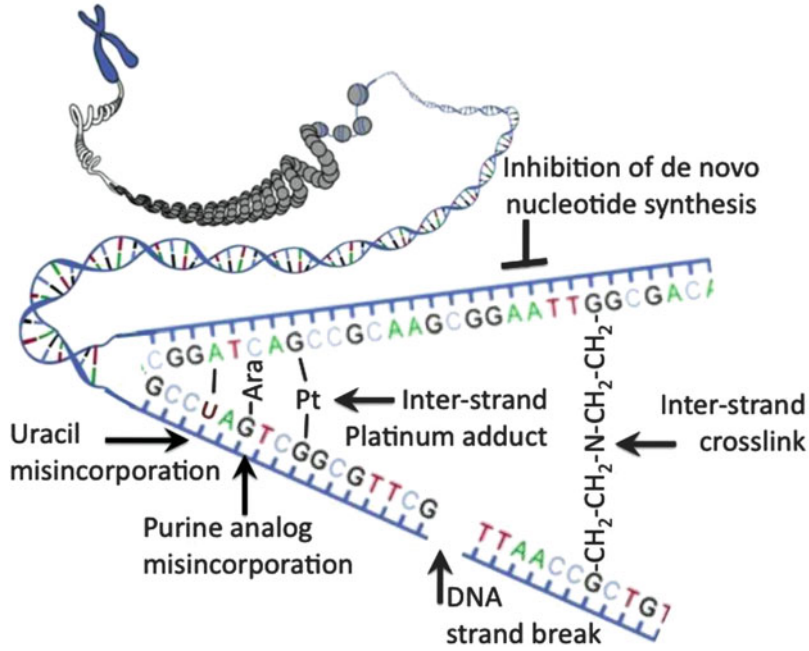

Fig. 2 DNA is a target for anticancer agents

of all DNA-modifying antitumor agents, these drugs exhibit widely different potency, toxicity, and tissue specificity. These differences can be attributed to structural features that affect the drugs' pharmacokinetics and biodistribution.

2.1.2 Purine and Pyrimidine Incorporation

Native purine and pyrimidine nucleotides are natural targets for rational drug design. Pyrimidines such as cytosine are incorporated into DNA as deoxycytidine triphosphate (dCTP), and competitive inhibition of this incorporation by Ara-C triphosphate (Ara-CTP) causes deregulation and inhibition of a wide range of enzymes including DNA polymerase and ribonucleotide reductase [10, 11].

Enzymes important to de novo purine synthesis such as phosphoribosyl pyrophosphate amidotransferase are inhibited by monophosphate derivatives of 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG). Misincorporation of ribonucleotide and deoxyribonucleotide metabolites of thiopurines can cause DNA strand breaks [12]. Cytosine arabinoside [13] and gemcitabine [14] are good examples of pyrimidine analogs used successfully in the clinic, whereas 6-MP [15] and 6-TG [16] are purine analogs with more limited application.

2.1.3 Dihydrofolate Reductase and Thymidylate Synthase

Thymidylate synthase (TS) is essential for the production of dTTP, and inhibition of TS leads to depletion of dTTP as well as increased levels of dUMP, which when phosphorylated leads to the misincorporation of dUTP into DNA causing DNA damage by uracil DNA glycosylase [17]. Dihydrofolate reductase (DHFR) is important in maintaining the reduced folate pool, which in turn is essential for the conversion of dUMP to dUTP by TS. Reduced folate pools are important for de novo

purine biosynthesis [18]. Both TS and DHFR are important targets and offer a degree of therapeutic selectivity. Both TS and DHFR are important for ongoing DNA replication and repair; thus, malignant cells can be more susceptible due to their rapid multiplication. Methotrexate is a commonly used anticancer agent that inhibits DHFR. On the other hand, 5-fluorouracil inhibits the TS enzyme in the folate synthesis pathway. Unfortunately, cancer cells can be selected to overexpress TS [19] and DHFR [20] as a mechanism to develop resistance to TS inhibitors such as 5-FU and methotrexate, respectively. Pemetrexed is a multi-targeted antifolate agent that blocks both of these enzymes as well as GARFT [21]. It may also have a secondary effect on inhibition of the AICART pathway of folate synthesis that can lead to subsequent inhibition of mTOR [22]. Pemetrexed is FDA approved for use in non-squamous NSCLC and in patients with pleural mesothelioma who are not surgical candidates.

2.1.4 Topoisomerase I and II

Eukaryotic DNA has a complex structure and is frequently supercoiled, knotted, or interlinked. Topoisomerase I and II are enzymes that modify the topological state of DNA by first cleaving the phosphodiester backbone of the DNA so as to allow the passage of another single- or double-stranded DNA, following which the topoisomerase reseals the strand, thus relieving DNA torsional strain [23, 24]. Relaxing the tertiary structure of DNA is essential for transcription, replication, and repair of DNA.

Two topoisomerases are current targets for anticancer drugs. Topoisomerase I causes single-stranded DNA breaks and is not ATP dependent, whereas topoisomerase II causes double-stranded DNA breaks and is ATP and Mg2+ dependent. Both topoisomerase I and II can be overexpressed in cancer, confirming their importance as valid targets. Successfully used topoisomerase I inhibitors include topotecan and irinotecan, whereas etoposide and doxorubicin are examples of topoisomerase II inhibitors that are used in the clinic [23, 24].

2.2 Microtubules

Microtubules are components of the mitotic spindles that are essential for dividing the replicated DNA into separate daughter chromatids during cell division. The microtubules are in dynamic equilibrium with the pool of soluble tubulin dimers present in the cell. There is a constant flux between incorporation of free dimers into the polymerized structures and release of the dimers into the soluble tubulin pool [25]. Although tubulin and microtubules are present in normal as well as tumor tissue, their fundamental involvement in cell division makes them important targets for anticancer drug development. Vinca alkaloids bind to distinct high-affinity binding sites on tubulin, causing alteration of microtubular dynamics at the ends of the mitotic spindle [26]. Clinically used vinca alkaloids include vincristine, vinblastine, and vinorelbine [27].

Taxanes bind mainly to polymerized tubulin. They decrease the lag time and shift the dynamic equilibrium between tubulin dimers and microtubules toward polymerization, thus stabilizing the microtubules [28]. Clinically, examples of drugs known to act as microtubule stabilizers include paclitaxel and docetaxel [29].

2.3 Nuclear Hormone Receptors

Hormones are known to influence a wide variety of malignancies. Most are steroids that enter the cell and bind receptors in the cytoplasm. Binding of the steroid hormone to its intracellular receptor then

allows the complex to translocate to the nucleus and activate intranuclear transcription factors. Examples of clinically relevant hormone receptors include those for estrogen, androgen, glucocorticoid, and retinoic acid.

2.3.1 Estrogen Receptors

Intranuclear estrogen receptors (ERs) have transactivation domains (AF-1 and AF-2) that when activated lead to transcription [30]. ER binds to specific DNA consensus sequences and also has considerable cross talk with the insulin-like growth factors and the oncogenic transcription factors c-Fos and c-Jun [31, 32]. Modulating ER by selective estrogen receptor modulators (SERMs) is standard treatment for breast cancers that express ER. Tamoxifen and raloxifene are SERMs that are also used in the preventative setting for reducing the incidence of contralateral breast cancer. Steroidal antiestrogens such as anastrozole and exemestane inhibit the conversion of estrogen to its active form, estradiol, by inhibiting the aromatase enzyme [33]. These drugs are approved for use in ER-positive breast cancers in postmenopausal women, where the majority of estrogen is formed by this peripheral conversion of the hormone, rather than secretion from the ovaries.

2.3.2 Androgen Receptors

Androgen receptors (ARs) bind both testosterone and the more potent dihydrotestosterone, which is formed by the action of the enzyme 5-alpha reductase [34]. Prostate carcinomas are heavily dependent on androgenic stimulation for growth and evasion of apoptosis, even when the receptor itself is expressed at seemingly normal levels [35]. Mutations of AR hormone binding domain, amplification of the AR gene, or alteration of AR coactivators lead to increased sensitivity to physiologic levels of androgens. Inhibition of AR signaling can be achieved clinically via reduction of testosterone (medical or surgical castration) or blocking the binding of testosterone to AR by agents such as flutamide and bicalutamide [36]. Constitutive cell-autonomous activation of the AR signaling pathway may allow escape from hormone dependence and resistance to antiandrogen therapy [37].

2.3.3 Glucocorticoid Receptors

Intracellular glucocorticoid receptors (GRs) are members of the steroid hormone superfamily and have both a hormone binding site and a DNA-binding domain. Activation and transcription of GR-dependent genes inhibits the growth of acute lymphoblastic leukemia (ALL) cells, whereas mutations in GR have been shown to confer resistance to dexamethasone [38]. Glucocorticoids are part of standard chemotherapy regimens in lymphoid malignancies such as ALL, Hodgkin and non-Hodgkin lymphomas, and multiple myeloma.

2.3.4 Retinoic Acid Receptors

Retinoic acid receptors (RARs) are also members of the nuclear steroid receptor superfamily. In acute promyelocytic leukemia (APL), a t(15;17) translocation fuses RAR-α, to a nuclear matrix protein PML (promyelocytic leukemia protein). The oncogenic fusion leads to constitutive expression of the PML protein, preventing differentiation and leading to a leukemic phenotype. Exposure of the cells to retinoic acid causes degradation of the PML–RAR fusion product, thus forcing the cells to terminally differentiate. Therefore, administration of all-*trans* retinoic acid (ATRA) effectively induces remissions in patients with APL [38].

2.4 Signal Transduction

2.4.1 Receptor Tyrosine Kinases

Receptors with tyrosine kinase activity are important targets for cancer. They may be overexpressed, mutated, or lie upstream of other signal transduction defects. Inhibiting the tyrosine kinase activity of key receptors has been successfully achieved using monoclonal antibodies and small molecule kinase inhibitors. Both strategies have been used against the epidermal growth factor receptor (EGFR). Cetuximab is a monoclonal antibody approved for head and neck squamous cell carcinoma and colon cancer that binds to EGFR and competitively blocks EGF from binding and stimulating its receptor [39, 40]. Erlotinib is a small molecule that specifically blocks the tyrosine kinase activity of the EGFR and is approved for use in non-small cell lung cancer and pancreatic carcinoma [39].

HER2 is a member of the EGFR family that has no known ligand, but when overexpressed, it signals by forming homodimers or heterodimers with other EGFR family members [41]. Trastuzumab is a monoclonal antibody licensed for treatment of HER2-positive breast cancer and gastric cancer. The antibody functions by preventing signaling through the dimerized receptors and/or by promoting antibody-dependent cellular cytotoxicity.

Proliferating tumor masses depend on new blood vessel formation in order to sustain growth. Thus, angiogenesis has been a promising target in several tumor types. Bevacizumab is a monoclonal antibody that inhibits angiogenesis by blocking vascular endothelial growth factor (VEGF) from binding to its receptor [42]. It is approved for use in glioblastoma multiforme, colorectal cancer, non-small cell lung cancer, and renal cell carcinoma.

2.4.2 Cytoplasmic Signaling Proteins

Imatinib is the first agent specifically designed to inhibit to an oncogenic intracellular kinase. In chronic myelogenous leukemia (CML), the (9:22) translocation results in the fusion protein BCR–ABL. The constitutively expressed and active kinase drives proliferation and survival through Ras, phosphatidylinositol 3-kinase (PI3 kinase), and Crkl pathways [43]. Imatinib is a potent inhibitor of BCR–ABL and achieves hematologic responses of 95 %, 53 %, and 29 % in chronic, accelerated, and blast phases of CML, respectively, which provided a basis for licensure [44, 45].

Sorafenib takes a broader approach to "targeted" therapy. This drug was developed to inhibit RAF kinase activity but also has activity against several other tyrosine kinases including VEGF receptor 2 (VEGFR2) [46]. Sorafenib is approved for use in renal cell carcinoma (RCC) as well as hepatocellular carcinoma (HCC). Its clinical utility in RCC most likely is a result of its activity against VEGFR2 [47]. In HCC, however, its efficacy is more likely due to its anti-RAF activity [48].

The mammalian target of rapamycin (mTOR) is a protein kinase that phosphorylates the initiation factor 4E binding protein (4E-BP1); this in turn binds eIF-4E, which is important for the translation of cyclin D1 mRNA. This crucial link to the cell cycle control pathway and the fact that it is downstream of the PI3 kinase–Akt pathway make mTOR an interesting target [49]. Rapamycin binds to the immunophilin FKBP12 that inactivates mTOR. This agent is approved for controlling solid organ transplant rejection due to its effects on T cell function. Rapamycin analogs temsirolimus and everolimus, however, are approved for RCC [50].

2.5 Chromatin Modulation

Histone deacetylases (HDACs) modulate chromatin structure and control other cellular functions. Four different classes of HDACs have been identified [51]. Studies in yeast in which specific HDACs

were deleted indicate that Rpd3, Sir2, and Hda1 have distinct functions in cell cycle progression, amino acid synthesis, and carbohydrate transport [52]. HDACs also have nonhistone targets and can regulate the deacetylation of p53 and E2F [53]. The binding of HDAC with the PML-RAR translocation product leads to inhibition of differentiation in the M3 subtype of AML. The treatment of such patients with retinoic acid, as mentioned previously, results in the displacement of HDACs and allows ligand-dependent coactivators such as SRC-1 to bind and activate transcription, which leads to reactivation of the differentiation process [54]. Two HDAC inhibitors, vorinostat and romidepsin, are approved for clinical use in cutaneous T cell lymphoma.

2.6 Protein Folding and Degradation

Rapid and irreversible proteasomal protein degradation is the key to the activation or repression of many cellular processes. The primary component of the protein degradation pathway in the cell is the 26S proteasome, which is where proteins marked with polyubiquitin chains are degraded [55]. The proteins are denatured to short (3- to 22-residue) polypeptides while the ubiquitin chain is recycled [56]. Proteins with tumor suppressor functions such as p27 (that inhibits CDK 2, 4, and 6) and IκB (that inhibits NF-κB) are degraded by the ubiquitin proteasome pathway. Proteasomal inhibition decreases cellular proliferation in vitro and in vivo in several cancer model systems [57]. The proteasome inhibitor bortezomib is approved for use in multiple myeloma and mantle cell lymphoma [58].

3 New Approaches to Established Targets

3.1 DNA Damage

The use of PARP inhibitors is an exciting and novel approach to exploit DNA damage in cancer cells. More than half a dozen highly potent and specific PARP inhibitors are currently undergoing clinical development in cancer populations. PARP (poly-ADP ribose polymerase) is a highly abundant nuclear protein that is activated when DNA is damaged [59]. The action of PARP1 is essential for repair of single-stranded DNA breaks, predominantly through the BER mechanism [59]. PARP also contributes to repair double-stranded breaks through NEHJ, which is further impaired when PARP activity is inhibited [60]. Small molecule inhibitors of PARP activity began development as sensitizers to DNA-damaging chemotherapy or ionizing radiation [61]. However, it was soon discovered that cells otherwise deficient in DNA repair pathways were strikingly more sensitive to PARP inhibition. For example, BRCA-deficient cells are 1,000-fold more sensitive to single-agent PARP inhibition than are wild-type BRCA1 and BRCA2 cells [62, 63]. BRCA1 and BRCA2 regulate repair of damaged DNA through HR [64]. In addition to BRCA1 and BRCA2 germline mutations, other inherited molecular defects may prevent effective HR DNA repair in cancer. Irreparable DNA damage triggers apoptotic cell death in cells with or without intact p53 [65]. Therefore, inhibiting PARP has been undertaken as a strategy to selectively kill cells with dysfunctional HR [66].

3.2 Microtubules

Epothilones are compounds derived from the myxobacterium Sorangium cellulosum that stabilize microtubules by interacting with the taxane-binding site. The epothilones improve upon

first-generation taxanes by overcoming multidrug resistance mediated by p-glycoprotein [67]. Ixabepilone is the first macrolide epothilone approved for clinical use in metastatic breast cancer [68]. Microtubule-stabilizing agents that bind to other sites are also under investigation, including vinflunine (vinca alkaloid binding site) and combretastatins (colchicine binding site) in order to improve on safety, tolerability, and efficacy.

3.3 Growth Factor Receptor Signaling and Tyrosine Kinase Inhibition

Resistance to first-generation targeted therapies is a common occurrence, and identification of these mechanisms of resistance has led to the development of second-generation targeted agents. In the case of tyrosine kinase inhibitors, cancer cells may develop a mutation in the ATP-binding pocket that lowers the affinity of the kinase for the drug and/or increases affinity for ATP. In CML, resistance to imatinib evolves in up to half of the patients, most often due to mutations in the BCR–ABL kinase. Dasatinib, a second-generation inhibitor, has a 350-fold greater potency against the unmutated ABL kinase and has activity against most of the imatinib-resistant BCR–ABL mutants, except the gatekeeper mutation T351I [69].

Tyrosine kinase inhibitors were originally developed as competitive inhibitors of the ATP-binding site. More recently, irreversible inhibitors have been developed to improve on the potency and sustainability of inhibition and to overcome acquired resistance to reversible TKIs such as erlotinib. For example, the EGFR T790M mutation is responsible for acquired resistance to erlotinib in many NSCLC patients who initially responded to erlotinib. Neratinib is an irreversible TKI that binds covalently to the ATP-binding cleft of EGFR and may show activity in cells with the T790M mutation due to its inability to be displaced by ATP [70]. Most irreversible TKIs also target other EGFR family members including HER2 and are therefore under clinical investigation in both lung cancer and breast cancer.

Resistance to trastuzumab develops in HER2-positive breast cancers. One mechanism for resistance is deletion of the extracellular domain of the HER2 molecule. This mutation prevents binding of the mAb trastuzumab but retains intracellular tyrosine kinase activity. Lapatinib is a dual inhibitor of both EGFR1 and HER2 that targets this intracellular kinase activity of the truncated HER2 protein. Lapatinib is approved for the treatment of trastuzumab-resistant HER2-expressing metastatic breast cancer [71].

3.4 Antiandrogens: MDV3100

MDV3100 is a novel antiandrogen that has three distinct mechanisms of action. It blocks testosterone from binding to its receptor, inhibits movement of the receptor to the nucleus, and prevents the binding of the complex to DNA [72]. This agent achieves clinical responses in over 50 % of patients in phase 1 and 2 trials and is currently under phase 3 investigation for the treatment of metastatic prostate cancer [73].

3.5 Tumor Blood Vessels

Vascular disrupting agents—drugs targeting existing endothelial cells—are classified as antivascular rather than antiangiogenic. Two classes of vascular disrupting agents (VDAs) are currently in

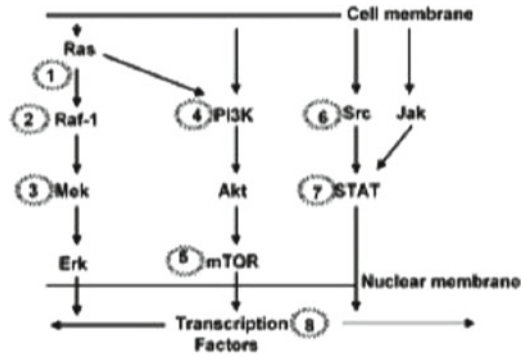

Fig. 3 Signal transduction pathways and nonreceptor sites targeted for anticancer drug development: (1) Ras prenylation, (2) RAF1, (3) MEK, (4) P13 kinase, (5) mTOR, (6) SRC, (7) STAT, (8) transcription factors

development [74]. The first class targets microtubules, and those binding to the vinca alkaloid domain appear to be more selective to endothelial cells. Drugs such as combretastatin A and its analogs destabilize vasculature, resulting in the rapid destruction of blood vessels [75]. The second class of VDA includes flavonoid compounds derived from flavone acetic acid (FAA) [74]. These agents appear to induce apoptosis in endothelial cells, via a TNF-related mechanism. The drug vadimezan (ASA404) is currently in phase 3 clinical trials in combination with carboplatin and paclitaxel for second-line treatment of NSCLC.

3.6 PI3K/Akt/mTOR

3.6.1 PI3 Kinase

PI3 kinase is a lipid kinase activated by Ras and a number of receptor tyrosine kinases including PDGFR and EGFR [76, 77] (Fig. 3). Downstream of PI3 kinase, Akt and mTOR are important kinases that are critical for cell proliferation [78]. The tumor suppressor gene PTEN (phosphate and tensin homolog deleted from chromosome 10) dephosphorylates and inactivates phosphatidylinositol 3,4,5-triphosphate (PIP-3), which is the lipid product formed by the activation of PI3 kinase [79]. PI3K is an attractive target for anticancer drug development based on several features: genes encoding PI3 kinases are amplified in certain cancers, PI3 kinase lies downstream of receptor tyrosine kinases that are overexpressed or mutated in cancer, PI3K is upstream of known oncoproteins such as PKB/Akt, and PTEN loss or mutation is the second most common tumor suppressor gene abnormality in human cancers [80].

3.6.2 mTOR

The mTOR pathway is a key checkpoint in the sensing of nutrients within the cell. Amino acids, glucose, and oxygen regulate the mTORC complex and upstream TSC proteins. Cancer cells appear to override the growth inhibitory signals of nutrient deprivation, perhaps by overactivation of the mTOR signaling complex. Similarly, resistance to rapamycin and analogs, which specifically inhibit mTORC1, may result from overactivation of mTORC2, or by feedback activation of Akt itself. Dual mTORC1 and mTORC2 inhibitors are under development, as are Akt inhibitors [81].

4 Novel Molecular Targets

4.1 Transcription Factor Pathways

4.1.1 NF-κB

NF-κB is an antiapoptotic transcription factor that is normally inhibited by IκB [82]. Amplification or overexpression of the NFκB gene has been seen in several hematologic malignancies and solid tumors, whereas inactivation of IκB has been demonstrated in Hodgkin's lymphoma. One approach to inhibit NF-kB activation is through blockade of the I-kappaB kinases, specifically IKK-beta. Several inhibitors of IKK-beta are in early phases of development [83].

4.1.2 JAK/STAT Pathway

Signal transducers and activators of transcription (STAT) are cytoplasmic transcription factors that are activated by growth factor receptors (interferons) and cytoplasmic tyrosine kinases such as JAK and SRC [84] (Fig. 3). STAT3 is required for the activation of v-SRC transformation, and a constitutionally active STAT3 mutation is sufficient to induce malignant transformation [85, 86]. Approaches to modulate STAT activity aim to target STAT dimerization, translocation, and DNA binding [84]. Inhibitors of JAK are also in clinical development, specifically for myeloproliferative disorders, which harbor the activating V617F mutation in JAK2 [87].

4.1.3 AP-1 Family

The activator protein-1 family (AP-1), consisting of Fos, Jun, and ATF proteins, plays an important role in development [88]. Overexpression of c-Fos has been shown to induce cartilaginous tumors, and absence of c-Fos is associated with reduced expression of matrix metalloproteinases, thus affecting angiogenesis and invasion [89]. c-Jun may transform mammalian cells but requires coexpression of other oncogenes such as Ras and SRC, but other Jun proteins may have tumor suppressor functions [90]. Better understanding of these transcription factors will help develop specific inhibitors. However, inhibition of protein–protein and protein–DNA interactions in which these proteins participate is technically challenging.

4.1.4 c-MYC

c-Myc is a prototype for oncogene activation by chromosomal translocation [91]. It is involved in several prooncogenic events such as protein synthesis, cell cycle progression by inactivation of cell cycle inhibitor p27, and activation of cyclin E and E2F [92]. Myc targets genes that regulate apoptosis such as p53 and affects cell adhesion by downregulation of LFA-1. Finally, c-Myc has been associated with a number of hematologic malignancies and solid tumors, making it an attractive target for anticancer drug development [93].

4.2 Cytoplasmic Kinases

4.2.1 SRC

SRC is a cytosolic nonreceptor tyrosine kinase that is activated by growth factor receptors and focal adhesion kinase [94]. SRC was the first identified oncogene and regulates many downstream cellular functions including proliferation, survival, adhesion, and angiogenesis. SRC kinase activity has been shown to be elevated in many types of cancer. Dasatinib, developed to treat imatinib-resistant CML, inhibits kinase activity of SRC in addition to its intended target, ABL [95]. Dasatinib is currently under clinical investigation as a SRC inhibitor in solid tumors.

4.2.2 Ras–RAF–MEK–ERK Pathway

Members of the Ras superfamily of proteins that are implicated in cancer include H-Ras, N-Ras, and K-Ras. Ras mutations are found in a variety of tumor types and have also been shown to be a marker for poor prognosis [96]. Ras undergoes prenylation, a lipid posttranscriptional modification required for proper localization to the inner surface of the plasma membrane. Some members of the Ras family require farnesylation, while others also undergo geranylgeranylation such as Rho, Rac, and cdc42, which are important to malignant transformation mediated by Ras. The enzymes that mediate farnesylation and geranylgeranylation are farnesyltransferase (FT) and geranylgeranyltransferase (GGT), respectively [97]. While H-Ras prenylation is inhibited by FT inhibitors, K-Ras is more difficult to inhibit and may require both FT and GGT inhibitors to block malignant transformation. There is increasing evidence that FT inhibitors may not act solely or even partly via Ras, since many other cellular proteins are farnesylated. Research to identify the downstream targets continues, with a potential opportunity for therapeutic intervention at the point of Rho-kinase (ROK) [98].

The RAF family of serine threonine kinases is activated downstream of Ras [96] (Fig. 3). Sorafenib, which predominantly targets RAF1, is in clinical use for treatment of RCC and HCC [47, 48]. Mutations in the genes encoding BRAF have recently been identified in a high proportion of melanoma and in a lower proportion of colorectal and other cancers [99]. PLX4720 specifically inhibits BRAF carrying the V600E mutation. A phase I clinical trial with this agent expanded enrollment of patients with melanoma harboring the V600E mutation, which occurs in nearly 50 % of melanomas. The BRAF inhibitor achieved an overall response rate of 70 % in this subset of patients. Thus, the V600E mutation provides a molecular marker predictive of sensitivity to this drug [100]. Unfortunately, responses are short-lived, and resistance to BRAF inhibition is reflected in reactivation of ERK phosphorylation in the cancer cells. Preclinical evidence suggests that acquired mutations in MEK1 could contribute to the resistance [101]. The BRAF mutation is present in approximately 8 % of other solid tumors, yet inhibiting the mutant BRAF is ineffective if K-Ras mutations coexist. The RAF inhibitors appear to induce a conformational change in RAF that interacts with the mutant Ras-GTP and promotes signaling through unmutated RAF1 [102]. Dual inhibition of RAF and MEK is being studied preclinically to overcome this phenomenon. MEK is phosphorylated by RAF and, once activated, phosphorylates the MAP kinases ERK-1 and ERK-2 [96]. Although MEK has not been identified as an oncogene product, no other substrates apart from ERK have been identified, thus making it an important focal point for mitogenic pathways activated by RTKs and/or oncogenes. Drug development programs are actively pursuing MEK inhibitors, and several are now undergoing clinical trials [103].

4.3 Mitotic Kinases

Cancer cells undergo unrestricted cell division, thus making mitosis a logical area of anticancer drug development. During mitosis, the chromosomes are separated by microtubules on the mitotic spindle. Therefore, microtubule-targeting agents were thought to kill cancer cells by inhibiting mitosis. Microtubules are essential to many other cellular processes, however, suggesting that these traditional chemotherapeutic drugs may kill the cells by other mechanisms as well [104]. In addition, the toxicities of these drugs in nondividing cells also point to their effects on intracellular protein trafficking as well.

Aurora kinases A and B and polo-like kinase 1 (PLK1) regulate distinct points in mitosis and have been explored as potential therapeutic targets in cancer [105]. Aurora kinase A acts at the point of centrosome separation and mitotic spindle assembly. Aurora kinase B is responsible for correct chromosome alignment and triggers mitotic checkpoint delay in the setting of misaligned chromosomes in normal cells. PLK1 functions in kinetochore–microtubule interaction and completion of cytokinesis. This protein may act as a tumor suppressor during development [106] but has been identified as a potential driver of K-Ras-mediated oncogenesis [107].

Specific inhibitors of each of these kinases are under development, but their ultimate use in cancer therapy is unclear [108]. Emerging preclinical evidence suggests that combined inhibition of these kinases may be antagonistic. Patterns of cell cycle arrest upon aurora kinase or PLK inhibition suggest that specific combinations may be optimal for treating cancers. For example, aurora kinase B inhibitors might promote killing by paclitaxel since they release the mitotic arrest induced by the taxane and could then accelerate cell death. Aurora kinase A inhibitors may promote killing by DNA-damaging agents, such as the platinums, by maintaining the cell in G2 arrest since this kinase is required to restart the cell cycle after G2 arrest. PLK1 inhibitors are able to induce mitotic delay and apoptosis as single agents, but may also cooperate in cytotoxicity of DNA-damaging agents as well [105].

4.4 Epigenetic Modifications

Methylation of cytosine residues in adjacent cytosine and guanine nucleotides in DNA (CpG) is achieved by DNA methyltransferase (DNMT) [109]. Consequences of CpG methylation include silencing of a variety of tumor suppressor genes including Rb, p16, p14, BRCA1, and MLH1 [110]. Although tumors tend to exhibit global hypomethylation, there are often large areas of CpG island hypermethylation in tumors [111].

DNMT inhibitors could be used as single agents with the aim to reactivate methylation-silenced tumor suppressor genes, or in combination with conventional cytotoxics, where reactivation of genes such as those encoding the mismatch repair protein MLH1 affects the sensitivity of cancer cells to carboplatin and epirubicin [110]. Two classes of DNMT inhibitors exist. Nucleoside analogs such as 5-azacytidine inhibit DNMT activity by incorporating into replicating DNA and irreversibly binding the DNMT enzyme [112]. This drug and its analog 5-aza-2-demethoxycytidine were approved for use in myelodysplastic syndrome. The second class of DNMT inhibitors includes non-nucleoside small molecule inhibitors of the catalytic site [112]. These compounds are in early phase clinical development.

4.5 Apoptosis

Apoptosis or programmed cell death is characterized by morphological changes (shrinkage, condensation of nuclei, and loss of microvilli) [113] and the biochemical hallmark of cleavage of

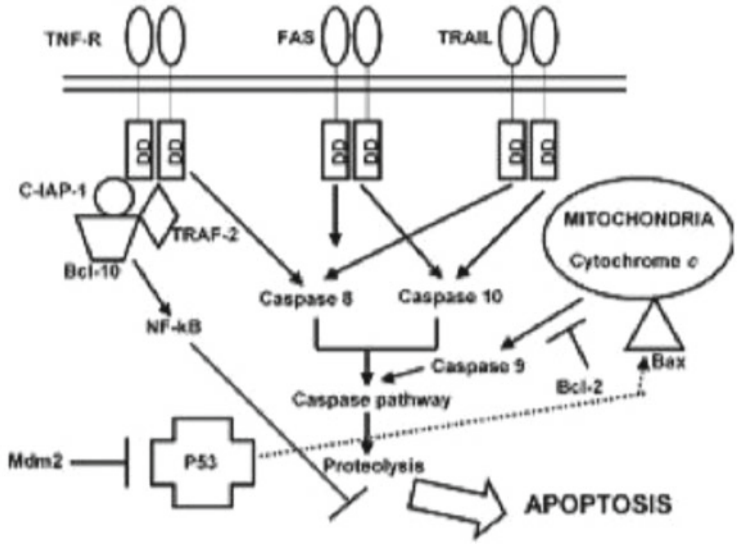

Fig. 4 Targets for apoptosis modulation

chromosomal DNA into nucleosomal units by caspases [114]. Apoptosis is governed by proapoptotic events, which include (1) death receptor signaling, (2) release of cytochrome c from the mitochondria, and (3) p53 activation. Antiapoptotic factors include (1) antiapoptotic protein Bcl-2, (2) cellular inhibitor of apoptosis 2 (c-IAP), and (3) NF-κB. Deregulation of any of these key factors can lead to inhibition of apoptosis and an inappropriate survival advantage to the affected cell [115] (Fig. 4).

4.5.1 Bcl-2 Family

Bcl-2 is an antiapoptotic protein that is overexpressed in a variety of malignancies [116]. Attempts to inhibit Bcl-2 by adenoviral vectors carrying a dominant negative gene or by an antisense approach have undergone clinical development with unsatisfying results [117]. More recently, specific small molecule inhibitors of Bcl-2 have been developed that directly bind to the BH3 domain of the anti-apoptotic proteins [118]. Navitoclax and its analog ABT-199 are undergoing early phase clinical development in hematologic malignancies that demonstrate Bcl-2 overexpression [119].

4.5.2 Apoptosis Inhibitor Proteins

TNF-related apoptosis inducing ligand (TRAIL) binds to the death receptors DR4 and DR5 and triggers the assembly of the death-inducing signaling complex (DISC), leading to apoptosis [120]. Synthetic TRAIL and an antibody to these death receptors have been used to target death receptors in malignant cells. Inhibitors of apoptosis, IAP1, IAP2, and XIAP block the apoptosis signaled through the DISC by inhibiting either caspase activation or directly blocking caspase activity. The secondary mitochondrial activator of caspase (SMAC) antagonizes IAP function by causing them to auto-ubiquitylate and target themselves for proteasomal degradation [121]. Small molecule mimics of SMAC are in preclinical and early clinical development.

4.6 Heat-Shock Proteins/Chaperones

Heat-shock protein 90 (HSP90) and its endoplasmic reticulum homolog GRP78 are important molecular chaperones involved in posttranslational folding of client proteins. Client proteins such as Erb-B2, BCR–ABL, SRC, RAF1, Akt/PKB, and CDK4/6 either are oncoproteins or are integral elements of signal transduction pathways that are deregulated in cancer [122]. The ability to affect multiple signal transduction pathways at different levels makes HSP90 an attractive target. The geldanamycin analog 17AAG has shown promising preclinical activity and is in clinical trials [123].

5 Targeted Chemoprevention

Given the challenges associated with inhibition of the multiple oncogenic abnormalities involved in the late stages of cancer, the concept of chemoprevention is an attractive one [124]. Chemoprevention can be primary, secondary, or tertiary. Primary prevention is aimed at healthy individuals, while secondary prevention is directed at patients with a preclinical or early stage disease. Tertiary prevention is aimed at patients who have undergone initial treatment and aims to prevent recurrence. Whereas targets for tertiary prevention have been covered under previous sections, primary and secondary prevention are discussed below.

5.1 Hormone Receptors

5.1.1 Breast Cancer

In 1998, tamoxifen was FDA approved for the prevention of breast cancer in women considered to be at high risk of the disease. Subsequently, the STAR trial (study of tamoxifen or raloxifene) resulted in the approval of raloxifene in 2007. Raloxifene was not as effective as tamoxifen in preventing invasive breast cancers but had a favorable side effect profile, with lower incidence of endometrial hyperplasia and thromboembolic events [125].

5.1.2 Prostate Cancer

Finasteride is a 5-alpha-reductase inhibitor that may prevent prostate cancer [126]. It has FDA approval for the treatment of benign prostatic hyperplasia but has not been approved for prostate cancer prevention, due to the concern for increased incidence of high-grade cancers that were detected in men taking finasteride. The value of 5-alpha-reductase inhibitors in prostate cancer prevention thus remains an area of debate.

5.2 Retinoic Acid Receptors

As described earlier, retinoic acid receptors belong to the nuclear receptor superfamily. They have been shown to influence the malignant potential of mammalian cells and are exploited in two principal ways in anticancer treatment: first to treat acute promyelocytic leukemia and second in secondary

prevention of a variety of cancers [127]. Epidemiological data suggest that geographical areas where vitamin A deficiency was endemic had an increased incidence of aerodigestive cancers [128], and underexpression of retinoic acid receptor β has been demonstrated in bronchial biopsies of chronic smokers [129]. Despite this rationale, retinoid supplementation has not been successful in large phase III cancer prevention trials. Nonetheless, retinoids are still under study as potential chemopreventive agents, either as single agents in specific populations or in combination with other agents [127].

5.3 Cycloxygenase-2

Cycloxygenase-2 (COX-2) expression has been linked to several cancers, and population-based studies showed a 40–50 % decrease in the relative risk of colorectal cancer in persons who were prolonged users of nonsteroidal anti-inflammatory drugs (NSAIDs) [130–132]. The selective COX-2 inhibitor celecoxib is FDA approved for the prevention of colon cancer in patients with familial adenomatous polyposis coli (APC) who are susceptible to developing colorectal cancer [124].

5.4 Novel Chemopreventive Agent: Metformin

Metformin is the most commonly used oral diabetes drug. Diabetics who take metformin, as opposed to other oral agents or insulin, have decreased incidence of several types of cancer [133]. This decreased cancer incidence may be related to inhibition of mTOR by metformin via activation of AMP-activated protein kinase [134]. Metformin is an effective chemopreventive agent in preclinical models of tumorigenesis that are not related to diabetes [135], which has raised the potential of metformin to be tested in humans at risk for cancer, irrespective of diabetes. Several clinical chemoprevention trials with metformin in high risk groups are planned or in progress.

6 Concluding Remarks

The development and use of new cancer therapeutics continues to progress at an exciting pace, accelerated both by the discovery of new molecular targets that have arisen from molecular oncology and genomics and also by the implementation of new discovery technologies. The rational development and application of new and established cancer therapeutics requires a thorough understanding and consideration of the mechanisms of action and molecular target involved, coupled to the principles of pharmacokinetics and pharmacodynamics. This chapter illustrates the plethora of molecular targets that are modulated by cancer drugs, from the relatively nonspecific effect on DNA to highly selective agents that are designed to attack particular loci responsible for malignant progression.

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Preclinical Screening for New Anticancer Agents

Angelika M. Burger and Heinz-Herbert Fiebig

Abstract Preclinical screening procedures for anticancer agents have evolved from empirical to target-oriented screens and have contributed to the approval of a number of molecularly targeted drugs over the past decade. This chapter reviews historical in vitro and in vivo screens, the currently used cell-based as well as cell-free high-throughput screens. Tailored, secondary predictive screening procedures employing primary patient tumors and clonogenic or nude mouse xenograft assays are also described. Examples of approved drugs that have been developed based on a particular screening approach and future perspectives for finding novel and more potent drugs are discussed.

Keywords Tumor models • NCJ 60 cell line • Marine models • Cell based screen • HTS • Xenografts • Patient derived xenografts • Hollow fiber assay • Clonojenic assay

1 Introduction

Cancer chemotherapy is a relatively young discipline of oncology. It has only been pursued with scientific vigor and multinational collaborations since the mid-twentieth century. To date, over 100 monographs of drugs used for the treatment of more than 200 different tumor types exist [1, 2]. Over the past decade, cancer has become a large therapeutic market, third only after central nervous system and cardiovascular drugs, and it is continuously growing. The number of blockbuster anticancer drugs with sales of $1 billion or more increased from 19 in 2007 to 24 in 2008. Nonetheless, the cure rate of 4 % for cancers that require systemic treatment remains very low [2].

Thus, the need for novel drugs is still pressing. Public institutions, the pharmaceutical industry, small business, and biotech companies create hundreds of thousands of compounds with potential anticancer activity. Only a certain number of drugs and concepts, however, can be evaluated clinically because of cost and ethical considerations. A preselection, called the screening process, is therefore required. The aim of screening efforts is to identify products that will produce antitumor effects

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matching the activity criteria used to define which compounds can progress to the next stage in the preclinical development program. Anticancer drug screening can be performed using various types of in vitro and in vivo tumor models. The ideal screening system, however, should combine speed, simplicity, and low costs with optimal predictability of pharmacodynamic activity.

2 History of Anticancer Drug Screens

Initial screening and drug development programs were small in scale and directed toward the evaluation of antitumor activity of small numbers and specific types of potential drugs [3]. Stimulated by the approaches of Ehrlich and Warburg, studies were conducted on the effects of dyes or respiratory poisons on tumor growth [4, 5]. In the 1930s, several researchers engaged in systematic studies of certain classes of compounds such as Boyland in the United Kingdom, who tested aldehydes in spontaneous tumors in mice, and Lettre in Germany, who studied colchicine derivatives and other mitotic poisons in tissue culture and ascites tumors [6]. In the United States, Shear, first at Harvard and then at the National Cancer Institute (NCI), inaugurated a screening program for testing and isolation of bacterial polysaccharides employing mice bearing sarcoma 37 as test systems for necrosis and hemorrhage. The program was quickly extended to plant extracts and synthetic compounds. In the early 1950s, the program had evaluated more than 300 chemicals and several hundreds of plant extracts. Two of these materials were tested clinically [7].

Larger-scale screens emerged around 1955, stimulated by the discovery that chemical agents, such as nitrogen mustard and folic acid antagonists, were capable of producing remissions of malignant lymphomas [8, 9]. As a result, the program of Shear at the NCI was extended to incorporate the evaluation of synthetic agents and natural products for antitumor activity. Further institutions that engaged in screening programs were Sloan–Kettering in New York, the Chester Beatty Research Institute in London, and the Southern Research Institute in Alabama [3]. In addition, screening, evaluation, and development programs were established at chemical and pharmaceutical companies, research institutions, medical schools, and universities in various countries in the world. As a result of these efforts, several agents were found with clinical activity, particularly against leukemias and lymphomas. Currently they still provide the mainstay of available drugs for systemic treatment of cancer and encompass alkylating agents (cyclophosphamide, bis(chloroethyl)nitrosourea [BCNU], 1-(2-chloroethyl)-3-cyclohexyl-L-nitrosourea [CCNU], antimetabolites (methotrexate, 5-fluorouracil [5-FU], 6-mercaptopurine), antitumor antibiotics (mitomycin C, adriamycin), and mitotic spindle poisons (*Vinca* alkaloids, taxanes) [3].

3 The NCI Screen

The NCI Developmental Therapeutics Program (DTP) anticancer drug screen has undergone several changes since its inception in 1955 [10]. It has become the foremost public screening effort worldwide in the area of cancer drug discovery, not the least because the experimental screening models were always adapted to novel emerging knowledge and technologies. The early philosophy from which the NCI endeavor proceeded was that the elucidation of empirically defined antitumor activity in a model would translate into activity in human cancers. The choice of specific screening models was guided by sensitivity to already identified clinically active agents and in the early period was exclusively focused on in vivo testing procedures [11]. Initially, three transplantable murine tumors were employed, namely, the sarcoma 180, the carcinoma 755, and the leukemia L1210. The latter was

found to be the most predictive rodent model among the available panel and was retained in 1975, when the NCI screening process was changed in that the P388 murine leukemia model was utilized as a prescreen and followed by a panel of tumors now also including human xenografts (breast MX-1, lung LX-1, colon CX-1) [12]. The human xenografts were utilized with the intent to achieve a better prediction for clinical response against solid human malignancies as compared to hematological malignancies.

For the same reason, starting in 1985, the human tumor cell line panel comprised of 60 different cell types, including mainly solid malignancies, was introduced and replaced the P388 in vivo leukemia prescreen in the 1990s (Fig. 1; see also http://dtp.nci.nih.gov/screening.html). This project has been designed to screen up to 20,000 compounds per year for potential anticancer activity. Selection criteria for preclinical drug candidates are cytotoxic potency and differential activity against particular tumor types and/or a few specific cell lines [13]. The screen is unique in that the complexity of a 60-cell-line dose response produced by a given compound results in a biological response pattern that can be utilized in pattern recognition algorithms [14]. Using these algorithms, it is possible to assign a putative mechanism of action to a test compound or to determine that the response pattern is unique and not similar to that of any of the standard prototype compounds included in the NCI database. Such agents are then tested against the sensitive cell line grown as subcutaneous xenografts in nude mice in vivo [15]. Because of the vast number of molecules emerging from the in vitro screen for nude mouse testing, in 1995 the preclinical development cascade was amended to include the hollow fiber (HF) assay [16]. The HF assay is a short-term in vivo assay combined with in vitro culture methods. It has been proven as a rapid and efficient means of selecting compounds with the potential for in vivo activity in conventional xenografts [10, 16].

In parallel with the implementation of the HF "in vivo filter system," a prescreen preceding the 60-cell-line screen was established in early 1995 as it became obvious that many agents were completely inactive under the conditions of the assay. Initially, the prescreen comprised three cell lines (MCF-7 breast, H460 lung, and SF268 brain cancer lines) tested against a range of drug concentrations. Currently, the prescreen assesses a new drug at one concentration of 10^{-5} M in all 60 cell lines. Only compounds which satisfy predetermined threshold inhibition criteria will progress to the five-dose screen. The threshold inhibition criteria for progression to the 5-dose 60-cell-line screen were designed to efficiently capture compounds with antiproliferative activity and are based on careful analysis of historical DTP screening data (http://dtp.nci.nih.gov/announcements/chg_to_screen.html). The actual NCI preclinical anticancer drug screening process is summarized in Fig. 2. Although the NCI drug development scheme is still empirical as it is based on selection of in vitro and in vivo antiproliferative activity, a number of new agents that are now in clinical use have been identified based on their unique patterns of and/or activity in the in vitro screen such as bortezomib (Velcade®, NSC 681239), romidepsin (depsipeptide, NSC 630176), a histone deacetylase (HDAC) inhibitory agent, and tanespimycin (17-AAG, NSC 330507) [17–19].

Recent insights into the molecular basis of human cancer and high-throughput profiling of the genome and proteome of the NCI 60-cell-line panel initiated a transition to rational molecular targeted discovery and development of anticancer agents in vitro and also in vivo [18, 19]. New programs such as the NCI Chemical Biological Consortium (CBC) have therefore been implemented. The CBC will select targets, actively screen for agents that affect these targets, and optimize the "drug-like" properties of hits, rather than focus on developing new agents submitted by outside investigators. The CBC drug discovery process is divided into four distinct stages including Exploratory Screen Development (ESD), Screening/Designed Synthesis (SDS), Lead Development, and Candidate Seeking with the goal to test the latter in phase 0/I trials. The CBC will mobilize a cancer drug discovery group on the scale of a small biotechnology concern, with an R&D pipeline linked to the academic community (http://dtp.nci.nih.gov/docs/CBC/cbc_index.html).

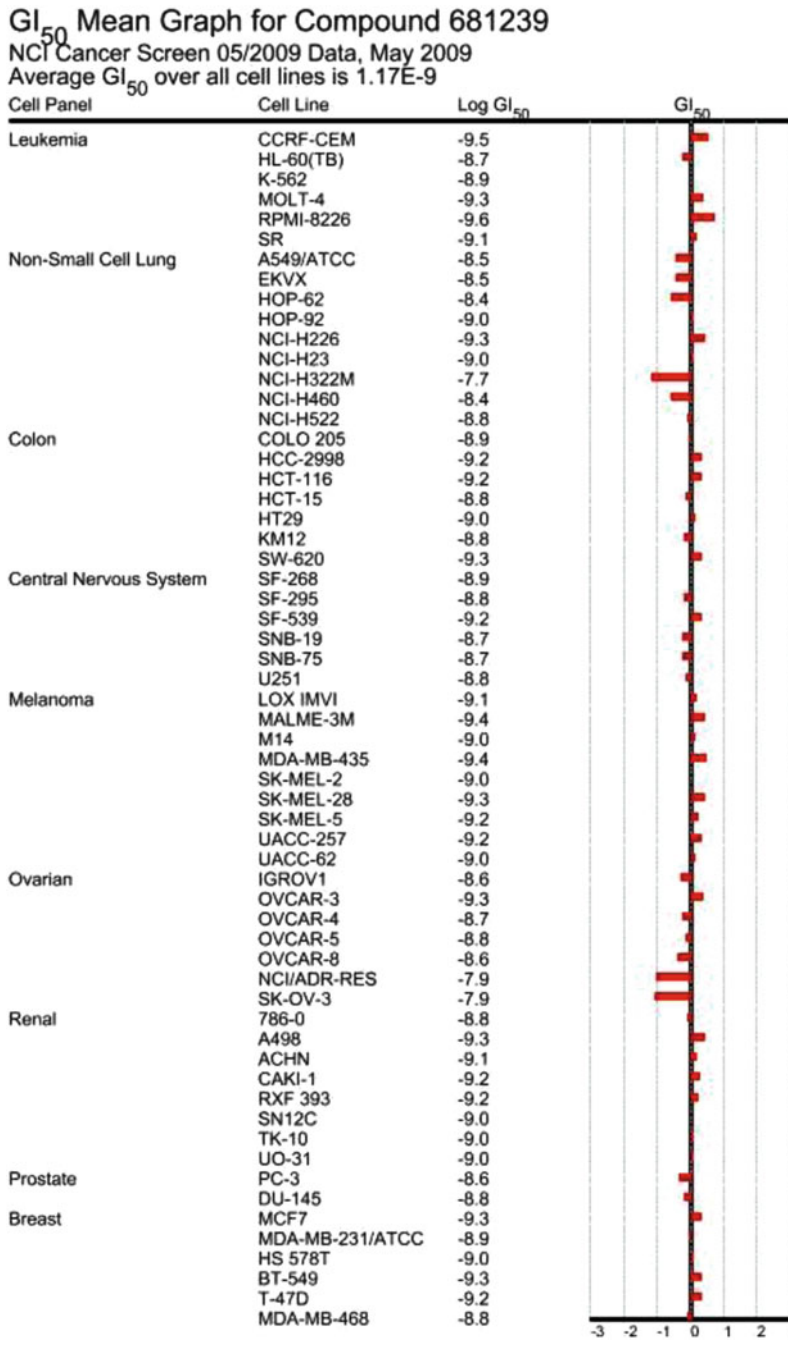

Fig. 1 Example of NCI 60-cell-line screening data. Shown is the sensitivity profile of bortezomib (681239) in 9 different tumor histologies on the basis of the 50 % growth inhibition (GI50). Bars to the *left* indicate more resistant and bars to the *right*, more sensitive cell lines

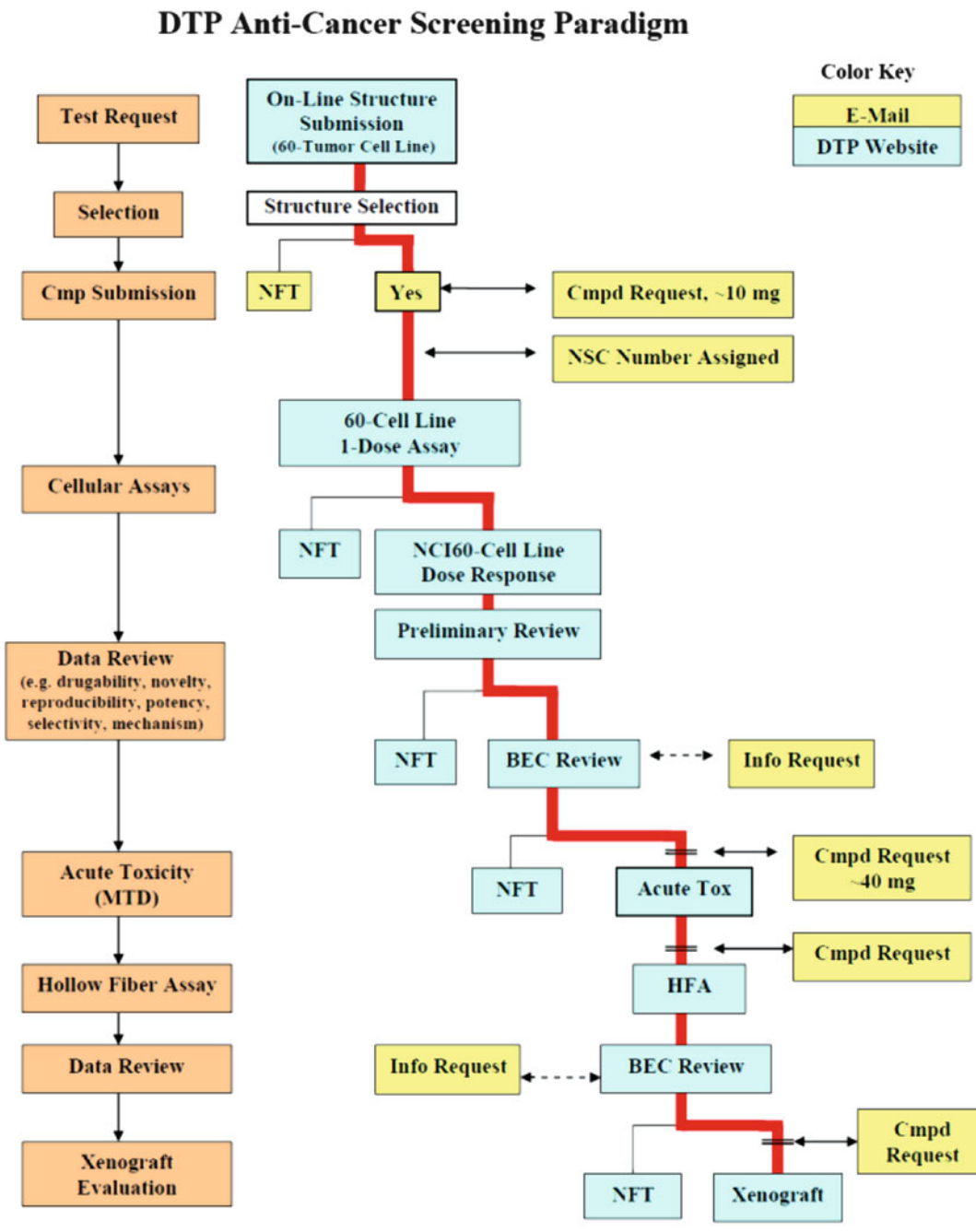

Fig. 2 Developmental Therapeutics Program (DTP) anticancer drug screening and decision-making process as of May 2008

4 Strength and Pitfalls of Cell-Based Screens vs. Cell-Free HTS on Isolated Targets

Large-scale screening using animal systems as practiced in the past (the P388 model; see above) is highly unethical and, particularly in Europe, strictly regulated. In the majority of cases, either cellular or target-based high-throughput assays will precede in vivo evaluation of potential anticancer drugs. High-throughput screening (HTS) plays an essential role in contemporary drug discovery processes. Miniaturization, robot-aided automatization, and data management by novel information technologies have provided the means of testing large compound libraries comprising several hundreds of thousands of molecules either from collections or combinatorial chemistry approaches [2]. Estimates of HTS screening capacity range from 100,000 to 1 million compounds per week. Whereas cell-based assay formats can be performed in 96- to 384-well plates, high-density formats such as 1,536-well plates with an assay volume of only 10 μL are suitable only for a cell-free isolated target-based screening setup [20].

4.1 Cell-Based Screening Assays

4.1.1 Conventional Cellular Screens

Cellular screens in cancer research employ mainly permanent human tumor cell lines; their immortal nature and hence manageable, reproducible growth behavior make them suitable test systems. Of critical importance, however, is the detection method, the choice of which depends on the cell number used and thus the desired sensitivity. Various procedures to determine cell growth are employed in screening laboratories. The earliest broadly used growth inhibition assays were developed by Mosmann and the NCI screening staff, namely, the methylthiazoldiphenyl tetrazolium (MTT) assay. The yellow MTT dye is reduced by mitochondria into a purple formazan, which can be read with ultraviolet/visible light scanners [20, 21, 49]. Its limitations are the use of large quantities of a hazardous solvent, dimethyl sulfoxide, which is required to dissolve the resulting formazan crystals and the varying number of mitochondria in cells. Currently employed in the NCI 60-cell-line screen is the sulforhodamine B (SRB) assay; SRB is a dye that stains protein [22].

Most industrial-scale cellular screens prefer the use of fluorescence or luminescence detection systems. The latter include, for example, the propidium iodide (PI) assay staining for DNA content [23] or use of a luciferase reporter [23–25]. They appear to offer the most advantages, such as high sensitivity and easy handling. The use of one-dimensional or monolayer cultures to measure cell growth is the most convenient and frequently applied method. Owing to tumor heterogeneity and three-dimensional in vivo growth, however, currently employed monolayer assays of human tumor (epithelial) cells are oversimplistic and have some disadvantages for the in vitro evaluation of certain anticancer agents:

1. Short-term culture conditions (2–6 days) may select for cytotoxic drugs.
2. Tumor cell growth can continue despite of the fact that clonogenic cells are reduced, missing certain classes of cytostatic agents (e.g., stem cell-targeted agents, differentiating agents).
3. Extracellular matrix and blood vessel targets (angiogenesis) are absent.
4. Gradients of oxygen tension, extracellular pH, nutrients, catabolites, and cell proliferation rate are a function of distance in solid tumors from blood vessels and are also not possible to mimic by monolayers.
5. Drug penetration barriers occur only in multilayered solid tumors.

Drugs that are affected by this list include signal transduction inhibitors, drugs targeting protein/protein interactions, antibodies, bioreductive drugs, antiangiogenic compounds, cancer stem cell-targeting agents, or telomerase inhibitors. These classes of drugs therefore might best be examined in either specially designed cell systems and tailored screens or biochemical assays.

4.1.2 Tailored Cellular Screens

Cancer stem cell-targeted drugs and inhibitors of pathways that regulate stem cell growth, such as Hedgehog and Notch inhibitors, are an emerging class of novel anticancer agents [26]. Examples of successful in vitro models that can be used to assess drug effects on cancer stem cells have been reported by Chang et al. [27] and Nakanishi et al. [28]. Cancer stem cells are a rare fraction of cells within a tumor which retain self-renewal properties. They also have self-protection mechanisms owing to the expression of high levels of drug efflux pumps [26]. Self-protection properties allow cancer stem cells to survive cytotoxic chemotherapy and their self-renewal capacity leads to the repopulation of tumors and, thus, recurrence [26]. Tumor recurrence is usually associated with development of resistance to the agents to which the patient initially responded. Conventional cellular screens are not suitable to evaluate stem cell-targeted treatments because they are aimed to measure tumor cell inhibition or kill the bulk cell mass.

Chang and coworkers have established an in vitro screen for the identification of drugs that can be used against treatment-resistant breast cancers. Their concept is based on growing mammospheres (tumorspheres) that can self-renew and grow in an anchorage-independent manner from tumor tissue biopsies. The mammospheres are then analyzed for breast cancer stem cell markers such as $CD44^{Hi}/CD24^{-/Low}/Lin^{-}$ or ALDH 1 (aldehyde dehydrogenase 1) and treated with drugs. While cytotoxic drugs such as adriamycin induced the fraction of $CD44^{Hi}/CD24^{-/Low}/Lin^{-}$ breast cancer stem cells, the HER2/EGFR tyrosine kinase inhibitor lapatinib was found to prevent the expansion of stem cells and led to a slight decrease [29]. Our laboratory also reported on an assay that can be used to identify drugs aimed to treat drug-resistant breast cancer stem cells [28]. The method is based on measuring the side population (SP). SP cells are characterized by having a high density of drug efflux pumps such as breast cancer-resistant protein (BCRP) or P-glycoprotein (Pgp), which causes these cells to efflux a fluorescence dye (Hoechst 33342), while mature bulk tumor cells take up the dye and can therefore be distinguished by fluorescence-activated cell sorting. The percent of side population cells in a given breast cancer cell line, including those resistant to tamoxifen, letrozole or trastuzumab, correlates with its ability to form colonies in soft agar [28]. We also demonstrated that inhibition of the HER2 family of growth factor receptors, particularly HER2 and HER3, by, e.g., trastuzumab can eradicate drug-resistant breast cancer stem cells. These examples show that cancer stem cell-targeted agents must be evaluated in a setting combining a stem cell-specific marker/characteristic with a growth assay format that allows only anchorage-independent and hence pluripotent or cells with self-renewal capacity to survive.

4.2 Biochemical Screening Assays

Biochemical assays are compared to "target-driven" cellular assays and provide the means for evaluating high numbers of compounds [30]. These screens are primarily employed in the pharmaceutical industry and institutions that harbor large compound libraries for systematic search of novel agents. Figure 3 summarizes the procedure for such an approach. An important advantage of biochemical screens is that they can be fully automated; thus, most steps can be performed by robot or computer

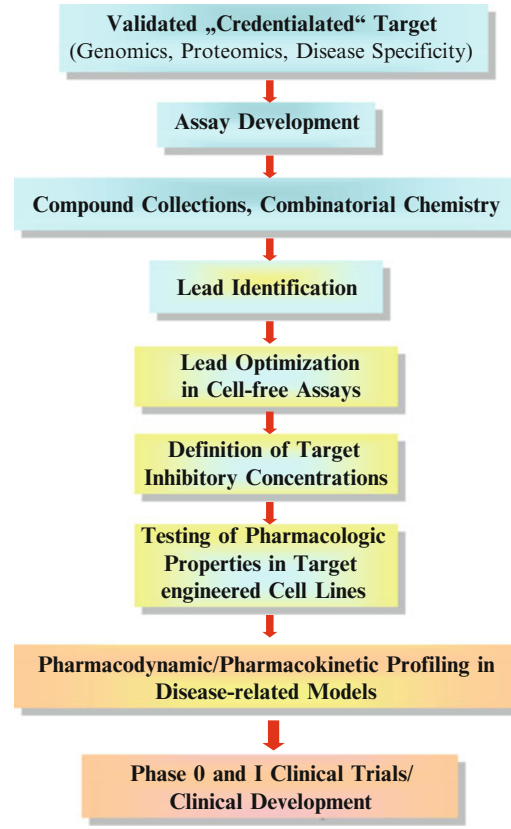

Fig. 3 Contemporary preclinical drug development cascade

systems such as dispensing of targets, addition of drugs and detection reagents, as well as compound library storage and management. Key requirements for target-oriented screening are:

1. The molecular target must be validated, shown to be causally linked to disease initiation or progression.
2. The target required for in vitro assays must be made available in large quantities, for example, by recombinant DNA techniques.
3. Defined, pure compound libraries comprising hundreds of thousands of structures derived from combinatorial approaches or collections of natural substances should be available.
4. Simple, cost-effective, highly reproducible assay and detection systems, which can be performed in microplate formats.

Suitable platforms have been proven to be enzyme-linked immunoadsorbent assays (ELISA) or other enzyme-based colorimetric methods. Further technologies that are frequently used are (1) radiometric assays dependent on scintillation proximity counting by employing scintillant-coated beads in microtiter plates, (2) time-resolved fluorescence based on highly fluorescing rare-earth metal–ligand chelates (europium, samarium, terbium), (3) fluorescence polarization, and (4) luminescence detection including chemiluminescence or electrochemiluminescence [2].

More recently, fluorescence resonance energy transfer (FRET) techniques have become a preferred method in high-throughput screens. FRET biosensors can readily be engineered and are suitable for cell-free and cellular systems [31].

Prominent targets for which these strategies have been employed and led to drugs that have progressed to advanced clinical development or even FDA approval are the protein kinases. For example, imatinib was found in an effort to develop bcr–abl kinase inhibitors after going through a biochemical screen using a panel of recombinant kinases. Bcr–abl is a chromosomal translocation product causing chronic myeloid leukemia. Imatinib has proven to be able to produce complete hematological and cytogenetic responses in this disease in patients [32]. Only careful testing of imatinib and its analogs in in vitro kinase assays and structural optimization of pharmacologic properties led to its success. If the agent would have been evaluated in a conventional cellular screen, it would have failed common activity criteria. In the NCI 60-cell-line screen, for example, only one cell line, namely, K562, possesses the bcr–abl abnormality; in addition, imatinib antiproliferative activity as a means of IC50 concentration is rather low. Mow et al. found, even in the K562 cell line, values for colony formation in the order of 12 μM and IC90s of target and growth inhibition of approx 20 μM [33, 34].

4.3 Combination of Target and Cell Screens

Both cell- and target-based screening procedures have clear advantages and disadvantages. While cell-based approaches will miss agents with certain defined modes of action owing, e.g., to lack of cytotoxic potency in short-term assays or the targeting of a rare subpopulation of cells in a bulk tumor mass, they might, on the other hand, identify compounds as active with previously unknown targets and hence allow for identification of novel mechanisms of action as well as the elucidation of their interplay in certain pathways. An example of this from the NCI 60-cell-line screen is bortezomib (Fig. 1).

Adams and colleagues synthesized a series of boronic acids as potential potent and selective inhibitors of the proteasome [35]. They submitted the compounds to the NCI 60-cell-line screen for evaluation. The average growth inhibition of 50 % (GI50) value for bortezomib across the entire NCI cell panel was 7 nM. Moreover, when 13 dipeptide proteasome inhibitors from the boronate series were examined, a strong correlation (Pearson coefficient, $r^2 = 0.92$) was noted after plotting K_i vs. GI_{50} values. Using the NCI's algorithm COMPARE, the bortezomib 60 cell line "fingerprint" was compared to the historical file of 60,000 compounds and found it to be unique, with little correlation to other "standard" or investigational agents, prompting further exploration of its activity in cell culture and in murine and human xenograft models. In these models, bortezomib exhibited many of the properties seen in preclinical studies of proteasome inhibitors such as lactacystin: activity as a single agent. They included enhancement of apoptosis induced by chemotherapy or radiation and specificity for transformed cells [17]. Subsequently, fluorogenic kinetic assays for measuring the inhibition of both chymotryptic and tryptic activities of the proteasome were developed [36] and found that bortezomib was a reversible, selective proteasome inhibitor [35, 36]. Phase I clinical studies found significant activity in multiple myeloma, with patients showing reductions in myeloma-related immunoglobulins and marrow plasmacytosis, which led to the development of bortezomib as an agent for the treatment of multiple myeloma and its approval by the FDA [17]. The mechanism(s) of action of bortezomib were identified retrospectively. One mechanism that is believed to contribute most to myeloma sensitivity to bortezomib is the dependence of this tumor type on the constitutive activation of NF-kappa B. If the proteasome is inhibited, I-kappa B, which binds to NF-kappa B, is not degraded and prevents the release of free NF-kappa-B which then can induce transcription and myeloma cell growth [37].

Another advantage of compounds identified in cellular screens is their proven cell-permeable properties, which might be missing in cell-free systems. In addition, ligand interactions might be more appropriate in the biological environment. Considering these facts, a combination of rational biochemical and "more" empirical cellular screening systems seems therefore the most optimal methodology in new cancer drug discovery.

5 Using Model Organisms for Screening

Nonmammalian organisms as systems for anticancer drug screening arose in the late 1990s as a potential alternative to human models in the light of advances in genomic research. A group at the Fred Hutchinson Cancer Research Center in Seattle headed by Steven Friend proposed to use yeast (*Saccharomyces cerevisiae*), the nematode *Caenorhabditis elegans*, or the fruit fly *Drosophila melanogaster*, because they share similar signaling and growth regulatory pathways with humans [38]. The advantage, particularly of yeast, is that the complete genome comprises only 6,250 defined genes, and, most importantly, many genes that are altered in human tumors have homologs in this model organism. For example, the *p53* tumor suppressor gene has its structural homolog in *RAD9*, the mismatch repair genes *MSH2* and *MSH1* in *MSH2Sc* and *MLH1Sc* or the *cyclins D* and *E* in *cyclin DDm* and *cyclin EDm*, respectively [38]. These models are therefore thought to provide a valuable resource to achieve a greater understanding about human cancer and hopefully give insights into new approaches for therapy. Friend and coworkers have chosen to employ DNA damage response elements/pathways to delineate mechanisms of actions of known, very effective anticancer agents (e.g., cisplatin in germ cell tumors) and to find novel targets for therapy by defining molecular changes underlying genetic instability of cancers, which they believe are mainly defects in DNA repair pathways, cell cycle checkpoints, and cell cycle transition. The group has determined the effects of cancer mutations on sensitivity or resistance to various chemotherapeutic agents in a panel of isogenic yeast strains, each defective in a particular DNA repair or cell cycle checkpoint function. Widely different toxicity profiles were observed for 23 standard anticancer agents and X-ray treatment, indicating that the type of DNA repair and cell cycle checkpoint mutations in individual tumors could strongly influence the outcome of a particular chemotherapeutic regimen [39]. While cisplatin was specifically toxic to yeast strains defective for the Rad6/Rad18-controlled pathway of damage tolerance during the S-phase, sensitivity to the ribonucleotide reductase inhibitor hydroxyurea was seen in the intra-S-phase checkpoint-deficient *mec1* and *mec2* strains. Hence, some of the commonly used anticancer agents showed significant specificity in their killing in yeast, and this provides strong evidence that new molecular diagnostics could improve the utility of the standard therapies [39]. However, screening and predicting activity of anticancer agents in yeast is limited by some differences in biology of yeast and mammalian cells such as tubulin. Spindle poisons are not toxic to *S. cerevisiae* and are therefore not active against yeast tubulin. Hormones, growth factors, and prodrugs requiring metabolic activation also cannot be modeled in yeast [38, 39].

Nonetheless, the yeast studies have brought about new useful anticancer agents based on the concept of synthetic lethality: two genes are synthetic lethal if mutation of either alone is compatible with viability but mutation of both leads to death. Targeting a gene that is synthetic lethal to a cancer-relevant mutation should kill only cancer cells and spare normal cells [40]. This paradigm arising from yeast led to the chemical synthetic lethality approach of total cancer cell kill and to the development of poly(ADP-ribose) polymerase (PARP) inhibitors for the treatment of cancers that have BRCA1 and BRCA2 mutations. The PARP inhibitor olaparib, which was the first drug to reach the clinic and blocks nucleotide excision repair, has shown responses in patients with BRCA1 or BRCA2, genes important in homologous recombination repair [41]. Phase II studies in BRCA mutant breast cancers are currently ongoing and reported very promising preliminary activity in this tumor type that has an overall poor survival outcome. When tumors with a genetic defect in the double-strand repair pathway are treated with a DNA single-strand repair inhibitor such as olaparib, chemical synthetic lethality occurs.

6 Predictivity of Screening Data

One of the key criteria for the strength/power of screening programs is their predictiveness of clinical response. Unfortunately, these analyses are very time consuming, as the process of preclinical and clinical development requires several years, so that outcomes of screens employing novel strategies are not yet foreseeable.

6.1 NCI Analysis of Activity in Preclinical Models and Early Clinical Trials

6.1.1 Xenografts

The NCI has conducted a retrospective review of the predictivity of their in vitro and in vivo screening efforts based on the 60 human cell line panel and xenograft testing in the 1990s. At the time of the review, the NCI procedures were mainly empirical and disease rather than target based [10, 42]. Data were available on 39 agents with both xenograft data and phase II trial results. The analysts found that histology of a particular preclinical model showing in vivo activity did not correlate with activity in the same human cancer histology. However, drugs with in vivo activity in a third of the tested xenograft models did correlate with ultimate activity in some phase II trials. This and the fact that none of the currently registered anticancer drugs was devoid of activity in preclinical tumor models, but showed activity in the clinic, led to the conclusion that activity in in vivo models of compounds demonstrating in vitro activity remains desirable [10, 43]. The hollow fiber assay has proven a valuable interface for selecting development candidates from large pools of compounds with in vitro antiproliferative activity for expensive and time-consuming subcutaneous xenograft testing (Fig. 2).

6.1.2 Hollow Fiber Assay

The HF assay was developed by Hollingshead et al. [16] at the NCI and is composed of 2 cm tubes filled with tumor cell lines. These fibers are implanted into mice at two sites (intraperitoneal and subcutaneous). The fibers are removed after 4–6 days in the animal and processed in vitro for quantification of tumor cell growth. By determining net cell kill, one can examine whether drugs administered via different routes are bioavailable and can reach the tumor sites [16, 42]. Of 564 compounds tested in the HF model and that were also tested in in vivo xenografts, 20 % showing HF activity also responded in xenograft models. This response was most likely if the intraperitoneal fiber activity was found in more than six intraperitoneal fibers. While a positive HF result could correctly predict in vivo xenograft response in one-fourth of the cases, 60-cell-line screening activity was able to predict correctly HF response in the order of 50 %. Significant HF activity in more than six intraperitoneal fibers was likely if the mean IC50 for in vitro growth inhibition of a compound was below 10–7.5 M. These analyses showed that the HF assay is a very valuable, rapid model system with predictive value.

6.2 Predictive Value of the Colony-Forming Assay

Another combined in vitro/in vivo testing procedure is the soft agar colony-forming assay, also termed tumor clonogenic assay (TCA). The TCA can either be used for sensitivity screening of patient tumor material in vitro predicting direct clinical response or with fresh xenograft tissue for selecting the

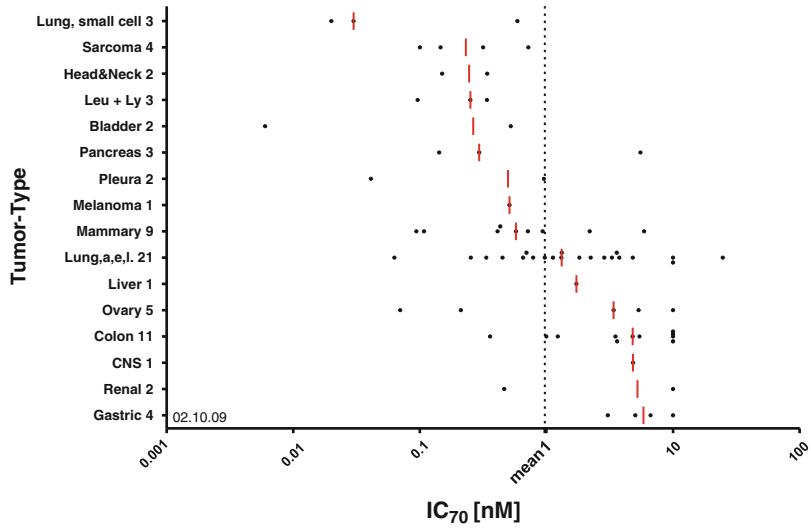

Fig. 4 Activity of trabectedin in 74 human tumor models in the clonogenic assay in vitro. The tumor types are listed on the *y*-axis; the numbers behind the tumor designation indicate the actual number of different patient-derived xenografts tested against trabectedin. The *x*-axis shows the IC70 values in nanomolar for each individual tumor in relation to the mean IC70 of all tumors (*dotted vertical line*). Data points to the left represent more sensitive and those to the right more resistant tumors

most appropriate in vivo model [44–47]. However, its high-throughput application is limited by lack of reproducibility (unique sample material) and the elaborative assay procedure.

A correlation between in vitro human tumor sensitivities and clinical responses of the same patients was first established by Salmon and coworkers. Their results demonstrated a highly significant correlation of in vitro tumor resistance to specific drugs with failure of the patient to respond to the same drugs clinically. Although the prediction for resistance was very high, that for sensitivity was less precise. Although in vitro tumor sensitivity was noted in every case where the patient responded, there was a significant fraction of false-positive tests resulting in clinical therapy failure [45]. Similar results were found in our laboratories when the response of xenograft tissue derived from patient tumors was compared to that of the patient. The TCA predicted correctly for tumor response in 62 % and for resistance in 92 % of the examined cases [45, 46]. The latter is mirrored by the even better response prediction of the Freiburg nude mouse xenografts if used in vivo. Figure 4 shows an example of the novel agent trabectedin (Yondelis®, ecteinascidin 743) and its activity in a panel of 16 tumor types in the TCA. Trabectedin is approved in Europe for the treatment of advanced soft tissue sarcoma. The European Commission and the US Food and Drug Administration (FDA) have granted orphan drug status to trabectedin and a registration dossier has been submitted to the European Medicines Agency (EMA) and the FDA for trabectedin when administered in combination with pegylated liposomal doxorubicin (Doxil, Caelyx) for the treatment of women with relapsed ovarian cancer.

The four xenografted soft tissue sarcomas, derived from patient explants that were tested in the TCA in our laboratories, were the second most responsive tumor type with a median IC70 of about 0.5 nM. All four sarcomas were more sensitive than the mean IC70. Sarcoma sensitivity is followed by head and neck cancers and hematological malignancies. Only the median response of small-cell lung cancers was superior to that of soft tissue sarcoma to trabectedin single-agent treatment (Fig. 4). Trabectedin was less active against 5 ovarian cancers with a median IC70 of 5 nM. However, 2 of the 5 ovarian cancers appeared to be very sensitive, whereas the others were more resistant than the mean IC70 of all tumor types combined. However, the combination of trabectedin and doxorubicin was not tested. Overall, the data in Fig. 4 demonstrate that the TCA is useful in predicting tumor response.

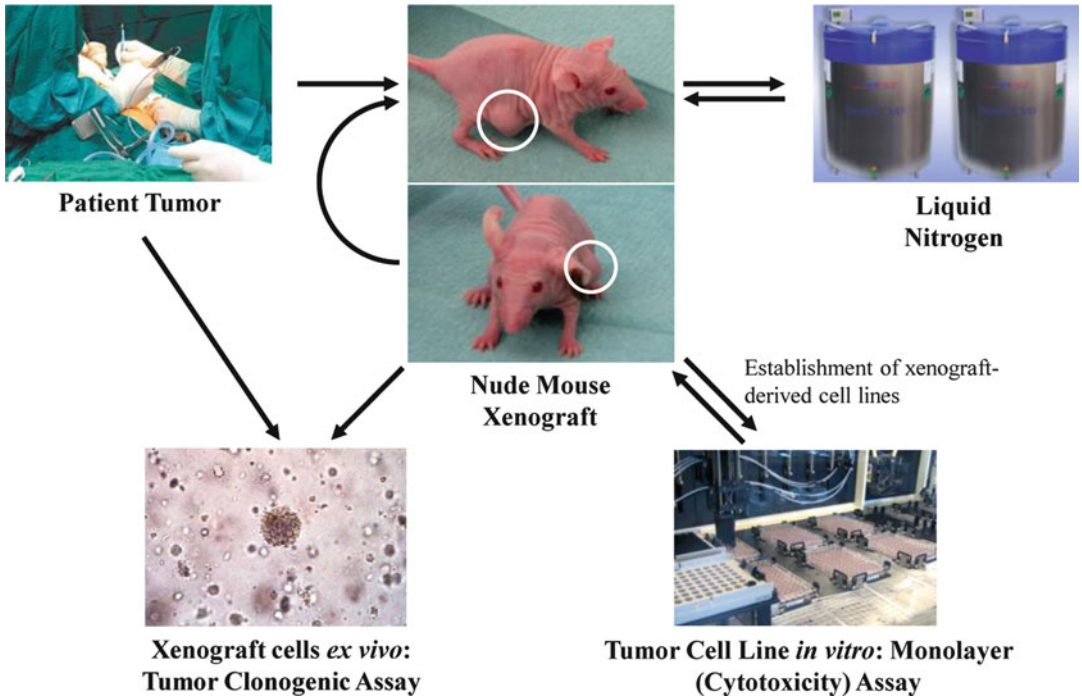

Fig. 5 Screening procedure using patient-derived tumors for the establishment of in vitro and in vivo models

Owing to the small-cell lung cancer responses and the poor treatability of this tumor type with chemotherapy, it should also be considered for studies of trabectedin efficacy.

6.3 Relationship Between Clinical Response and Patient Explants in Nude Mice

6.3.1 The Freiburg Experience

Unlike the NCI in vivo screen, the Freiburg xenograft panel is derived directly from patient explants and not established from permanent human tumor cell line material as detailed in Fig. 5. By comparing the efficacy of a standard-of-care drug or drug combinations in patients and their tumors grown in nude mice, a total of 21 patients reached a remission. The same result was observed in 19 tumors growing as xenografts. Fifty-nine patients did not respond to treatment and the same result was found in 57 cases in the nude mouse system. Overall, xenografts gave a correct prediction for resistance in 97 % (57/59) and for tumor responsiveness in 90 % (19/21) [46].

Although most analyses of predictivity and usefulness of in vitro and in vivo screening procedures indicate clearly a high value of anticancer drug screens, particularly if validated by employing agents that have made it to the clinic, it remains unclear how the new molecular targeted agents with no prior defined clinical activity will translate into patient benefit. It also seems to be certain that pure in vitro screening methodology will not be sufficient to delineate potential clinical activity, particularly because pharmacokinetics have a major impact on pharmacodynamic activity. Data derived from in vivo model systems deem necessary to ensure that drug concentrations inhibiting the target and in vitro cell growth to 100 % or at least 50 % can be reached.

7 Conclusions and Perspectives

Preclinical screening is necessary to prioritize compounds for further development. In the era of target-oriented molecular cancer therapeutics, screening procedures are tailored toward the desired mechanism of tumor inhibition. They require, however, careful design and validation. In the past, empirical screens designed to find highly potent cytotoxic agents produced an arsenal of clinically used drugs with low selectivity and efficacy in solid tumors. Although antiproliferative activity is generally a desirable effect, it might bias toward finding compounds poisoning DNA and the cytoskeleton in the commonly used short-term cultures rather than drugs with novel mechanisms. However, empirical screening approaches looking for compounds with novel profiles to which molecular mechanisms could be fitted retrospectively, such as the histone deacetylase inhibitor vorinostat, the proteasome inhibitor bortezomib, and the heat-shock protein inhibitor tanespimycin, led to the identification of subgroups of patients benefitting from these therapies. Thus, rational drug design or drug discovery approaches combined with novel knowledge from genome and proteome research as well as bioinformatics are the most promising ways toward individualized cancer therapy. Our drug screening and discovery pathways have evolved into an integrated approach which combines the use of cell line and tumor xenograft models that resemble very closely the patient characteristics and response (Fig. 6). They are molecularly profiled for most of the validated targets using state-of-the-art genomic and proteomic technologies as shown in Fig. 6. Drug–target interactions are assessed and tumor tissues pre- and posttreatment are used to explore and develop gene signatures or biomarkers of tumor response (Fig. 6) [48].

Target-driven drug development has led to the availability of many useful cell signal transduction inhibitors and antibodies targeting growth factor receptors. The next challenge in preclinical anticancer drug screening and development is to find the means to disrupt protein–protein interactions and to control deregulated transcription with small molecules. To accomplish the latter, molecular in vivo imaging procedures and drug delivery technologies need to be incorporated particularly into preclinical screening processes.

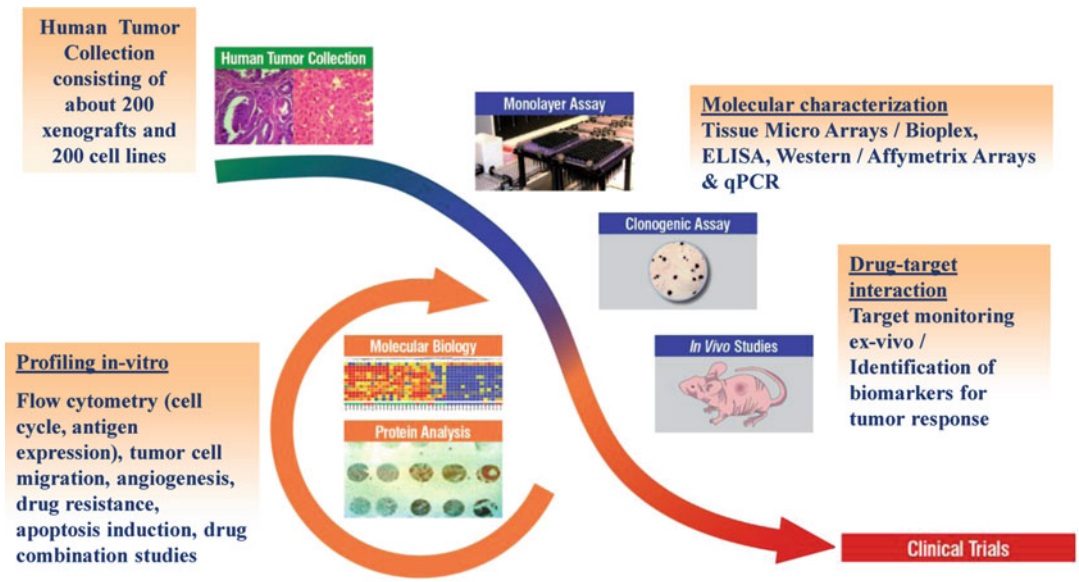

Fig. 6 Example of an integrated approach to anticancer drug screening as used by the Institute for Experimental Oncology, Freiburg, Germany

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Natural Product Screening

Tawnya C. McKee, Albert W.W. Van Wyk, and Emily L. Whitson

Abstract Natural product screening marries the search for new medicines with the search for new molecules from natural sources. The rationale for natural products as a source for new hits from which to develop new drugs will be discussed, and a brief overview of screening methods and techniques including how these are modified for the screening of crude natural product extracts will be described. This chapter will also provide a summary of the importance of natural products to drug discovery and development, the results from screening assays developed, and the natural products isolated utilizing these screens.

Keywords Screening • Natural products • Assay development • Phenotypic screen • Biochemical screen • Nuisance compounds

1 Introduction

Throughout recorded history, humans have been probing nature for cures for illness and disease. Among the earliest recorded use of natural product-based drugs includes the pharmacopeia of traditional Chinese medicine (or TCM) [1] and Ayurvedic practices [2] from India. Nature has produced a rich diversity of structurally complex compounds or secondary metabolites with a wide variety of biological activities that can be exploited as medicines. Natural products chemistry, that is, the study of the isolation and structural characterization of individual compounds from naturally occurring sources such as plants, invertebrate animals, and microbes, gave rise to the field of organic chemistry [3]. Natural product discoveries, along with the identification of their relevant biological and biochemical mechanisms, have been pivotal in advancing organic and medicinal chemistry studies [4] and are critical components in the development of a variety of therapies [4]. Natural products have profoundly impacted the course of modern medicine, and their central role in the drug discovery process is unmatched [3]. Approximately 63 % of anticancer compounds are derived from or inspired by natural products (products of natural origin, semisynthetic natural product analogs, or synthetic compounds based on natural product scaffolds) [5].

However, despite years of searching there is still a significant number of diseases and other medical needs that are unmet by currently available, approved drugs. In medicine, to screen someone is to look

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for the presence of disease when there may or may not be overt symptoms. Many screenings are routine such as mammogram or colonoscopy; that is, they are performed on a recurring basis after a person reaches a specific age or has certain symptoms. In drug discovery, screening has a similar meaning; it is a process whereby scientists test or examine compounds or mixtures for certain therapeutic properties. This can be the ability to kill specific cells or to inhibit a specific target enzyme or other protein interaction. It is a primary way to identify chemical structures that are developed into new medicines to fight disease. Billions of dollars are spent each year in the search for new medicines, and screening is an integral part of this research undertaking.

Natural product screening marries the search for new medicines with the search for new molecules from natural sources. As technology has advanced, the ability to screen for new drug leads has also increased. Speed, the number of compounds that can be interrogated, the types and complexity of questions that can be asked, and the level of detail at which data can be analyzed have all been increased. Today, much of the screening is done as "high-throughput screening" or HTS. High-throughput screening involves screening thousands of compounds per day against a target. It generally requires robotics, advanced liquid handling instruments, computer analysis of results, and very sensitive detection methods since HTS involves small volumes to maximize throughput while minimizing reagent and compound consumption. The active compounds (or "hits") identified by HTS are then confirmed, further tested using lower throughput methods, and generally improved by chemical modification (medicinal chemistry), resulting in a drug lead for clinical trials.

Throughout this chapter, the rationale for natural products as a source for new hits from which to develop new drugs will be discussed, and a brief overview of screening methods and techniques including how these are modified for the screening of crude natural product extracts will be described. Since 2001, the authors' laboratory at the NCI has been focused on developing both biochemical and cell-based molecularly targeted screens, to discover natural product modulators of these targets utilizing the NCI natural products extract repository. This repository is the world's largest and most diverse collection of natural product extracts. This chapter will also provide a summary of the importance of natural products to drug discovery and development, the results from screening assays developed and run in the lab, and the natural products isolated utilizing these screens.

2 Historical Importance of Natural Products

Evolution would dictate that organisms do not produce natural products to specifically interact with or bind to human proteins, so it is reasonable to wonder why they have such profound pharmacological effects on human disease [6, 7]. Biological space, that is, the structural space occupied by macromolecules involved in cellular processes, is relatively limited. While the human genome is comprised of roughly 30,000 genes, only a small percentage of these genes or their protein products is targeted by existing therapeutics [8]. This space is further limited by the three-dimensional space of protein folds. Current findings estimate that there are roughly only 1,700 separate folds and 4,000 structural superfamilies within proteins [6]. In effect, biologically relevant human targets of disease may be composed of the same fundamental protein folds or analogous structural domains as the biological targets of naturally produced molecules, thereby eliciting the same or a similar response in an entirely different setting [9–11]. Chemical space, conversely, is vast, estimated at approximately 10^{60} organic molecules with molecular weights below 500 [8]. The ability to target the intersection between these two distinctly different yet analogous spaces is the basis of the discovery and development of new drugs.

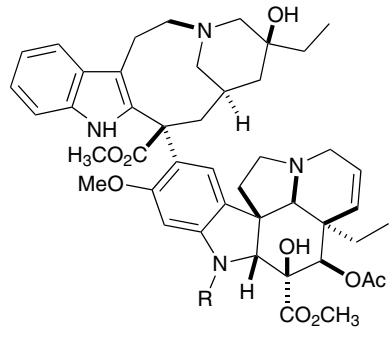
Natural products provide unique structural elements as products of biological systems. This includes exquisite detail in not just two-dimensional space but in three-dimensional space through stereochemistry. It is this structural diversity of chemical space from nature that not only stimulates

new discoveries about organic chemistry but also leads to a better understanding of the biochemistry so critical to drug discovery. In essence, every natural product identified can be appreciated both for adding to the diversity of known chemical structures and the possibility that it may be exploited through lead optimization to provide new drugs. Due to their evolutionary importance, effective use of chemical space, and documented therapeutic usefulness, natural products will continue to play an essential role in the drug discovery process.

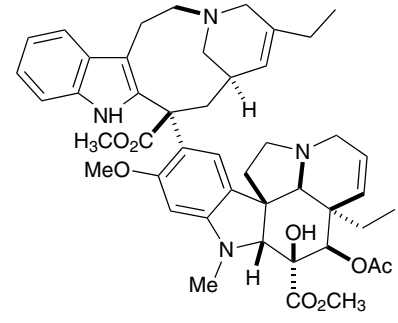
3 Natural Product Cancer Drugs

The majority of current cancer therapeutics can be traced back to isolated compounds of plant, microbial, or marine origin. Examples of natural product-derived cancer drugs and their natural product inspiration are briefly summarized below. Among the most important plant-derived antitumor compound classes are the vinca alkaloids, taxanes, podophyllotoxins, and camptothecins.

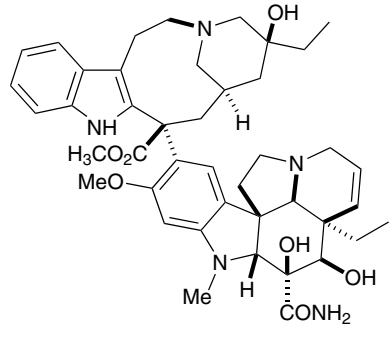
The Madagascar periwinkle (*Catharanthus roseus*) was originally investigated as a source of oral hypoglycemic agents, resulting in the isolation of the vinca alkaloids (Fig. 1), vinblastine (**1**, Velban®)



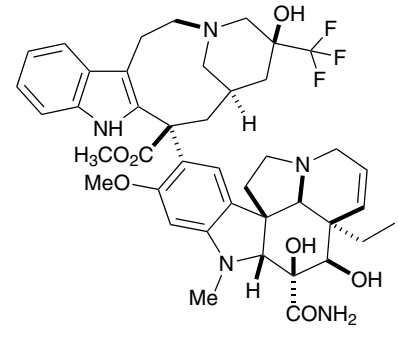
1 vinblastine R = CH_3
2 vincristine R = CHO



3 vinorelbine

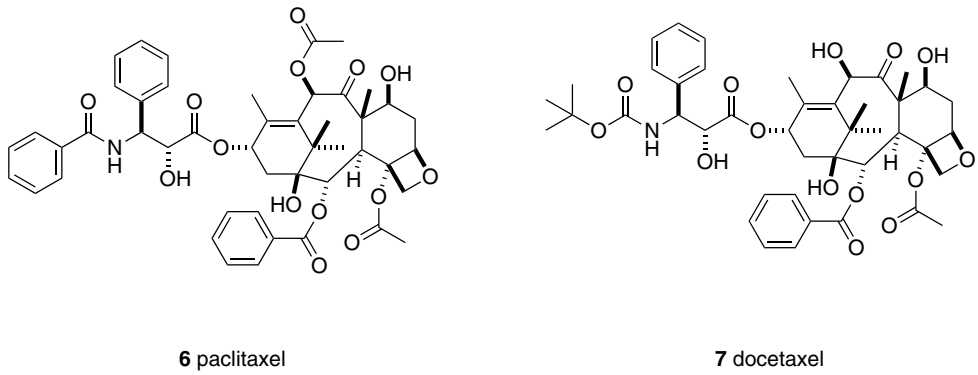


4 vindesine



5 vinflunine

Fig. 1 Important vinca alkaloids



6 paclitaxel **7** docetaxel

Fig. 2 Paclitaxel and docetaxel

and vincristine (**2**, Oncovin®, Vincasar PFS®) [12–14]. Further biological evaluation led to the discovery of their utility as anticancer agents. Vinca alkaloids bind to β-tubulin and prevent polymerization with α-tubulin to form microtubules [12, 15–18], disrupting the mitotic spindle assembly and mitosis [12, 15]. Vinorelbine (**3**, Navelbine®) and vindesine (**4**, Edelsine®) are semisynthetic derivatives of vincristine that were later developed for clinical use [12, 15]. New vinca alkaloids continue to be developed. Vinflunine (**5**), a bis-fluorinated analog of vinorelbine, is currently being studied in clinical trials as a single use agent and in combination for bladder, breast, and non-small cell lung carcinoma (NSCLC) [15, 19]. The vinca alkaloids are still very important in cancer chemotherapy today; vincristine (**2**) is used in combination with other anticancer drugs for the treatment of acute lymphoblastic leukemias and lymphomas [12, 15]; vinblastine (**1**) is used in combination to treat breast, testicular, and bladder cancers, as well as Hodgkin's disease [12, 15]; vinorelbine and vindesine are used for the treatment of NSCLC and metastatic breast cancer [12, 15].

Paclitaxel (**6**, Taxol®) and docetaxel (**7**, Taxotere®) are the two FDA-approved members of the taxane family of compounds (Fig. 2). Paclitaxel was first isolated from the bark of the Pacific yew (*Taxus brevifolia*) in very small quantities in the late 1960s [20]. The limited amount of material available slowed paclitaxel development, but it now is produced by semi-synthesis from 10-deacetylbaccatin III from the leaves of the closely related *Taxus baccata* and by cell culture. Docetaxel, a related compound, is also synthesized from 10-deacetylbaccatin III. In contrast to the vinca alkaloids, paclitaxel and the other taxanes promote tubulin polymerization and stabilize microtubules [21–23], disrupting normal microtubule dynamics that are required for cell division and other vital processes, leading to cell death [24]. Paclitaxel and docetaxel are used for the treatment of breast, lung, ovarian, head, and neck cancers and also for AIDS-related Kaposi's sarcoma [24]. Additional taxane derivatives continue to be developed and evaluated in clinical trials [5, 15, 25].

Podophyllotoxin (**8**, Fig. 3) was isolated from the roots of the podophyllum plant (*Podophyllum peltatum*), which was traditionally used by Native Americans for its emetic, cathartic, and anthelminthic properties [15, 26]. The epipodophyllotoxins [etoposide (**9**, Toposar®) and teniposide (**10**, Vumon®)] (Fig. 3) are synthetic analogs based on the original podophyllotoxin scaffold. Etoposide and teniposide bind to DNA topoisomerase II [5, 15]. Topoisomerase II alters the tertiary structure of DNA through a transient double-stranded breakage of the DNA backbone to allow for an intact DNA duplex to pass through the break, unpacking the condensed DNA structure and allowing transcription [15, 27]. Etoposide and teniposide stabilize the topoisomerase II–DNA complex, preventing the religation of the double-stranded breaks in the DNA and, ultimately, causing cell death [15, 26]. Etoposide is a drug frequently used for the treatment of lung, ovarian, and testicular cancer; choriocarcinoma; lymphoma; and acute myeloid leukemia, while teniposide is used for the treatment of central nervous system tumors, malignant lymphoma, and bladder cancer [15, 26, 28]. However, development of drug

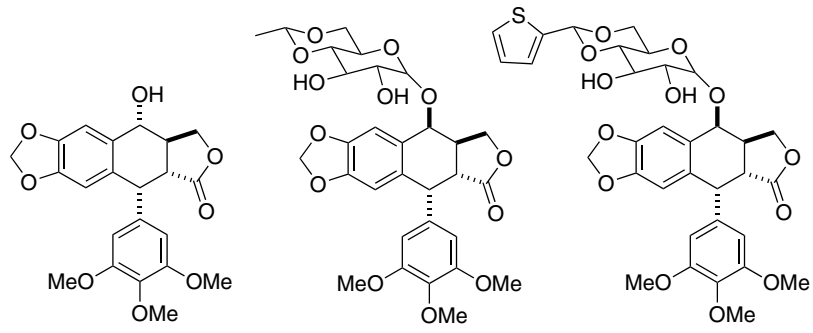


Fig. 3 Podophyllotoxin and *epi*podophyllotoxin cancer drugs

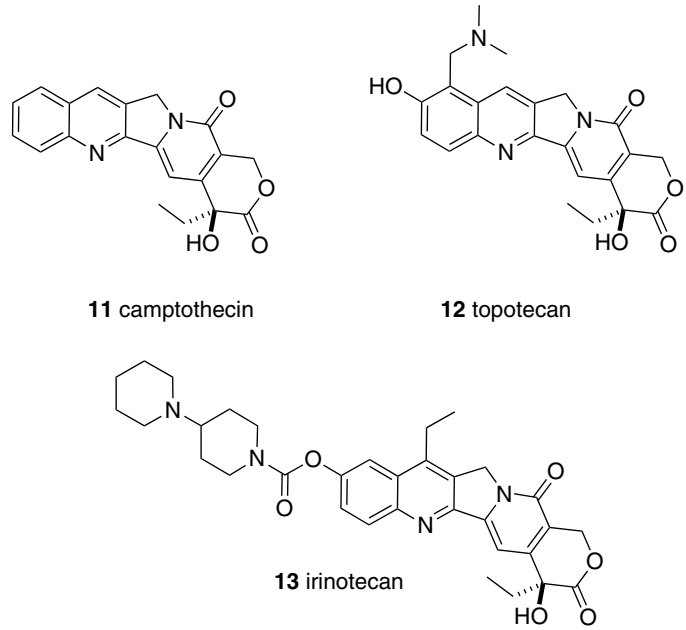


Fig. 4 Camptothecin and camptothecin analogs

resistance and poor water solubility are major shortcomings of this class of compounds that are being addressed with a new series of analogs undergoing clinical trials [5, 26].

Camptothecin (**11**, Fig. 4), originally isolated from the Chinese ornamental tree (*Camptotheca acuminata*), was too toxic and insoluble for clinical use [29]. However, the semisynthetic camptothecin analogs, topotecan (**12**, Hycamtin®) and irinotecan (**13**, Camptosar®), have since been developed and are currently used in the treatment of colorectal and ovarian cancer [15, 30]. The camptothecins stabilize the topoisomerase I–DNA complex and prevent resealing of the DNA break, leading to cell death [28, 31, 32]. There are also a large number of newer synthetic camptothecin-based compounds in clinical evaluation [15, 33].

There are many microbe-sourced antitumor antibiotics in use, including the anthracyclines (Fig. 5) doxorubicin (**14**, Adriamycin®, Doxil®, Rubex®), daunorubicin (**15**, Cerubidine®, Daunoxome®), epirubicin (**16**, Ellence®), pirarubicin (**17**, Pirarubicin®), idarubicin (**18**, Idamycin®), valrubicin

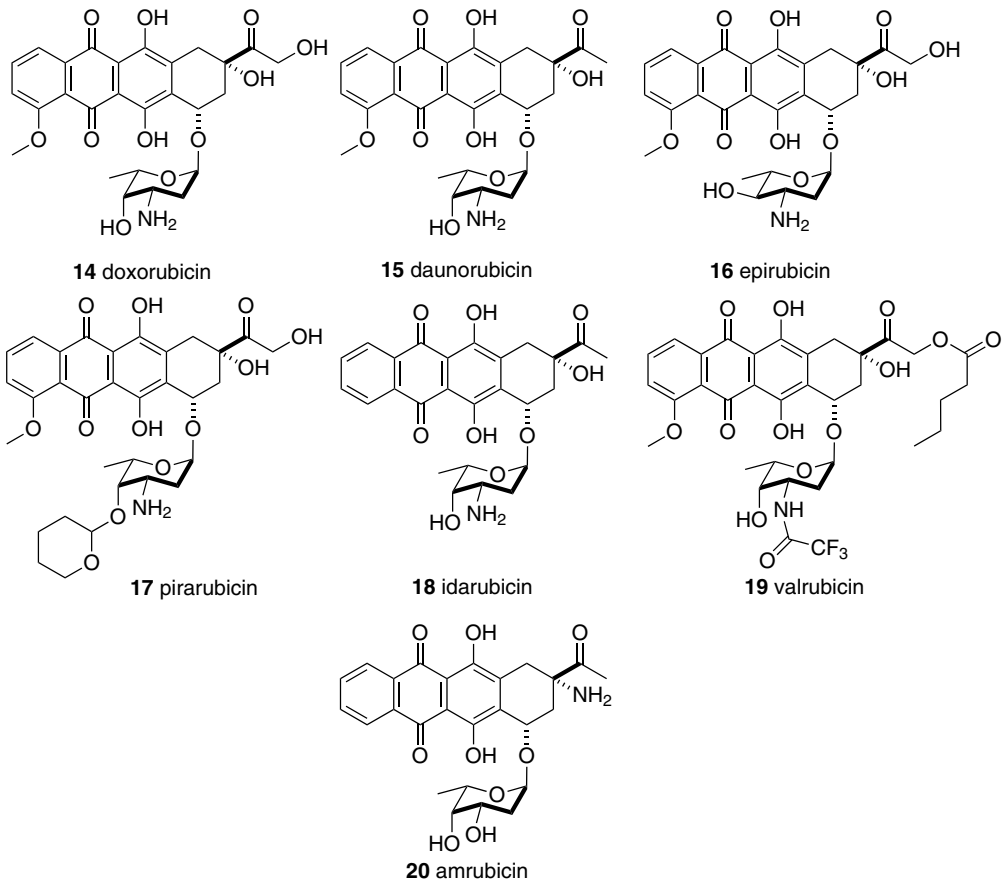


Fig. 5 Anthracycline microbial antitumor antibiotics

(**19**, Valstar®), and amrubicin (**20**) [34]. Both daunorubicin and doxorubicin are used in combination protocols for the treatment of acute nonlymphocytic leukemia, Hodgkin and non-Hodgkin lymphomas, breast cancer, and sarcomas [34, 35]. Epirubicin and idarubicin are less toxic than daunorubicin and doxorubicin and are used for the treatment of leukemia, breast and ovarian cancer, as well as other diseases [34, 36]. Other microbial-derived cancer therapies (Fig. 6) include the bleomycins [e.g., bleomycin (**21**, Blenoxane®)] [37], actinomycins [e.g., dactinomycin (**22**, actinomycin D, Cosmegen®)] [38], mitomycins [e.g., mitomycin C (**23**, Mitozytrex®, Mutamycin®)] [39], and enediynes (e.g., calicheamicin **24** and gemtuzumab ozogamicin, Mylotarg®) [30, 40]. Bleomycin is used as a single agent for the treatment of squamous cell carcinomas and in combination for the treatment of Hodgkin and non-Hodgkin lymphomas, testicular cancer, and germ cell ovarian cancers [37, 41, 42]. Dactinomycin is used for the treatment of trophoblastic tumors [43], metastatic testicular cancer [44], Wilms' tumor [45] and Ewing's sarcoma [40]. Mitomycin C has been used in combination with other anticancer drugs against a variety of cancers, the most common treatments being gastric, pancreatic, and cervical cancers [39, 46, 47]. Mylotarg®, a calicheamicin analog, is the first antibody-targeted chemotherapeutic agent, which is used for the treatment of acute myelogenous leukemia [40, 48].

Ecteinascidin-743 (**25**, Fig. 7, ET-743/trabectedin, Yondelis®), originally isolated from the ascidian *Ecteinascidia turbinata* [49], is the first marine-derived anticancer drug approved by the FDA. The ET-743 is now produced by semi-synthesis from cyanosafracin B, which is produced by large-scale fermentation of *Pseudomonas fluorescens*. ET-743 binds to the minor groove of DNA, disrupting the

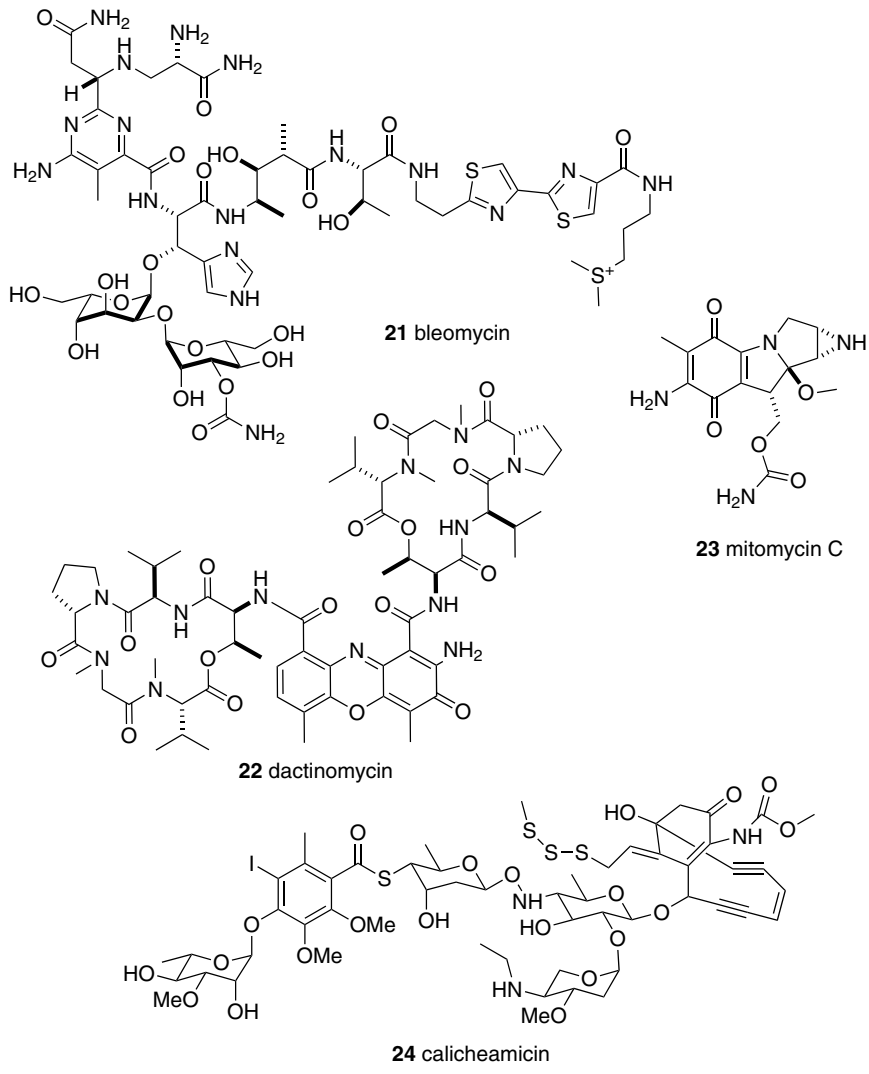


Fig. 6 Additional microbial-derived cancer therapies

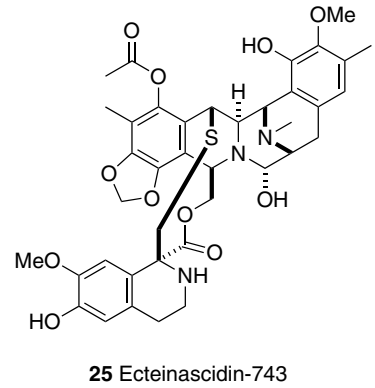


Fig. 7 Ecteinascidin-743

cell cycle and inhibiting cell proliferation, and it also interferes with transcription [50, 51]. ET-743 has been approved for use in Europe for the treatment of soft tissue sarcomas and is also being evaluated in a number of US clinical trials [5, 15, 51].

In addition to the currently approved anticancer natural product drugs, there are a number of natural products that are currently undergoing clinical trials for cancer and show promise as cancer therapeutics [5, 15, 30]. There is also an abundance of potential antitumor agents which warrant further investigation [52–54].

4 Current State of Natural Products Research in Industry

Pharmaceutical companies are under constant pressure by investors to maintain double digit profit each year. Given that most drug candidates fail, it is imperative that these companies maintain a robust pipeline of candidates progressing through development, both as potential new sources of revenue and treatments, but also to replace existing drugs as their earnings decrease with the end of patent protection and competition from generic equivalents.

Despite the proven effectiveness and reliance of physicians on natural product-derived drugs, from the mid-1990s, there was a diminished interest in natural product discovery for pharmaceutical use [55] due to the challenges associated with natural products research. Because the lead compounds come from organisms occurring in nature, the first step in natural product research involves collection of organisms. Permission and agreements to collect including compensation for any discoveries that result from collections must be in place from the countries of origin prior to collection. These organisms need to be collected in sufficient quantities to be able to identify even low-abundance compounds. Detailed collection notes provide location information in order to allow for recollections to resupply the isolated compounds unless (or until) the compounds can be synthesized. Taking a complex mixture in an extract composed of literally thousands of compounds and separating it into individual, biologically active compounds can be a multistep, challenging process. When one is using biological activity as the method of selectivity, it can be slow. Each step in the purification process is followed by reassay of resulting fractions in order to determine which fractions will continue to be pursued. Next, there is the time required for the determination of the chemical structure, including stereochemistry, of the isolated molecules. Last, many natural products as isolated are structurally unsuitable as drugs and require chemical modification to improve efficacy and/or reduce toxicity. These challenging realities made natural products seem too slow and too costly to many within the pharmaceutical industry [55].

A second significant factor in this shift away from natural products research was the introduction of high-throughput screening (HTS) [56]. Effective screening of biological targets is accomplished through exposure of a target to the widest variety of chemical structures possible, with the intention of finding those unique molecules that will bind to the specific target of interest. HTS, by screening thousands of compounds per week, encouraged many companies to seek out well-defined synthetic chemical libraries that were more compatible with the sample turn around demands of HTS [56]. In contrast, natural product extract libraries, with their complicated mixtures, made the isolation and identification of active compounds comparatively time consuming and laborious—a less than ideal pairing for the short timeline of HTS programs where assays are run for weeks, not months or years. The concomitant development of synthetic combinatorial chemistry—which allowed for the production of thousands of compounds at a significantly lower cost than would be spent generating natural product libraries of the same magnitude—was a third contributing factor.

Combinatorial chemistry offered access to a preselected range of molecules centered on a common chemical core (or pharmacophore), providing large compound libraries in a relatively short period of time. The chemical diversity of these earliest combinatorial compounds, however, has been shown to

be limited in stereochemistry. Additionally, molecular biology was identifying therapeutic targets at a rapid rate (months instead of years), making chemistry *the* rate-limiting step in discovery programs [11]. Natural product chemists could not continuously supply the huge numbers of new compounds required by screens. The traditional method of bioassay-guided fractionation appeared to take a great deal of time and resources, and it offered no guarantee that a screening hit would be an ideal candidate for lead optimization or, more significantly, that it would be patentable. It was due to these factors that natural products fell out of favor in the pharmaceutical industry [11].

Many companies terminated their natural product programs as they concurrently embraced the new field of combinatorial chemistry. This decision has not been the unqualified success envisioned as the number of new molecular entities (NME, a drug that contains no active moiety previously approved by the FDA) declined rapidly from 35 in 1999 to 17 in 2009 [57]. With drug development timelines clustering around the decade mark, the correlation between this low number of NMEs and the emergence of combinatorial chemistry as the source of new drugs becomes obvious. Regardless of initial appeal, combinatorial chemistry has had little positive impact to date on the discovery of NMEs or lead scaffolds [6]. And although combinatorial chemistry has been effectively applied to lead optimization, only one de novo combinatorial compound, sorafenib (Nexavar®), has been approved in the last 25 years [58]. With its insufficiently diverse pool of structures and deficiencies in the biological application of compounds generated by its screening libraries, early results from combinatorial chemistry have fallen short of expectations [7].

As combinatorial chemistry's promise to fill drug development pipelines with de novo synthetic small-molecule drug candidates remains as yet unfulfilled, the interest in natural products has been rekindled. Natural product chemistry has reemerged as a highly reliable source of refined, naturally selected, specific, and potent lead molecules [10, 59, 60], although active research programs have not been reinstated within the pharmaceutical industry.

Modern technologies have continued to develop and change the way isolation and structure elucidation are carried out, and novel, minor metabolites are becoming easier to analyze. Minute quantities of material can now be identified with astonishing speed [55, 61]. Modern structure elucidation methods have made it easier to explore the unique and innovative structures that natural product extracts harbor. Complete characterization of these natural products, within the bounds of what is possible given current technologies, is essential to an understanding of the three-dimensional shapes they take and how these structures interact with biological targets.

Although combinatorial chemistry as yet has not proven fruitful as the discovery tool envisioned, it has shown promise in the further development of active compounds [11]. Combinatorial chemistry has been used to optimize the drug-like properties of natural products (bioactivity, pharmacokinetics, solubility, etc.). These optimized natural products are in all phases of drug development [11]. More recently, researchers have looked at combining the potential of natural products with synthetic chemistry through combinatorial total synthesis of analogs, chemical investigation of natural product scaffolds, and the design of unnatural molecules inspired by natural products [9, 62–64].

5 Screening

With the continued rapid expansion of molecular and cellular biology, the number and the variety of assays that are being developed is increasing rapidly. Regardless of the type of screen utilized, the ability to detect an output that reflects the desired activity requires the availability of a variety of methods (Table 1). Each method has advantages and disadvantages; many can also be utilized in a variety of assay formats. The purpose of screening is to expose the largest number of test materials to assess their effect on a biological activity. Screens can be either cell-based or biochemical (cell-free). The activity of a screen can be general or phenotypic, such as cell death, or it can be more

Table 1 Definitions of common screening terms

Term	Definition
Assay	Assay is a procedure to test or measure a response or activity of a compound
Screen	A process used to identify or select for compounds that possess desired activity
Format	The arrangement of an assay that indicates conditions and detection method used
Phenotypic screen	The unbiased testing of compounds in cells without knowing their targets and assaying for varying effects
Molecularly targeted screen	The testing of compounds against a specific target and identifying compounds that modulate the target
Scintillant	A substance that flashes light upon stimulation
TRF	Time-resolved fluorescence; detection of fluorescence from sample is delayed for a period of time so that the fluorescence of the fluorophore is greater than that of the background material
FRET	Förster (fluorescence) resonance energy transfer; both the material absorbing light and that to which the energy is transferred both fluoresce. The distance is generally <10 nm. Lanthanide elements are used because of their unusually long fluorescence lifetimes (milliseconds), which allow long-range energy transfer between fluorescent lanthanide energy complexes and their corresponding resonance energy acceptors and remove the background
Fluorescence anisotropy	Fluorescence polarization is based on the principle that the rotational speed of molecules is related to their size. Molecules rotating quickly depolarize light to a greater extent than those rotating slowly; thus, when small fluorescent molecules bind to larger molecules, their rotation speed decreases, giving rise to a larger polarized signal
ELISA	Enzyme-linked immunosorbent assay; systems where a protein (often an enzyme) is bound by a specific antibody linked to a second enzyme whose presence is measured by the colorimetric or fluorescent endpoint produced
Luciferase	Commonly used luminescent detection enzymes that are available from a wide variety of organisms such as fireflies and sea pansy
HCS	High-content or image-based screening. Involves the direct observation of endpoints and looks in detail at changes within individual cells through imaging; examples include movement of subcellular organelles and formation/depolymerization of microtubules
Dynamic range	Difference between the positive and negative controls of a given screen
Hit	A compound that displays the desired activity in a given screen, generally a minimum of three standard deviations from the negative control
Lead	A hit confirmed in more than one assay related to the target and can be taken forward towards drug development
Nuisance compound	Compounds or families of compounds that test positive in a screen but that are not suitable for medicinal purposes
Z' factor	Z' factor gives an indication of the degree of separation between the positive and negative controls in an assay and consequently how easily a "hit" can be reliably picked out from inactive compounds

focused, looking for a response from a single target or pathway. Regardless of the activity measured, the initial screen must be followed up with additional tests to confirm and further define the initial activity observed.

5.1 Assay Detection Methods

Much of the diversity of the available screens is due to the large number of methods used to detect the assay's endpoint or readout. These methods can be organized into several large general categories that will be briefly described below. All of these methods can be used to screen natural products, so the selection of which method to use often is based on the target or cell line to be interrogated, the reagents that are readily available, and the methods used by the individuals or lab in the past.

5.1.1 Radioisotopes

Early cell-free assays primarily utilized radiometric technology, in which a target molecule was immobilized onto a solid support system containing a scintillant. Subsequent binding of a radiolabelled molecule to the target would then bring the scintillant and radioisotope into close proximity and hence allow for energy transfer in the form of light [65]. As assays have increased in throughput and consequently undergone miniaturization, use of radioisotopes in HTS is now primarily in scintillation proximity assays (SPA) [66–68]. The reduced attractiveness of radioisotopes is due to their purchase and disposal costs and has limited their application, particularly with the advent of alternative detection methods (such as fluorescence).

5.1.2 Fluorescence

The development of a large number of different fluorescence techniques has largely replaced radioisotopes in HTS. Fluorescence is the emission of light by a substance (fluorophore) that has absorbed light at a different wavelength. Fluorescence has the added advantage of being useful in both cell-free and cell-based assays and the availability of a large number of fluorophores with differing absorption and emission wavelengths. Many reviews covering these methods are available in the literature [69]. The use of simple fluorescence in HTS is often not feasible due to interferences such as background signals from light scattering and quenching, but many strategies are available to overcome these limitations. One strategy is the use of red-shifted labeling agents that absorb light at wavelengths greater than 520 nm and allow them to be distinguished from typical background materials that fluoresce at lower wavelengths. Another technique is time-resolved fluorescence (TRF), in which the delay in fluorescence after excitation of the fluorophore is greater than that of the background material [65, 70–73]. FRET, or Förster (fluorescence) resonance energy transfer, methods are widely used not only for assays but also for a variety of cellular studies [74–76]. In this method, both the material absorbing light and that to which the energy is transferred fluoresce. The distance the energy is transferred is generally less than 10 nm. Europium and other rare earth or lanthanide elements are used in chelates in protein-based assays because of their unusually long fluorescence lifetimes (milliseconds instead of nanoseconds), which allow long-range energy transfer between fluorescent europium energy complexes and their corresponding resonance energy acceptors, and this, in turn, removes the resulting background fluorescence [65, 72, 73, 77, 78]. Fluorescence polarization, also known as fluorescence anisotropy, is another useful detection method based on the principle that the rotational speed of molecules is related to their size. Molecules rotating quickly depolarize light to a greater extent than those rotating slowly; thus, when small fluorescent molecules bind to larger molecules, their rotation speed decreases, giving rise to a larger polarized signal [77]. Fluorescence is perhaps the most common method of detection used in HTS [77].

5.1.3 Colorimetry/Luminescence

Other detection methods include colorimetric and luminescence methods. Colorimetric methods are employed in many different assays and are generally very simple to run. These methods measure color intensity as a function of activity. MTT, SRB (sulforhodamine B), Alamar blue, and XTT (2,3-bis-[2-methoxy-4-nitro-5-sulphophenyl]-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) are common reactants used in cell-based colorimetric assays. For example, Alamar blue, XTT, and MTT require metabolically active cells to generate the colored reaction product, while SRB is a protein stain. Color intensity correlates to cell number and is assessed using a spectrophotometer. Finally, ELISA or enzyme-linked immunosorbent assay systems [79] are also very common, where a protein (often an

enzyme) is bound by a specific antibody linked to a second enzyme whose presence (observed through its activity) is measured by the colorimetric or fluorescent endpoint produced. Horseradish peroxidase (HRP), alkaline phosphatase, and *β*-D-galactosidase are common enzymes used in ELISA and all are methods that work by similar mechanisms. For example, *β*-D-galactosidase (ß-gal)-labeled antibodies are quantified based on the concentration-dependent color produced when the substrate ONPG (*o*-nitrophenyl-*β*-D-galactoside) is hydrolyzed producing a yellow *o*-nitrophenol metabolite [80].

Luminescence involves an enzyme-catalyzed chemical reaction that emits light as a product of the reaction. Luciferases are the commonly used detection enzymes and are available from a wide variety of organisms such as fireflies (*Photinus pyralis*) and *Renilla* sp. (sea pansy). These reactions require O_2 and ATP and the luciferin substrate to produce light. Depending on the source of luciferase used, the light produced can range from yellow-green to red. Luciferase is widely used in both cell-free and cell-based assays [81–84]. In multi-well plates, it generally outperforms fluorescence because of its lower background and therefore higher signal to noise ratio and sensitivity [85]. In cell-based screens, cell lines are engineered where luciferase is coupled to a regulatory element of a gene (or receptor) such that stimulation of the regulatory element (or receptor) turns on luciferase production. This is also known as a reporter gene assay [81]. One major advantage with luminescence is that the cell lines are also amenable to in vivo evaluation with whole body imaging [81].

5.1.4 Image-Based Screens (or High-Content Screening)

Advances in cellular engineering have allowed for the creation of cell lines with built-in reporter genes, such as *β*-lactamase, luciferase, or green fluorescent protein, and provide a distinct endpoint linked directly to the target/pathway under analysis. The direct observation of these endpoints looking in detail at changes within individual cells through imaging is called high-content screening. For example, a change in chromosome distribution could be indicative of a compound acting on tubulin formation, while other details such as subcellular distribution of receptors or abnormal nuclear morphology would also be valuable information.

For high-content screens, advances in imaging systems have been of great importance. These screens are still run in microtiter plate format but document changes to or locations of components within individual cells with imaging systems [82]. One can look at an entire well, individual cells (live or fixed) or subcellular components. Data are normalized to whatever scale is being imaged. The requirement for sophisticated and expensive imaging systems and a greater degree of expertise required to develop, implement, and interpret [83] these screens are perhaps the greatest disadvantages. However, it is the detail available from these images and the ability to work in a three-dimensional environment that are also its greatest advantages [82]. In each well in a microtiter plate, multiple fields are sampled. There have also been extraordinary cameras designed, CCD (charge-coupled device) cameras, that are capable of imaging the entire plate using simultaneous illumination of each well with an intense laser beam to maximize signal [73, 82, 84]. Special telecentric lenses are required as the walls of the wells otherwise interfere with focusing [84]. Whether detection is by individual cells, wells, or whole plates, computer software that calibrates and quantifies the readings has also been developed. The large number of images generated in these screens requires concomitant development of computer analysis and storage methods.

5.2 Overview of Screening Design Categories

As noted, there are two basic screening designs: cell-based or biochemical (cell-free). A cell-based assay is defined by the addition of the test components (treatment) to living cells which is then

followed by a measurement of the cells' response. These assays can be a simple homogenous screen where one just "adds and reads." They can also be multiple step assays with the addition of several reagents with or without incubation times and/or wash steps between steps. They can involve cell treatment followed by lysis or fixation of the cells and detection based on the contents of a well, or even treatment, lysis and then transfer to an assay plate for reading. Unless one is looking at a cell surface phenomenon, cell-based assays require the test materials to be able to traverse at least one membrane. In this way, cell-based assays better replicate the in vivo environment since the activity takes place within an intact cellular environment and therefore subject to effects of the microenvironment of the cell. The screens can be used to detect a phenotypic response (proliferation, differentiation, invasion/migration, cytotoxicity, apoptosis, cell cycle) or the effect on a specific target or pathway at the level of transcription or translation (activation or inactivation of intracellular enzymes, gene or protein expression, receptor activation or inactivation, receptor binding, and membrane transport).

Biochemical or cell-free assays on the other hand look at the effect of interactions between a specific target and the detection readout and how the test materials (compounds or extracts) enhance or disrupt these interactions. One can look at enzymatic activity, receptor–ligand interactions, protein–protein, or other macromolecular interactions. The advantage is that the material tested interacts directly with the target, but the disadvantage is that there are frequently nonspecific interactions that have to be distinguished from the specific interactions that are desired.

5.2.1 Phenotypic Screens

Historically, screening was based on the observation of a change in a phenotypic or cellular characteristic, often cell death. For example, the earliest cancer screens focused on cytotoxicity (cell death) as the desired outcome. Ultimately, phenotypic screening proved effective since it led to the discovery of most of the early drugs used in cancer therapy, even though their molecular targets and mechanisms of action were undefined. Unfortunately, it also results in many compounds with side effects because these drugs target the fastest growing cells: cancer cells, hair cells, cells lining the gastrointestinal tract and immune cells leading to hair loss, nausea and vomiting, and neutropenia. As the ability to culture cells derived from solid tumors developed and techniques in molecular biology and genetics advanced, the field began to desire more specificity from screens, that is, a more targeted approach. Early "targeted" screens identified compounds that were active against a single solid tumor cell type or a panel of cell lines derived from a single type of cancer. One of these screens looking at tumor cell type is the NCI-60 screen [86–90].

5.2.2 Example of a Phenotypic Screen: The NCI 60

The NCI 60 is a screen that utilizes 60 different human tumor cell lines to identify compounds with antitumor activity. The screen was designed to exploit what was then a relatively new ability to culture solid tumor cells as a way to identify lead compounds with activity specifically against solid tumors. The 60 cell lines are divided into eight panels representing cancer derived from different cell types: leukemia, melanoma, breast, kidney, prostate, central nervous system, colon, and lung. It is a 2-day screen that detects any activity that reduces or stops cell growth or causes cell death for cell lines in a given panel. A colorimetric endpoint is used to define three endpoints for each cell line: GI_{50} or 50 % growth inhibition indicates the concentration at which the cell line grows at 50 % of the untreated control cell line (this is what many people record as IC_{50}); TGI or total growth inhibition is the concentration at which the cell neither increases nor decreases in number (the same number of cells as when the experiment began); and LC_{50}, the concentration at which the number of cells is 50 % less than at the beginning of the screen (50 % net cell killing over time). Once the three points for each cell

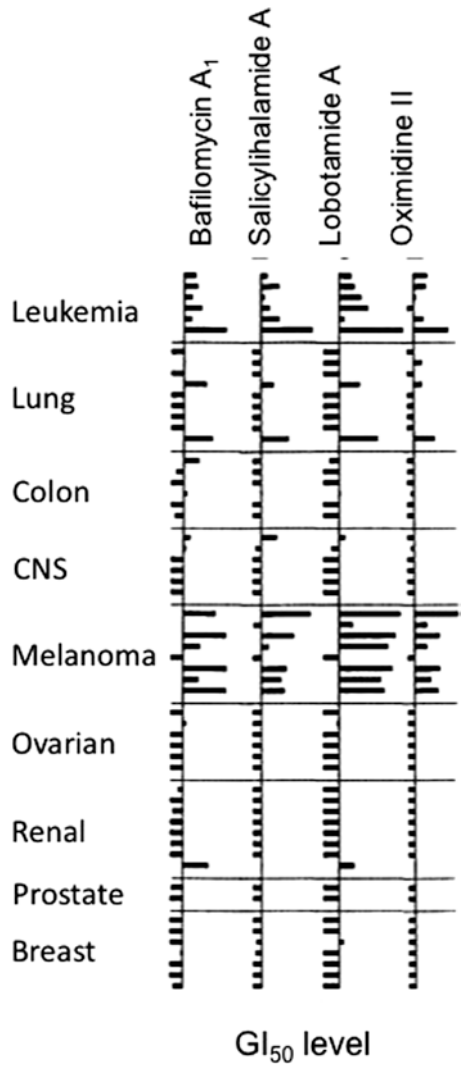
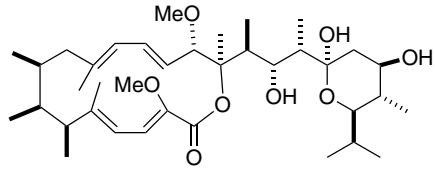
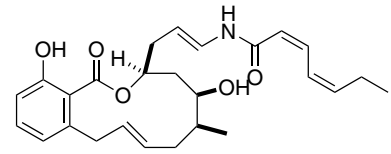


Fig. 8 Mean bar graph at GI_{50} for the V-ATPase inhibitors bafilomycin, salicylihalamide, lobatamide A, and oximidine II

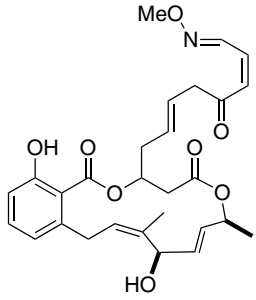
line are obtained, a mean is calculated for each point and the data is presented both as dose–response curves for each cell line and as a bar graph. The calculated mean for each point is the vertical center of the bar graph, and, for each cell line, the difference from the mean is shown by a bar extending from the vertical centerline. By convention, cell lines that are less sensitive (resistant compared to the mean) fall to the left; cell lines that are sensitive fall to the right (see Fig. 8 for examples). Each tested compound gives a fingerprint set of bar graphs that allows a quick visual determination of selectivity and/or panel specificity. Over time, it became clear that compounds that acted through the same mechanism had similar patterns. Kenneth Paull, in the Developmental Therapeutics Program, at the NCI used this information to develop the COMPARE algorithm (http://dtp.cancer.gov/compare) [86–89] that identifies compounds with similar patterns and provides a Pearson correlation coefficient to identify matches (1.00 is an identical match; the number decreases as pattern differences increase; with greater than 0.6 as significant) [87]. An example of this is seen by comparing the mean bar graphs (Fig. 9) of bafilomycin A1 (**26**), salicylihalamide A (**27**), lobatamide A (**28**), and oximidine II (**29**), all of which inhibit V-ATPase (Figs. 8 and 9) [91].



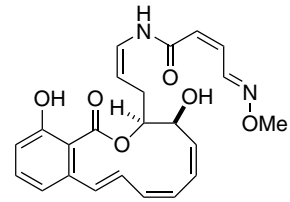
26 bafilomycin



27 salicylihalamide A



28 lobatamide A



29 oximidine II

Fig. 9 Structures for V-ATPase inhibitors

The 60-cell screen was extensively utilized in the authors' laboratory at the NCI for more than 10 years. During that time, several hundred compounds were isolated from natural product extracts. A sample of compounds isolated is shown in Fig. 10. A public database with the complete list of compounds isolated by this lab can be found at https://ccrod.cancer.gov/confluence/display/CCRMTDPBeu/MTL+PUBLIC+COMPOUNDS. The assay continues to be widely used and is a valuable resource for discovery and characterization of anticancer agents.

There are many other phenotypic screens that are widely used. They include assays that focus on cell migration or invasion, neurite outgrowth, angiogenesis, apoptosis, cell cycle alterations, gene expression, and differentiation. What differentiates these screens is that they look for a response that does not depend on knowledge of a specific molecular target or pathway within a cellular environment. It also typically has the advantage of requiring that test compounds pass at least one cellular membrane in order to exert its effect on the intact cell [92].

5.3 Molecularly Targeted Screens

With continued advances in molecular and cellular biology came the ability to develop methods to look at specific intracellular and extracellular targets thought to be important in cancer and other diseases such as HIV. Targeted screening can be done in both cell-free and cell-based environments. There are advantages and disadvantages to each type, and with natural product extracts, there are additional considerations.

5.3.1 Cell-Based Molecularly Targeted Assays

Cell-based assays are advantageous for investigating the effects of compounds on generalized pathways. They may represent a more physiologically relevant representation of the in vivo activity of the

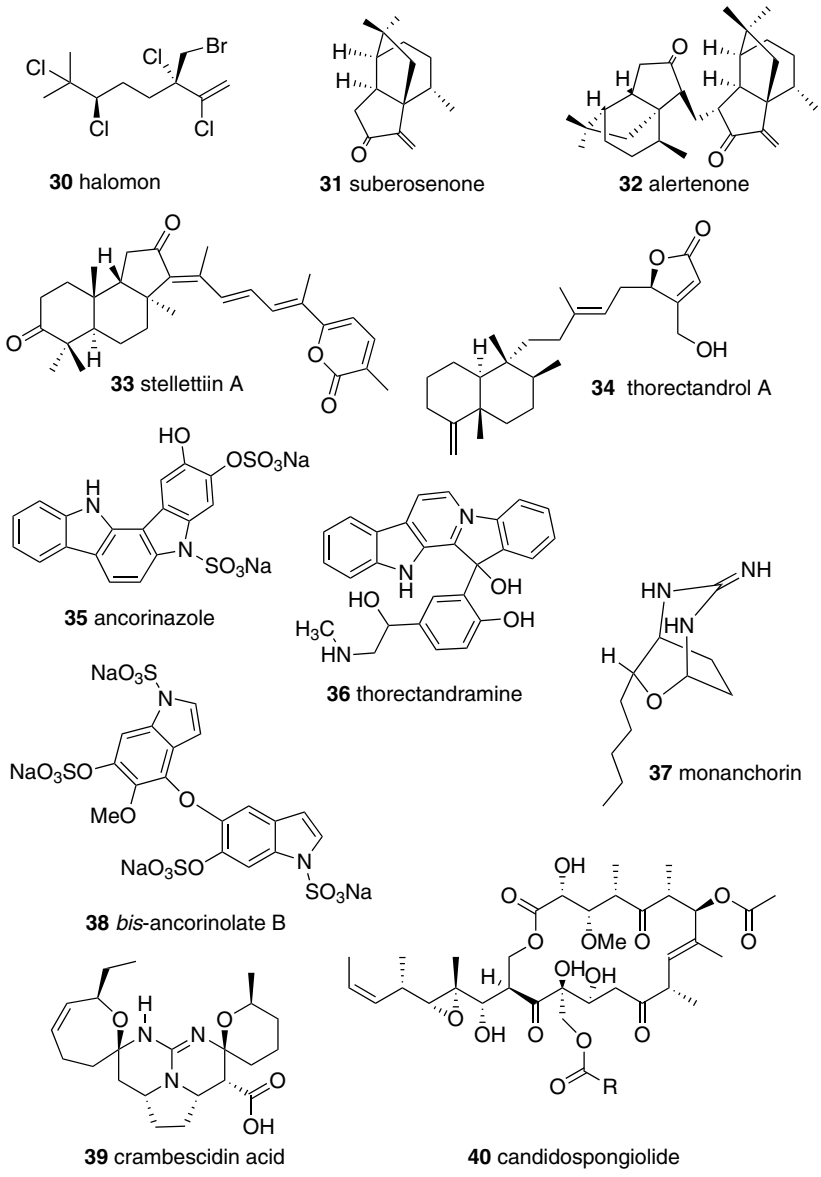


Fig. 10 Examples of natural products isolated using the NCI 60 empirical screen

compounds being evaluated [81]. Since cells can replicate, sufficient testing materials (cells) can be grown in culture, making them more available than proteins, which need to be purified and perhaps chemically modified before being suitable for use. Cell-based screens can be designed to assess secondary messenger systems or transcriptional or translation events with reporter gene engineering or look at phenotypic events like proliferation, migration, or cell killing.

5.3.2 Example of a Molecularly Targeted Cell-Based Screen: ABCG2

The ABCG2 is a member of the adenosine triphosphate (ATP)-binding cassette family of multidrug transporters associated with resistance of tumor cells to many cytotoxic agents. Other members of this

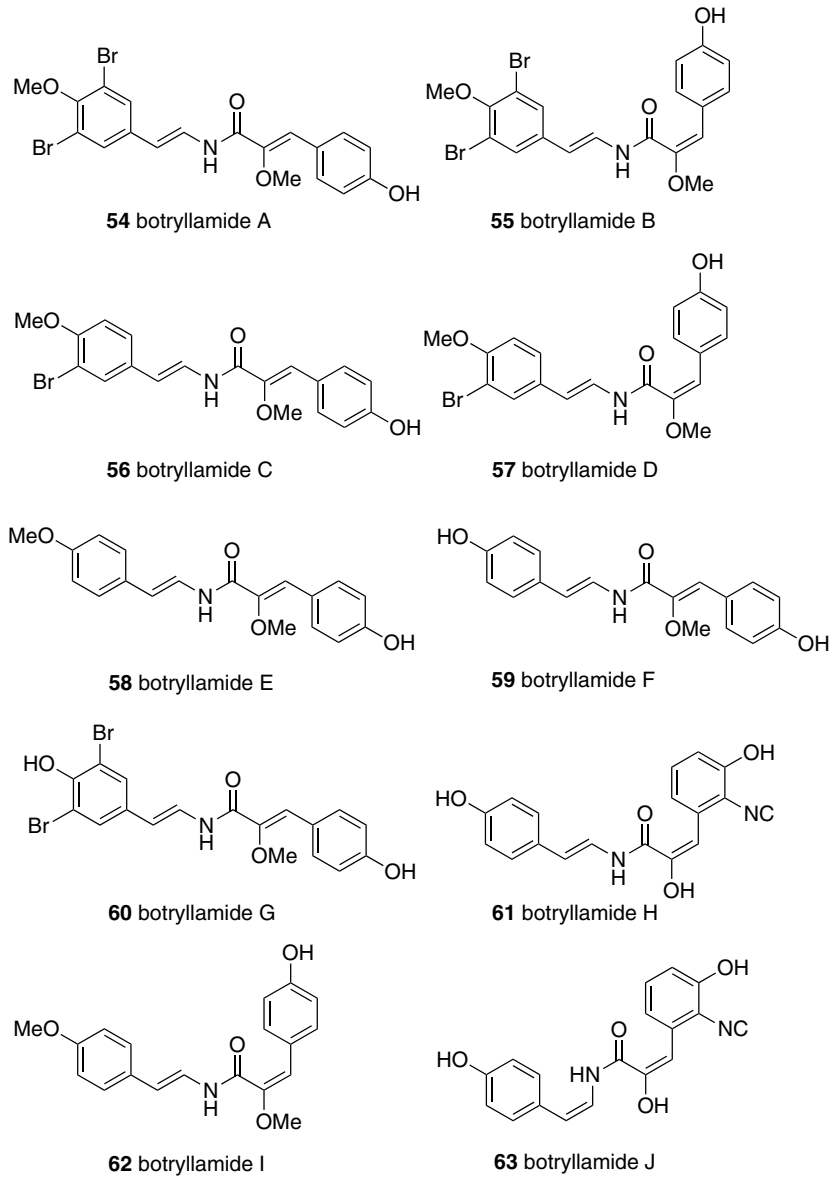


Fig. 11 Examples of natural products active in the RNase H assay

transporter family include P-glycoprotein (Pgp) and multiple drug resistance gene 1 (MDR1). The ABCG2 transporter is thought to be especially important to cancer stem cell resistance to chemotherapy [93]. To search for novel inhibitors of ABCG2, a fluorescent cell-based assay was developed. In the screen, the accumulation of pheophorbide a (PhA) by H460 cells overexpressing the ABCG2 protein occurs over several hours in the presence of an ABCG2 inhibitor. Cell-associated fluorescence is measured after overnight incubation of cells in the presence of PhA and the test compound/extract. The positive control used was fumitremorgin C (FTC, from *Aspergillus fumigatus*), among the first ABCG2 inhibitors identified [94]. A "hit" in the screen was defined as $\geq$ 50 % of FTC activity (after subtracting negative control background). Hits were confirmed by quadruplicate reassay.

From this screen an extract of the ascidian *Botryllus tyreus* was identified as active [93]. Isolation of the active components led to elucidation of a series of botryllamides (**54**–**63**) (Fig. 11). Follow-up

evaluation of these metabolites included the analysis of their inhibition of ABCG2-mediated transportation of BODIPY-prazosin in transfected HEK293 cells, where all but two of the botryllamides inhibited the efflux two- to threefold. Studies were also done to compare the ability of the botryllamides to compete with a radiolabeled prazosin ([^{125}I]-IAAP) labeling of ABCG2. Botryllamides were found to decrease IAAP's ability to interact with ABCG2 17–37 %, indicating that they compete for the same binding site. Finally, the botryllamides were tested to see if they could stimulate ATPase activity associated with ABCG2. All the compounds were able to do so, indicating that they directly interact with the transporter. The compounds were also able to reverse ABCG2-mediated resistance, increasing the clinically used anticancer agent mitroxantrone's ability to kill ABCG2-expressing cells. Of the isolated botryllamides, botryllamide G (**60**) is the most potent, suggesting its structure may be the starting point for the development of additional analogs with increased potency and selectivity [93].

5.4 Molecularly Targeted Biochemical Assays

Cell-free assays directly measure the interaction of compounds with the specific molecular target being investigated, such as a protein outside of its natural (cellular) environment. While this form of assay provides reassurance that a specific target is being affected, it does not provide any information regarding the specificity of the activity to that target within the context of an intact biological system [77, 95, 96]. Cell-free assays can also be prone to a high proportion of "hits" due to nonspecific binding. Thus, it is necessary to determine the specificity of each hit with subsequent testing. Additional confirmation by means of a cell-based assay is used to determine the ability of a given compound to cross membrane barriers. Many of the most robust cell-free screens have been commercialized as kits making them widely available (but costly) to incorporate into any given research program. Additionally, kit-based assays generally do not require sophisticated imaging equipment or a great deal of specialized knowledge to utilize.

5.4.1 Example of a Molecularly Targeted Biochemical Assay: RNase H

The RNase H is a separate RNA cleaving activity found on the HIV-1 enzyme reverse transcriptase (RT) p66/p51 heterodimer. Identification of selective HIV RNase H inhibitors could potentially be an added component of HIV chemotherapy in which two drugs could be included that acted at two distinct epitopes on HIV RT. As designed, this is a cell-free, enzymatic assay for inhibition of the ribonuclease H (RNase H) using FRET [97]. The substrate is an 18 nucleotide RNA/DNA duplex labeled with 6-FAM (fluorescein) on the 3′ end of the RNA and DABCYL on the 5′ end of the DNA. As a duplex, the fluorescein fluorescence is quenched by the close proximity of DABCYL. After reverse transcriptase is added to the reaction, the RNase H activity of reverse transcriptase cleaves the RNA four nucleotides from the 3′ end. This RNA fragment dissociates into solution, and the fluorescence of the label is no longer quenched [97].

The RNase reaction is terminated by the addition of EDTA which removes the required Mg^{2+} cofactor from solution. Based on the assay design, compounds that inherently quench fluorescence or bind to the oligonucleotide substrate could cause false positives. From previous work with HIV-active natural product extracts, it was clear that tannins were strong inhibitors of enzymatic activity and required dereplication. To reduce the hit rate due to tannins and other nonspecific protein binding compounds, 0.5 % bovine serum albumin is added to the buffer when screening natural product extracts. Several concentrations of *N*-(3,4,5-trihydroxybenzoyl)-2-methoxy-1-naphthaldehyde

hydrazone (KMMP), a known inhibitor of RNase H, serve as a positive control. EDTA added to the reaction at time zero is used for a negative control. For pure compounds, >50 % inhibition was required for "hit" selection; for extracts, >80 % inhibition was required for subsequent follow-up [97].

A variety of natural products were identified in this assay either from pure natural product libraries or from isolation work on the active extracts from the natural product extract libraries. The compound classes included tropolones (**41–47**) [98], dimeric lactones from *Ardisia japonica* (**48, 49**) [99], phenolic glycosides from *Eugenia hyemalis* (**50**–**52**) [100], and 1,3,4,5-tetragalloylapiitol from *Hylodendron gabunensis* (**53**) [101]. Examples are shown in Fig. 12. Secondary follow-up assays included RNase H selectivity testing (RNase H from HIV-1, HIV-2, and *Escherichia coli*). Compounds that were selective for HIV-1 or HIV-2 but not human RNase H activity were the most desirable. Compounds selective for HIV RNase H were also tested for their inhibitory effects in a cell-based HIV cytopathicity screen [102].

6 Practical Considerations in Screening

These considerations are summarized in Table 2.

6.1 Throughput

Naturally, one of the first considerations when planning a screen is the scale at which it will be undertaken. If the screen will be run on a limited number of samples (<1,000), one can use a benchtop scale assay. These screens have several advantages due to the low number of samples that will be run at any given time. One advantage is that the assay scheme can be complex with multiple variables including time, dose, temperature, and addition and subtraction of reagents. They can also be done using either 24 or 96 well plates. While these require larger amounts of reagents, they also generally have higher signals simply from the large amount of reagents used. Reagents can be added and removed by hand. Assay format can also accommodate a dose–response analysis with the lower number of samples and an individual can easily analyze all of the data the screen generates. These sorts of assays are typically used to define initial conditions for HTS assays.

As one moves to higher throughput screens, many of these options disappear. Economics begins to play a major role, since the reagents and materials required are expensive and, moreover, sometimes difficult in large amounts to source.

6.2 Miniaturization

Miniaturization is the process by which assays are run using higher density plates (384, 1,536, or even 3,456 wells/plate) allowing smaller volumes to be used, thus decreasing costs per sample. Miniaturization also often requires investment in robotics in order to ensure consistent, reproducible, and accurate dispensing of small volumes. In addition, assay protocols become simpler, often measuring results at a single dose, a single time point, and a single read of each well, and with a protocol that contains a limited number of steps overall. Signal strength also becomes an issue as volumes decrease. Reproducibility and robustness becomes paramount in order to be able to compare data plate to plate as well as day to day.

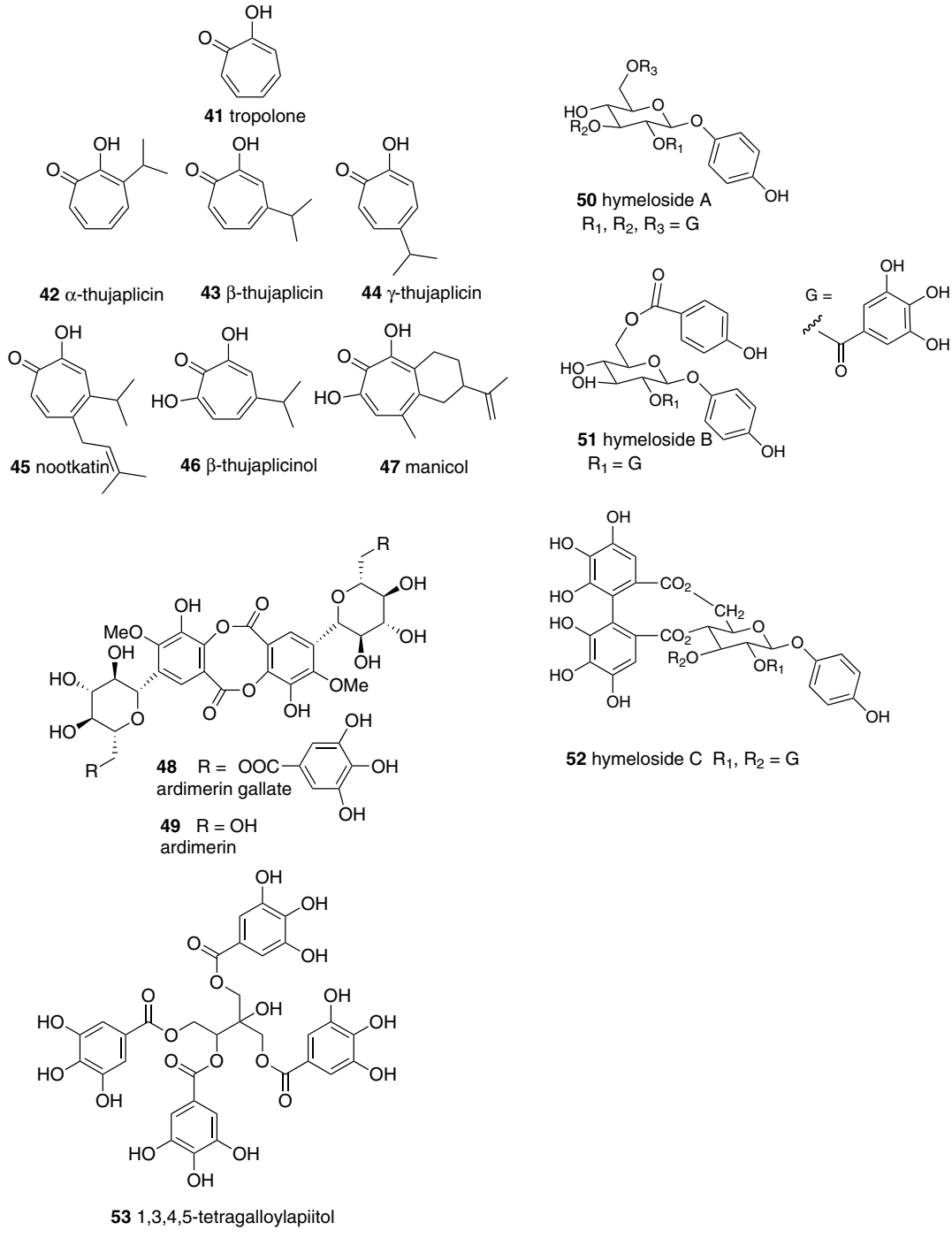


Fig. 12 Structures of the botryllamides, ABCG2 active natural products

Table 2 Comparison of high- and low-throughput screening considerations

Low-throughput screen (benchtop)	High-throughput screen
Generally academic laboratories, small research groups	Pharmaceutical companies, large research groups, academic cores
Fewer than 1,000 samples	Thousands to millions of samples
Low degree of automation required	High degree of automation required
Reagents can be expensive, but relatively small amounts are needed; commercial kits may be available and affordable	Large amounts of reagents required; in-house assay development/optimization the norm
Use 24 or 96 well plates, which require larger volumes of reagents; reagents can be added by hand	Use 384 or 1,536 (or 3,456 rarely) well plates, requires sophisticated readers and micro-dispensers
Complex, multistep assay protocols allowed	Need to simplify assay steps
Multiple variables/conditions can be explored	Single set of conditions for final assay
Dose–response curves normal output	Single read output is norm (dose–response is a follow-up)
Individual attention can be paid to each data point	Massive amount of data generated, software required for analysis. Large databases and facilities for archiving data also required
Secondary screens necessary	Secondary screens necessary

6.3 Selection of Control Compounds

The selection of the positive control for a given assay is a critical step in its development. When possible, it is best to use an inhibitor that specifically inhibits the target of the assay. However, there are many times, such as when investigating a newly described protein, that an inhibitor is not available or even known. In these instances, one by necessity must use a nonspecific inhibitor. For example, for reporter gene screens one can use a nonspecific transcription poison like actinomycin D or a general kinase inhibitor for a kinase assay. It is the positive and negative (background) control wells that are used to define the dynamic range of the assay and to monitor the assay's reproducibility and variability [103]. These are critical requirements that, when met, allow interpretation of the assay data.

6.4 Definition of Hits and the Hit Rate

A "hit" in an assay is a compound or extract that displays the desired activity, at the level that is specified for a given assay. Therefore, definition of what constitutes a "hit" becomes critical. In reality, hit criteria are often quite subjective. A general guideline is that a hit is a minimum of three standard deviations from the negative control as the minimum required for reproducibility. For many assays, this would be an overwhelming number of hits to confirm and work with. The other reality is that at the beginning of a screen, one does not know the proportion of "hits" that will turn out to be due to false positives or due to nuisance compounds. Because of this uncertainty, one often sets an arbitrary but scientifically defensible point (e.g., 50 % inhibition) at the beginning of an assay and then monitors over time the number of "hits" that set point gives you. The hit criteria can then be modified to provide adequate numbers of "hits" for further evaluation. It is important to keep in mind the difference between a "hit" in a screen vs. a "lead" for development. One definition from an article detailing HTS results by Fox et al. [104] nicely describes the difference:

> "A 'lead' is defined as a hit confirmed by more than 1 assay in vitro, and if possible in vivo, in a manner that shows biologically relevant activity that correlates to the target. To be a lead, the compound must show evidence that SAR (structure–activity relationship) can be built around it."

Table 3 Variables optimized during assay development

Category	Item	Values tested	Optimal value
Assay variables	– Cell number – Reagent concentration(s) – Incubation time(s) – Wash steps – Order of reagent addition – Sensitivity to DMSO – Stability of signal over time – Volumes of addition – Microtiter plate – Cofactor concentration – Addition of BSA to buffers	Range selected based on benchtop assay value	Maximizes signal output

One other factor, which is not intuitive, is that the higher the library size of a given screen, the lower the desired observed hit rate is. Ideally, this observed hit rate should be significantly less than 1 %. For example, for a screen utilizing 500,000 samples, a 1 % hit rate is 5,000 compounds. Each of these not only needs to be confirmed as active, and hits must then be further evaluated in order to identify the most promising leads for further development.

6.5 Robustness

For any assay, a traditionally useful tool for assessing the robustness and feasibility is the so-called Z' factor. The robustness of an assay can be defined as the degree to which external factors, such as temperature or length of incubation, affect the assay. The Z' factor is defined by Zhang et al. [105] as "the ratio of the separation band to the signal dynamic range of the assay." In simpler terms, the Z' factor gives an indication of the degree of separation between the positive and negative controls in an assay and consequently how easily a "hit" can be reliably picked out from inactive compounds and is calculated by the following equation:

$$Z' = \frac{1-\left(3\sigma_{C+} + 3\sigma_{C-}\right)}{\left|\mu_{C+} - \mu_{C-}\right|},$$

where σ is the standard deviation, μ is the mean, and $C+$ and $C-$ are the positive and negative controls, respectively. It is generally accepted that a Z' factor between 0.5 and 1 (1 being the maximum value possible) denotes an excellent assay and anything between 0 and 0.5 a marginal assay. Negative values indicate that too much overlap exists between the positive and negative controls for any usable information to be gathered from the assay. The Z' factor is calculated for each plate in each run of the assay. Plates that do not have a high enough value fail and must be repeated. This method provides a continuous snapshot of the assay's performance over time. Z' may also be useful for monitoring quality during a screen.

6.6 Optimization

In addition to calculation of the Z' factor, there are a number of other parameters that are explored during the process of assay optimization prior to the commencement of screening. Some of these are listed in Table 3. The assay is analyzed at each step and each component, and variable is optimized to

Table 4 Final considerations during assay development

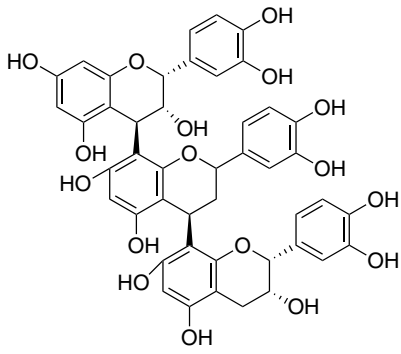
Category	Item	Initial output	Final output
Robustness	Each optimized variable	Vary optimal values ±50 %	Defines range of acceptable values
Repeatability	≥3 repeats/day × 3 days	– Z' – CV w/in plate – Same-day plate to plate – Day to day	Defines normal dynamic range; defines plate quality pass/fail criteria
	Controls	Dose–response curve	IC_{50}, EC_{50} calculations
	Sample types	– Pure compounds – Extracts	Identify potential interfering compound classes
Data analysis	– Calculations performed – Definition of "hit" criteria	– Definition of data output	– Data output format
SOP or protocol	– Identify suppliers – Key variables – Reagent sources – Reagent QC – Assay timeline – Step-by-step procedure – Variable limits – Definition of hit criteria – Explanation of data output	Complete summary of assay and conditions	– Definition of hit criteria – Explanation of data output to individuals

yield the highest and most reproducible output signal. Once each variable is optimized, the general robustness and reproducibility of the assay must also be determined. A general summary is provided in Table 4. It is also during this time that a detailed protocol or standard operating procedure (SOP) is written to document the assay as completely as possible. This document contains a complete step-by-step detail of the assay itself, identifies the suppliers and ordering details for all reagents, identifies the key variables and their limits of reliability, documents reagent quality control, and details the assay timeline. The SOP also defines the criteria used to identify a "hit," and it explains the output and data analysis of the assay for end users. Whenever possible, application of the assay to other known active and inactive compounds provides additional assurance of reliability.

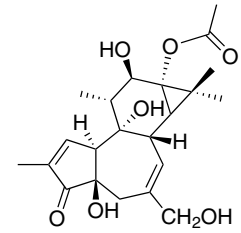
6.7 Nuisance Compounds

Once optimization of the screen has been completed and screening has commenced, the appearance of nuisance compounds is the next factor to consider. Nuisance compounds are compounds or families of compounds that test positive in a screen but that are not suitable for medicinal purposes. Often, the same compound families test positive in multiple assays due to nonspecific protein binding or other nonspecific mechanisms. Nuisance compounds occur in both cell-based and cell-free assay systems. One general method of decreasing the amount of nonspecific binding in an assay is to add a protein like BSA at a relatively high concentration (0.5–3 %). There are other compounds that give false positives or negatives due to a number of reasons, including, but not limited to, precipitation, aggregation, or degradation by the solvents used in the assay, potential to oxidize proteins [106, 107] or being strongly charged [71, 108]. Finally, compounds that strongly absorb light in the range of an assay's endpoint or auto-fluoresce can also be considered nuisance compounds.

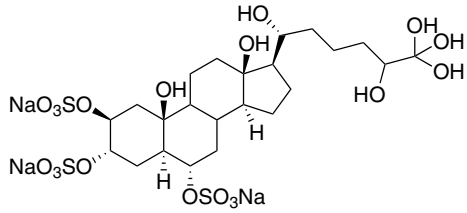
There are potential nuisance compounds for any particular type of assay, the key to efficiency is to identify them quickly so that those compounds, or the natural product extracts that contain them, can be eliminated from further analysis as quickly as possible. Examples of common classes of nuisance compounds are shown in Fig. 13 and include tannins (e.g., **64**) [71], phorbol esters (e.g., **65**) [109],



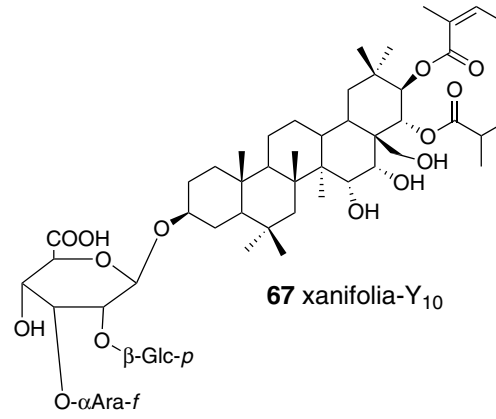
64 proanthocyanic C_1



65 sapintoxin D



66 halistanol sulfate



67 xanifolia-Y_{10}

Fig. 13 Examples of nuisance compounds encountered in natural product screening

sulfated polysaccharides and sulfated sterols (e.g., **66**), and saponins (e.g., **67**) [71]. Tannins [71], for example, are common components in extracts from plants and algae and will bind to many different types of protein. They are of particular annoyance in cell-free assays, employing proteins; but they have also been found to be active in cell-based assays where the assay end product can be inhibited through interactions with proteins at the cell membrane. Should tannins be suspected as the active components of an extract, a simple method for confirming this suspicion is to pass the crude extract through polyamide stationary phase column. Tannins will bind irreversibly to the polyamide, so if the crude extract is active while the polyamide purified fractions are not, the activity can be attributed to tannins without any further testing being required [110].

Phorbol esters are another class of nuisance compound which can easily be identified, as they are produced exclusively by plants of the Euphorbiaceae and Thymelaeaceae families [71] and are known protein kinase C (PKC) activators and downregulators and thus modulate many different cellular pathways. It is thus likely that a phorbol ester is an active component of an extract if the extract from one of the aforementioned families tests positive in a cell-based screen. Most phorbol esters have tumor-promoting properties [111] and are not suitable drug candidates.

Saponins (glycosylated sterols and diterpenes) are another class of nuisance compounds that are produced by plants and marine animals. Saponins cause cell lysis and this may lead to false positives in cell-based assays. Cell lysis due to saponins is much quicker (minutes) than other cell killing mechanisms (hours) and can thus be eliminated as hits if the cells are monitored carefully in the early stages of the assay [71], or if a time course experiment is run on saponin-containing fractions.

Sulfated polysaccharides and sterols are known to be active in PKC and HIV assays [112–115]. They are found in the aqueous extracts of marine invertebrates, and they can easily be removed by

means of an ethanolic precipitation of the crude extract. Sulfated sterols can easily be identified by the characteristic proton shift and appearance in an NMR spectrum.

Finally, many other strongly charged species can also act as nuisance compounds. These compounds do not belong to a select class, but share the property of aggregation in aqueous buffers. These aggregates then sequester and inhibit protein targets [116]. Steep dose–response curves, flat structure–activity relationships, and high sensitivity to assay conditions can point to aggregation. A simple method to confirm that aggregation is taking place is to add a small amount of detergent to the assay plates containing compounds suspected to be aggregating [116]. A change in activity upon the addition of a detergent may be considered diagnostic for the presence of aggregators.

7 Summary

As the practical difficulties of natural product drug discovery are overcome by advances in technologies—particularly in the speed and sensitivity of structure elucidation—natural products are returning to the forefront of medical science as primary suppliers of the unprecedented structures that will further drug discovery efforts once again. Screening, especially with natural product extracts, is by its very nature a high-risk endeavor, but it also has the potential for high rewards, based on both historical and current results. There are significant challenges, but these challenges can and are being addressed with careful selection of assay methods and readouts chosen for a given target. Future prospects for the discovery of new treatments for cancer and other diseases have only increased with the ongoing expansion of "-omics" research. Coupling the "-omics" with the wider chemical space occupied by natural products and our growing ability to access even the non-expressed biosynthetic pathways and the pathways from "unculturable" organisms can only lead to new discoveries. These areas hold great promise for the continued advancement of drug discovery and development.

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Defining the Starting Dose: Should It Be mg/kg, mg/m^2, or Fixed?

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Abstract Background: Traditional cytotoxic drugs are characterized by a narrow therapeutic window and significant interpatient variability in therapeutic and toxic effects. The new targeted therapies have a larger therapeutic window and some have different drug clearance mechanisms. Objective: To provide an insight into history, rationales, and limitations of current dosing methods in traditional cytotoxic drugs and new targeted therapies and to suggest a practical framework for dose calculation and a basis for future research and clinical studies. Methods: Review of relevant literature related to dose calculation of anticancer drugs. Results: Body surface area (BSA) or weight-based dosing and fixed dosing fail to standardize systemic anticancer drug exposure between individuals. Strategies using clinical parameters, genotype and phenotype markers, and therapeutic drug monitoring all have potential and each has a role for specific drugs. However, no one method is a practical dose calculation strategy for many or all drugs. Neither body size nor fixed dosing alone can be used for currently available drugs. Conclusion: Dosing strategies for anticancer drugs should be individualized according to elimination mechanisms and individual patient characteristics. Ways to determine these factors require further investigation and should be a component of early phase studies.

Keywords Body surface area • Interindividual variability • Pharmacokinetics • Drug disposition • Flat dose • Toxicity-adjusted dosing • Therapeutic drug monitoring • Dose individualization

1 Introduction

There are three issues that set the scene for defining the starting dose of novel anticancer drugs:

1. There is a revolution in the understanding and identification of drug elimination mechanisms at the molecular level.
2. Some of the new targeted therapies have a larger therapeutic window than traditional cytotoxic agents.

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3. The traditional monopoly held by body surface area for dose calculation of cytotoxic agents is inaccurate for many drugs.

Cytotoxic drug disposition is minimally affected by body size. At best, body size accounts for less than 30 % of the interindividual variation in drug exposure. Most of the variation is due to genetic and phenotypic differences in elimination and absorption processes. Drug elimination is largely determined by mechanisms that are unrelated to body size and other methods that account for these variations are needed for dose calculation. Even with targeted therapies that may have a larger safety margin, reduction in interpatient variability in drug exposure is critical to minimizing underdosing.

2 History of BSA in Dose Calculation

In 1916, when Delafield and Eugene DuBois developed a formula to approximate body surface area (BSA), they would not have realized the implications that this would later have on the millions of cancer patients treated with cytotoxic chemotherapy [27]. BSA is the two-dimensional surface area of an individual's skin, using height and weight. They developed the nomogram to normalize measurement of basal metabolic rate among individuals, but in the late 1950s, it was suggested after minimal investigation that BSA should be used to normalize cytotoxic drug calculation.

One of the first uses of BSA in drug dose calculation was in 1950, when Crawford et al. [16] showed that plasma drug levels for sulfadiazine (an acetylated and renally excreted antibiotic) and acetylsalicylic acid (a renally excreted analgesic) linearly correlated with administered dosage per unit of BSA in patients varying widely in size. BSA has also been used to extrapolate preclinical animal toxicology data to allow an estimation of a safe starting dose for phase I studies of cytotoxic agents in humans [31, 40]. In 1958, an attempt was made to define a more accurate method of dose calculation for cytotoxic drugs in children [97]. Pinkel examined the literature and found that the "conventional" dose of five cytotoxic drugs (mercaptopurine, methotrexate, mechlorethamine, triethylenethiophosphoramide, and actinomycin) for pediatric and adult humans and for experimental animals was similar if corrected for "representative" BSAs for humans and animals. Pharmacokinetic analyses were not performed and actual patients were not included in the study so comparison of other variables such as antitumor effect or toxicity could not be undertaken. Three of the drugs are renally excreted (mercaptopurine, methotrexate, and actinomycin), and the apparent relationship may have been due to the known correlation of BSA and renal function. Pinkel recommended that the potential use of BSA for dose calculation should be further investigated, but this was not undertaken until the last decade. In the meantime, the use of BSA for dose calculation in oncology became dogma, without further investigation into the relationship between dose and BSA or other parameters of body size.

3 Does Body Size Correlate with Drug Disposition?

3.1 Drug Disposition

Drug disposition or blood concentration is determined by absorption, distribution, and clearance (Fig. 1). Absorption and clearance are largely determined by activity of transmembrane transporters and metabolizing enzymes in the gut, kidney, and liver. For some drugs, hepatic and renal blood flows are also important. Drug distribution is dictated by the degree of plasma protein binding and whether the drug freely distributes into extravascular tissue. For instance, drugs that are highly plasma protein bound such as warfarin, tolbutamide, and ibuprofen have a low volume of distribution (approximately

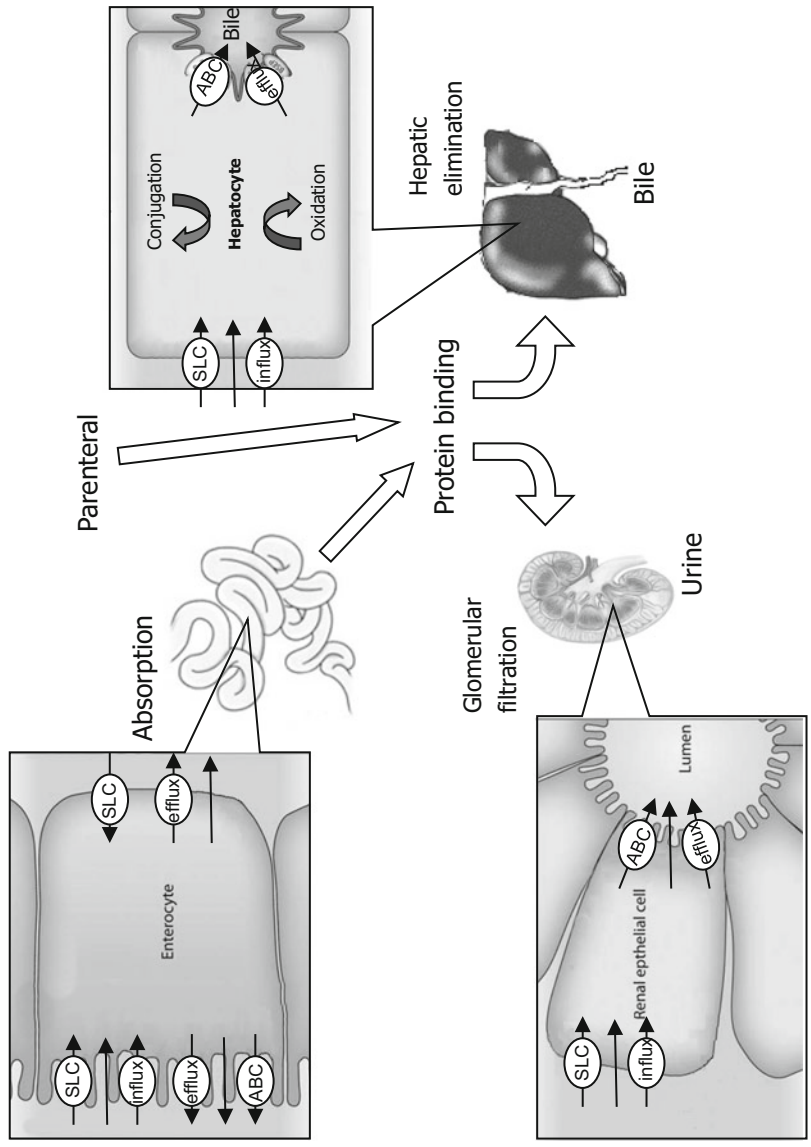

Fig. 1 Schematic representation of drug disposition. ABC: ATP-binding cassette; SLC: Solute carrier. Reproduced with permission from [36]

Table 1 Pharmacokinetic correlation with toxicity and tumor response

Toxicity	Tumor response
AUC, CL, or steady-state concentration	
Etoposide	Teniposide
Carboplatin	Methotrexate
Vincristine	Etoposide
Vinorelbine	5-Fluorouracil
5-Fluorouracil	Docetaxel [7]
Docetaxel [7]	
Doxorubicin	
Irinotecan and SN-38	
Topotecan	
Trimetrexate	
N-Methyl-formamide	
Hexamethylene bisacetamide	
Menogaril	
Plasma concentration	
Cisplatin	Doxorubicin
6-Mercaptopurine	
Paclitaxel [56]	
Methotrexate	

[a]See reference Gurney [44]

0.1 L/kg) which roughly equates to blood volume [105]. Since blood volume and the amount of total body water is related to body size, volume of distribution may relate to body size in some circumstances [1]. Aminoglycosides, phenobarbitone, ibuprofen, carboplatin, vinorelbine, irinotecan, and tacrolimus are some drugs where measures of body size have correlated with the apparent volume of distribution [74, 80, 86, 91, 109, 127, 138].

However, it must be remembered that volume of distribution (V_d) is not a physiological measurement but a pharmacokinetic ratio. It is the theoretical volume into which a drug is distributed and is described by the formula; V_d = Dose/Concentration. Therefore, the volume of distribution for an intravenous dose is determined by peak blood concentration. The possible relationship between body size and volume of distribution may be important in circumstances where peak plasma concentration determines toxicity or drug efficacy. Intuitively, one would expect a relationship between peak plasma concentration and toxicity for cytotoxic agents. However, limited information is available. No correlation was found between toxicity and peak concentration of irinotecan SN-38 or epirubicin [107, 124]. A correlation has been shown for oral etoposide [129]. However, for this drug and also for paclitaxel, the time above critical plasma concentration, rather than peak concentration, appears to be more important [37, 56, 79]. Where a relationship has been shown between a pharmacokinetic parameter and drug efficacy or toxicity for anticancer treatment, it is usually the area under the time–concentration curve (AUC) or steady-state plasma concentration rather than V_d or peak plasma concentration that correlates (Table 1).

The AUC is determined by dose and clearance and defined by the formula, AUC = Clearance/Dose. As mentioned before, metabolism and elimination by the kidneys and liver determines the drug clearance of most drugs. Very few of these processes would be expected to be determined by body size. A few drugs such as aminoglycosides are almost solely eliminated by glomerular filtration. It has been suggested that GFR correlates with body size [113], and dose of gentamicin and tobramycin is now determined by adjusting for body weight. However, even for carboplatin, a cytotoxic drug that is mostly eliminated by glomerular filtration, dose calculated using GFR is more accurate than using BSA [57].

Table 2 Correlation of body size with drug clearance

Drug	Correlation/comments	References
Docetaxel	Interpatient variability of CL correlates with BSA	[8]
Paclitaxel	BSA explains 53 % of interpatient variability in CL	[114]
Temozolomide	BSA reduced interpatient variability of CL from 20 to 13 % on day 1 and 16 to 10 % on day 5	[50, 60]
Oral busulfan	CL correlates with BSA (r^2=0.28) and weight (r^2=0.3)	[39]
Vinorelbine	CL correlates with BSA (r^2=0.27)	[91]

Table 3 No correlation for body size with clearance or AUC

Class	Drug	References
Topoisomerase inhibitors	Etoposide	[90]
	Irinotecan	[76, 80]
	Topotecan	[71]
Antibiotics	Epirubicin	[23, 45]
	Pixantrone	[44]
Spindle poisons	Vinorelbine	[46]
	Paclitaxel	[43]
Antimetabolites	5-Fluorouracil	[44]
	Methotrexate	[44]
	Trimetrexate	[44]
	Dichloromethotrexate	[44]
Alkylating agents	Ifosfamide	[44]
	Busulfan	[44]
	Cisplatin	[18, 81]
	Carboplatin	[12]
Miscellaneous	*N*-Methyl-formamide	[44]
	Hexamethylene Bisacetamide	[44]
	Menogaril	[44]
	Brequinar	[44]

3.2 Body Size and Cytotoxic Drug Clearance

Giving a larger dose to a larger person makes intuitive sense, and to some extent this is true, but overall body size is a minor determinant of drug exposure. BSA is known to be proportional to blood volume [1]. It has been claimed that BSA is also proportional to glomerular filtration rate (GFR) [113], but a more recent assessment has questioned this relationship [24]. Liver function decreases with advancing age in parallel to the loss of liver volume [110]. Liver volume as determined by helical CT scanning has been shown to correlate with BSA (r^2=0.54) and total body weight (r^2=0.61) in 21 patients with a history of cancer but without liver metastases [87].

Over the last decade, the relationship between BSA and drug disposition of cytotoxic drugs has been revisited [43, 44, 99, 101]. Table 2 is a list of drugs that have been reported to show a correlation between drug clearance and BSA. Even for some of these drugs the correlation coefficients are low, indicating that BSA accounts for less than 30 % of the variability in clearance between individuals. For most cytotoxic drugs, no correlation can be seen with BSA and drug clearance (Table 3). The most compelling evidence against the use of BSA alone for dose calculation is the fact that a large interpatient variability in drug exposure remains despite "normalization" of dose by BSA.

In adult populations the extremes of BSA vary from approximately 1.4 to 2.3 m^2—a little over a 1.5-fold range, but the majority of individuals fall into a range much less than this. Even for drugs

where the use of BSA may reduce variability, there is minimal contribution in reduction in variability from the use of body size for a person 1.7 m^2 compared to 1.8 m^2. Assuming a maximum contribution of BSA to drug disposition of 30 %, it is only in the situations of extreme BSA (e.g., an individual of 150 cm and 40 kg compared to one of 185 cm and 120 kg) where this parameter may become a significant factor in dose calculation.

The issue of substantial variation in body size is amplified in pediatric oncology where body weight ranges from a few kilograms to adult size. In these situations of extreme difference, body size must come into play. This is more akin to the interspecies scaling of chemotherapy dose, such as in estimating the dose for humans based on toxicology studies in rodents. BSA has proved useful in this situation of interspecies scaling of dose [31, 40]. It would therefore be reasonable to use BSA to scale an approximate starting dose of a drug for clinical trials in children based on adult data. However, even here it is unreasonable to use BSA as the sole determinant of dose for individual infants. The same inaccuracies of using BSA alone would hold when differentiating dose between children within a small range of body size.

3.3 Body Size and Targeted Therapies

3.3.1 Small Molecules

A few studies have looked at the relationship between body size and dose or drug exposure for small molecules. The pharmacokinetics of tyrosine kinase inhibitors (TKI) has recently been reviewed by van Erp et al. [130]. The effect of body size on small molecule inhibitors is summarized below.

Sunitinib

In a predominantly pharmacogenetic study, van Erp et al. found no correlation between BSA and toxicity in 183 patients who received at least one cycle of 50 mg single-agent sunitinib [131]. In a population pharmacokinetic meta-analysis of sunitinib, body size was found to affect the volume of distribution (V_d/F) but not clearance of sunitinib [54]. However, simulated exposure of sunitinib varied considerably and it was predicted that body size effect on AUC was minimal. Clearance and AUC rather than V_d/F is more likely to affect the steady-state level of sunitinib.

Imatinib

Two studies in Japanese populations have suggested an effect of body size and dose of imatinib [63, 108]. Sakai et al. found that trough imatinib concentration did not correlate with body weight or BSA but did correlate with imatinib dose and dose adjusted for BSA or weight. Kawaguchi et al., in 31 patients in complete cytogenetic response (CCR), found that BSA was significantly smaller in patients receiving a reduced dose due to toxicity compared with those receiving a standard dose, pointing to a weak effect of body size on "optimal dose" (defined as the dose of imatinib that could achieve and maintain a CCR with acceptable adverse effects). Again there was no relationship between BSA and imatinib trough level. Both these studies indicated that reduced dose of imatinib may be sufficient in smaller patients to achieve adequate drug exposure and clinical benefit. However, both studies showed a wide interpatient variation in imatinib exposure regardless of the dose taken. In the Sakai et al. study, even among those taking the same dose of 400 mg/day, the imatinib concentration was widely distributed (582–2,420 ng/mL) regardless of body size. In other words, an individual was just as likely to get an

effective (or ineffective) drug concentration regardless of whether they ingested 300, 400, or 500 mg of imatinib daily. Just like cytotoxic chemotherapy, factors other than dose of imatinib are more important in determining drug exposure and body size has a weak effect.

Erlotinib

Lu et al., in 1,047 patients with non-small cell lung cancer, showed that erlotinib clearance did not correlate with body weight but was affected by total bilirubin, α1-acid glycoprotein, and smoking status [73]. Interestingly, occurrence of skin rash has been associated with survival in erlotinib-treated patients [133] and erlotinib AUC correlated with occurrence of skin rash in the study of Lu et al., indicating a correlation between drug exposure and efficacy. More recently, Thomas et al., in a study of 42 patients with head and neck cancer, showed that erlotinib clearance was partly explained by patients' age, hepatic function, ABCG2 genetic polymorphism, and smoking status but not by body weight [123].

3.3.2 Monoclonal Antibodies

Ten mAbs are currently approved by FDA for the treatment of cancer and all of them are of the IgG class. It is important to understand different clearance pathway between mAbs and traditional cytotoxic drugs. mAbs are given intravenously and once in the systemic circulation, entry to the extravascular compartment (intestinal fluid and tissue) is primarily driven by hydrostatic pressure, osmotic pressure, endothelia pore size, and vessel tortuosities [88]. The distribution is limited to 1–2 times plasma volume, indicating a poor penetration into tissue spaces, including tumors [139].

Unlike small molecules, IgG antibodies are large (150 kDa) and are therefore not filtered by the kidney or excreted in urine [3]. The dominant route for elimination of antibodies is via uptake and catabolism by the reticuloendothelial system (RES). The neonate receptor FcRn, expressed on macrophages and natural killer cells, binds to the Fc portion of IgG antibody and plays a major role in antibody clearance. Since blood volume correlates with body size, there is some logic to using weight or BSA to estimate dose for these agents.

Bevacizumab

For antibodies like bevacizumab, which targets soluble antigens, the pharmacokinetic profile is characterized by a linear two-compartment model with a rapid elimination phase from a short distribution and more prolonged elimination half-life, as a result of the nonspecific clearance by the RES and interaction with FcRn. In a population pharmacokinetic study of bevacizumab, Lu et al. demonstrated that body weight and gender were the covariates with the greatest influence on bevacizumab central compartment volume of distribution (V_c) and clearance (CL), which support the body weight-based dosing [72]. Despite that, covariate effects of all factors only explained about 40 % of interpatient variance for V_c and 60 % of interpatient variance for CL.

Trastuzumab, Rituximab, and Cetuximab

For antibodies targeting membrane-associated internalizing antigen, the total clearance is a combination of two different clearance pathway: (1) the nonspecific, linear pathway attributed to the RES and (2) the specific, nonlinear, and saturable antigen-mediated clearance pathway, which is mediated by

the binding of the antibody to the antigen and subsequent internalization of the antibody–antigen complex, followed by degradation of the internalized antibody and antigen complex [121]. The contribution of antigen to mAb clearance depends on various antigen-related factors, such as antigen concentration, distribution, and turnover rate. These effects were demonstrated in a pharmacokinetic modeling of 476 patient with metastatic breast cancer treated with trastuzumab, where Bruno et al. found that body weight as well as burden of disease and serum level of extracellular domain of the Her 2 receptor affected trastuzumab clearance. However, these covariate effects on trastuzumab exposure were only modest in comparison with the large interpatient variability of CL which was 43 % [9]. Similarly Ng et al. showed that BSA accounted for about 19.7 % of interindividual CL variability of rituximab and that adjusting the dose as a function of body surface area does not seem to improve the predictability of rituximab exposure [89].

In a review of data from two studies of 143 patients with head and neck cancer treated with cetuximab, Dirks et al. found a fourfold variation in trough cetuximab level while receiving BSA-based dosing [22]. Together ideal body weight (not actual weight or BSA) and WBC accounted for almost 35 % of the total variability in maximum elimination rate, a parameter that determines trough level. A comparison of the trough concentrations of underweight patients (dosed according to body size) showed that these were lower than other patients (median 48.2 vs. 62.4 μg/mL, $P=0.014$) and the authors questioned the use of the current practice of use of BSA for cetuximab dose.

4 Alternative Body Size Measures and Obesity

Lean body mass (LBM) consists of body cell mass, extracellular fluid, and nonfat connective tissue and is essentially fat-free mass [87]. LBM is commonly measured by dual-energy X-ray absorptiometry which distinguishes fat, fat-free mass, and bone.

It has been suggested that LBM correlates with systemic drug clearance, but so far this has not yet been substantiated [83]. Nawaratne et al. showed that LBM correlates with liver volume and antipyrine clearance, a nonspecific quantitative test of hepatic drug oxidation [87]. However, in this study there was no correlation between liver volume and antipyrine clearance indicating that other unknown factors account for the relationship. Further studies are required to determine the importance of LBM in dose calculation of hepatically eliminated drugs.

Ideal body weight (IBW) is the weight that insurance companies consider appropriate for height and is determined by a formula. The use of IBW for dose calculation (sometimes as a function for BSA) attempts to account for excess adipose tissue. Body constitution in the obese is characterized by a higher percentage of fat and a lower percentage of lean tissue and water. The effect of obesity on some cytotoxic drugs has been examined. In obese patients methotrexate clearance is increased, busulfan and ifosfamide clearances are unchanged, and doxorubicin and cyclophosphamide clearances are reduced [29, 39, 69, 98, 104]. Hepatic oxidative metabolism is unaffected by obesity as measured by antipyrine clearance or erythromycin breath test [13, 57]. It would be expected that the volume of distribution be affected by obesity especially for drugs that are lipid soluble. However, this expected relationship is variable with some lipid-soluble drugs increasing the volume of distribution (e.g., benzodiazepines, verapamil), while others have no effect (e.g., cyclosporine, propranolol) [14].

Obesity is an extreme of body size and in this special situation size may become an important determinant of drug disposition and so higher doses are required. Curiously, it is often in the obese patient where the strict practice of BSA dose calculation is abandoned and other arbitrary rules are applied such as capping of BSA or dose. However, retrospective studies of breast cancer patients have shown that obese patients seem to be less likely to develop neutropenic sepsis even if actual body weight (ABW) was used and that obese patients who receive arbitrary dose reductions had a worse outcome [15, 42, 61, 106].

Sparreboom et al. assessed actual plasma pharmacokinetics of eight anticancer agents in 1,206 lean and obese adult patients and found the disposition of some, but not all, drugs was significantly altered by obesity [118]. For example, absolute clearance of cisplatin, palitaxel and troxacabine ($P<0.023$) were increased but decreased for doxorubicin ($P=0.013$) and unchanged for carboplatin, docetaxel, doxorubicin, irinotecan, or topotecan. The selection of a better prediction of pharmacokinetics among alternate weight descriptors for dose calculation in obese, including actual body weight, predicted normal weight, lean body mass, (adjusted) ideal body weight, and the mean of ideal and actual body weight, is drug specific and sex dependent and seemed unrelated to the intrinsic physicochemical properties or route of elimination.

In conclusion, obesity may affect drug clearance and treatment outcome in a drug-specific manner, and empiric decrease in drug dose in obese patients (e.g., dose capping or by using IBW) should be discouraged because they may compromise efficacy in this group of patients. However, it is important to remember that variation in drug exposure still occurs even in the obese patient [48]. Obesity may not be the dominating factor in dose calculation for a patient who has reduced drug elimination for other reasons such as intrinsic variations in metabolism and/or drug transporter function. In other words, it is important to realize that drug elimination for all drugs varies widely between individuals, obese or otherwise, and often this variation eclipses any contribution body size has to drug disposition. In many individuals, accounting for obesity may be of minor importance compared to the normal overriding factors of interpatient variation in drug effect. Obesity is probably of most significance in the situation where the patient has "average" drug metabolism and elimination.

5 Significance of Getting the Wrong Dose

A common argument in support of the continued use of BSA for dose calculation of chemotherapy is that the degree of inaccuracy is not clinically significant. The obvious consequence of incorrect dose calculation is overdose and excessive toxicity, a situation most oncologists have learned to accept. But perhaps a more common but less appreciated consequence of using BSA alone is underdosing and reduced drug effect.

Individuals vary in their capability to eliminate xenobiotics by four- to tenfolds [44]. For drugs with a wide therapeutic window such as some antibiotics, this problem is not crucial since the recommended dose can be pitched towards the high end of the dose range without fear of significant dose-related toxicity. On the other hand, most cytotoxic drugs have a narrow therapeutic window. The dose that causes unacceptable or even fatal toxicity is not much higher than the optimal dose needed for anticancer effect for many drugs. For this reason, the main endpoint of dose-finding studies has traditionally been prevention of unacceptable toxicity. Coupling this with the wide interpatient variability in drug disposition, conservatism becomes intrinsic to the dose recommendation process for anticancer drugs. Mean dose is pitched towards the low range to minimize the number of patients with severe toxicity, and consequently a substantial proportion of cancer patients may be inadvertently underdosed (Fig. 2) [48].

A number of studies have shown a significantly worse antitumor effect for those patients who failed to develop myelosuppression after treatment compared to those who did in patients receiving chemotherapy for breast cancer, ovarian cancer, advanced testicular cancer, and lung cancer [21, 47]. Similarly, lack of skin rash has been associated with worse outcome for lung cancer patients treated with erlotinib and for lack of hypertension with sunitinib therapy for renal cell cancer [103, 128].

Pharmacokinetic evidence for inadvertent underdosing and its consequence in cytotoxic drugs have been demonstrated clearly in a series of studies by Gamelin et al. [33]. This group first established an $AUC_{0\text{-}8}$ of 20–25 mg h/L as the optimal level with a regimen using 5 FU in a dose of

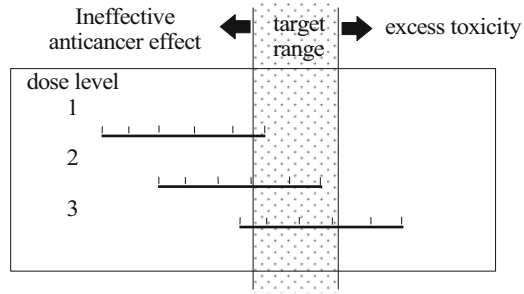

Fig. 2 Scheme of a phase I study for a drug with linear pharmacokinetics. The *horizontal lines* represent the variation in systemic exposure at various dose levels. At dose level 3, those patients with lower drug elimination capability develop dose-limiting toxicity and subsequently that dose level is defined as the maximum tolerated dose. Dose level 2 is recommended for phase II studies since it causes tolerable toxicity in all patients. However, due to the variation in drug handling, a proportion of patients will be relatively underdosed since they are more capable of eliminating the drug. This means the wide distribution of systemic exposure is skewed towards the ineffective range when dose is calculated using BSA

Table 4 Correlation of pharmacokinetic parameters and clinical outcomes in selected kinase inhibitors and monoclonal antibodies

Drug	PK parameters	Outcomes	References
Imatinib	Trough level	Response	[96]
	Dose	PFS, response	[132]
	AUC	Response	[135]
	AUC	Neutropenia	[19]
Gefitinib	AUC	Diarrhea not rash	
Erlotinib	AUC, C_{max}	Rash	[73]
	C_{ss}, C_{min}	Survival	[116, 117]
Lapatinib	C_{min}	Response	[11]
Sunitinib	AUC	OS, TTP, Response	[55]
Sorafenib	Trough level	PFS	Sorafenib IB
Rituximab	Trough level, AUC	Response	[125]
Trastuzumab	C_{min}	Progression	[2]
Cetuximab	Trough level	Response	[30]

1,300 mg/m^2 infused over 8 h every week [32, 35]. In a group of 81 patients treated with dose calculated using BSA, 80 % of patients were found to have an ineffective 5 FU plasma concentration after the first dose [33]. In a subsequent study in 2008, they showed that pharmacokinetically guided 5 FU dose adjustment (targeted AUC 20–25 mg h/L) led to significantly improved objective response rate, a trend to higher survival rate, and fewer grade 3/4 toxicities, comparing to fixed BSA-based dosing [34].

Small molecule inhibitors and monoclonal antibodies (mAbs) that target key components of the signal transduction pathways that are commonly activated in cancer are transforming the care of patients with cancer. Some of these therapies, particularly the antibodies, have a larger therapeutic window than conventional chemotherapy and therefore have minimal acute toxicity at levels of drug exposure that causes an anticancer effect. Examples include the CD20 antibody rituximab, the EGFR antibody cetuximab, and the Her2 antibody trastuzumab. On the other hand, some of these new agents have significant toxicity at doses not far above those required for an effect on the tumor. Examples in this category include the kinase inhibitors sunitinib, sorafenib, and imatinib. But clear exposure–effects relationships have been reported consistently both in animal model and clinical studies (Table 4). During the early pharmacokinetic studies of imatinib, La Coutre et al. treated Bcr–Abl tumor-bearing nude mice with a regimen that assured a continuous block of Bcr–Abl

kinase activity [66]. Administration of imatinib three times per day, over an 11-day period, cured 87–100 % of treated mice, whereas administration once or twice a day did not. This suggested that continuous adequate exposure is critical to the success of this inhibitor as a therapeutic agent [25]. Several studies showed that the mean plasma imatinib concentrations in nonresponder were significantly lower than those in responders for CML patients receiving a fixed standard dose [96, 108, 112]. In mouse xenograft models, sunitinib inhibited target receptors when plasma concentrations reached between 50 and 100 ng/mL, and similar results were obtained in a functional assay of VEGF-induced vascular permeability in vivo [77]. Subsequently, this concentration was selected to be the target for clinical applications. In a phase 1 study, sunitinib trough levels above 50 ng/mL were associated with tumor response [28]. In a study of sunitinib in patients with gastrointestinal stromal tumors (GIST), at the currently recommended dose of 50 mg/day, 21 % of patients (10 of 48) achieved trough concentrations of sunitinib and its major active metabolites SU12662 below 50 ng/mL [20].

6 Can Fixed Dose Be Used?

6.1 Cytotoxic Drugs

Since body size is not useful for the majority of anticancer drugs, is it reasonable to use a fixed dose for all patients [18, 76]? The advantages for using fixed doses of cytotoxic drugs are many including financial and safety issues. For example, what is the additional cost of prescribing 305 mg of paclitaxel instead of 300 mg? Can 215 mg of DTIC (instead of 200 or 220 mg), 85 mg of docetaxel (instead of 80 or 90 mg), or 63 mg of methotrexate be accurately compounded? The decimal point can easily be missed by an inexperienced technician when 2.2 mg of vincristine is prescribed.

Flat-fixed dosing has been studied for several cytotoxic drugs, including irinotecan [17], capecitabine [111], cisplatin [18], and paclitaxel [78, 85, 114] with or without comparison with BSA-based dosing. As predicted, fixed doses may result in comparable pharmacokinetic variability in some drugs, but it is no more accurate than BSA-derived dose. A large interpatient variation in drug exposure will remain with all the implications of overdosing and underdosing as discussed above. Our group has examined the use of epirubicin (150 mg continuous infusion) and vinorelbine (60 mg every 21 days) in separate studies and found that this approach was safe for both drugs [45, 137]. However, interpatient variability in clearance was still eightfold and fourfold, respectively, for each drug which is similar to the variability if BSA was used for dose calculation [45].

Loos et al. compared flat-fixed dosing of cisplatin with BSA-based dosing in 25 Dutch patients with extreme BSA values (exceeding the average ± 1 standard deviation). The results suggested that a fixed dose calculated on the average BSA of all patients might lead to exacerbated toxicities in small patients and underdosing in large patients. This resulted in the recommendation of fixed dose per BSA cluster (e.g., 100 mg for patients with BSA $\leq$ 1.65 m^2; 130 mg for those with BSA between 1.66 and 2.04 m^2; 150 mg for those with BSA $\geq$ 2.05 m^2) [70].

6.2 Targeted Agents

6.2.1 Flat Dose in Targeted Agents

Small molecular inhibitors are mainly metabolized by the phase I liver enzyme CYP3A and are substrates for the ABC transporters [130]. Given the potential substantial interindividual variation in the activity of these mechanisms [52], it is not surprising to see the wide interindividual variation in

Table 5 Examples of pharmacokinetic variability expressed in coefficient of variation or folds at current recommended dosage in oral tyrosine kinase inhibitors

Drug	Dosage per day	C_{max}	AUC	$T_{1/2}$	Reference
Imatinib	400 mg daily	30–80 %	25–55 %	18–39 %	[65, 92, 95, 134]
	400 mg BD	27–39 %	19–39 %	13–22 %	[65, 95]
Gefitinib	250 mg	9.6-fold	15-fold		[68]
Erlotinib	150 mg		64 %		[51]
Sunitinib	50 mg	46 %	41 %		[6]
Sorafenib	400 mg BD	41–107 %	24–91 %	22–24 %	[120]
Lapatinib	250 mg	2.7-fold	5.2-fold	1.8-fold	[5]
Cetuximab	400 mg/m^2	29 %	39 %	21 %	[30]
Trastuzumab	6 mg/kg q3w	7–12 %	10–35 %	92–183 %	[67]
Rituximab	375 mg/m^2	63 %			[75]

systemic exposure of most small molecular inhibitors (Table 5). For example, one study showed a fivefold variation in estimated imatinib clearance (CL/F) on day 1 with fixed dose (600 mg/day) in patients with CML and GIST [49]. This implies that a fixed dose should not be used for these agents unless they have a very wide therapeutic window. In general terms, the small molecules are less specific in their action compared to antibody therapies and are associated with more off-target toxicity. Strangely, the more toxic small molecules are given as a fixed-flat dose, while the antibody therapies, which have less acute toxicity, are dosed according to body weight or BSA. For example, in phase III studies of 4 weeks on 2 weeks off schedule of sunitinib, 38 % of patients with metastatic renal cell carcinoma and 28 % of patients with GIST required dose interruption, whereas a dose reduction was necessary in 32 % and 11 %, respectively [20, 84]. However, some small molecules are less toxic. A maximum tolerated dose for imatinib was never reached in the phase 1 trials of imatinib [26] and some studies have used a high fixed dose (800 mg/day) so that almost all patients reach a pharmacokinetic threshold where the drug might be active if the target is willing.

For some of these targeted agents, the toxicity is so low that a "lack of toxicity trigger" for dose increase for these drugs cannot be depended upon. However, these same drugs may be suitable for a high fixed dose, ensuring an active drug concentration is achieved provided that drug exposure-dependent cumulative toxicity (such as cardiotoxicity) is not present.

Small molecule inhibitors are largely given orally and continuously which introduces additional factors that can increase variation in drug exposure apart from body size. Oral bioavailability of some small molecular inhibitors is highly dependent on gastrointestinal absorption and first-pass drug metabolism by the liver, two processes that both vary considerably among individuals. For example, high-fat meals can lead to more than threefold increase of AUC of lapatinib [100] and 82 % increase in bioavailability of nilotinib [122]. On the other hand, no obvious influence of food is found with sorafenib [120], imatinib [38], or sunitinib [4]. Additionally, patient adherence to oral antineoplastic agents is quite variable with reported adherence rates ranging from 20 to 100 % [93].

Chronic administration may cause induction of drug elimination pathways [10]. For example, imatinib clearance increased by 33 % after chronic exposure over 12 months in one study [62]. This may be a contributing factor in the amelioration of imatinib toxicity that occurs with time or partial overcome of tumor resistance by imatinib dose escalation in CML patients [115].

6.2.2 Therapeutic Drug Monitoring in Small Molecular Targeted Therapy

Therapeutic drug monitoring (TDM) entails the measurement and interpretation of drug concentration in biological fluids and the individualization of drug dosages or schedules to maximize the therapeutic effect and to minimize toxicities [82]. Trough drug level, an indicator for drug level at steady state,

has been used as a useful and practical TDM method and provided valuable guidance for dose adjustment in several selected drugs, including antibiotics, immunosuppressives, antiepileptic, and anti-HIV treatment. The use of TDM in traditional cytotoxic drugs, however, has been limited to few drugs only [41, 94, 136], due to several factors including lack of established "therapeutic ranges" and concentration/effect relationship, frequent use of combined drugs with overlapping therapeutic and toxic effects, and intermittent drug schedules [36].

Clear concentration and effect relationship has been shown in several small molecules (Table 4). For example, a number of studies have demonstrated that trough imatinib levels were strongly associated with efficacy in patients with chronic myeloid leukemia [96, 112]. Trough sorafenib concentrations were evaluated in 67 patients in early phase studies and were found to be moderately predictive of prolonged progression-free survival (sorafenib investigator brochure). Dose-limiting toxicities of sunitinib were associated with combined trough levels of sunitinib and SU 12662, an equipotent metabolite (Sunitinib investigator brochure).

Similarly, correlation of trough drug level and clinical outcomes were also found in monoclonal antibodies. In a Japanese study, serum trough levels of rituximab of responders were higher than non-responder [125]. Fracasso et al. recently reported a correlation between cetuximab trough levels and antitumor response on cetuximab monotherapy [30].

Based on these findings and the fact that small molecules are given chronically and usually as a single agent, trough level monitoring may be a useful tool to ensure an effective target concentration is maintained.

7 A Compromise

Since BSA-based dosing is inaccurate in most anticancer drugs and it is unlikely that using a single fixed dose for all patients is the answer, consideration should be given to using a range of "fixed doses" for a particular drug that could be used as the starting dose and for dose adjustments. However, the original question remains. How should we determine the starting dose for anticancer drugs? The answer must be in defining ways to predict drug handling in each individual. We do this currently when carboplatin is dosed using GFR. However, as previously stated, the use of simple formulae for other drugs will not be possible because of complex elimination mechanisms. Complex formulae using obscure parameters also should not be favored. Dose calculation must be kept relatively simple to allow the busy clinician to adopt any new system.

Studies are underway to define the drug handling genotype and phenotype before drug administration so an individualized dose can be given on the first cycle [58, 102, 119]. Assessment of both hepatic metabolism and active biliary excretion is essential since these are the important elimination processes for the majority of cytotoxic drugs. Such in vivo tests of drug handling would have the advantage of being applicable to a range of cytotoxic and non-cytotoxic drugs, cleared by similar mechanisms.

Tamoxifen is activated to endoxifen by CYP2D6 and breast cancer patients with certain polymorphisms of this gene have lower endoxifen levels and may have worse anticancer outcome [53, 64]. We are undertaking a trial to determine whether dose escalation in such patients will overcome the detrimental effect of possessing particular CYP2D6 polymorphisms (ClinicalTrials.gov Identifier: NCT01075802). As we learn more about the pharmacogenetics of other drugs, similar fixed dose-range system for dose calculation could be applied for other anticancer drugs based on genotype. A number of polymorphisms of the UGT1A1 and other genes are associated with a variation in irinotecan exposure and toxicity, but so far a dose cluster recommendation based on different genotypes has not been made [59, 126].

One scenario worth investigating is whether pretreatment in vivo tests of genotype or phenotype can identify the estimated 20–30 % of patients who fall into the extremes of drug elimination

Table 6 Development of a dose grid for drug Z

1. In a phase 1 study using a different fixed dose levels for drug Z, the effect of various parameters such as body size, drug elimination measures, and protein binding on drug disposition was examined. It was found that:
   (a) Drug exposure varied by 4- to 6-fold between individuals
   (b) BSA contributes 30 % of variation in drug disposition and effect
   (c) Drug elimination contributes 70 % of variation in drug disposition and effect
2. It was also confirmed that drug Z is metabolized by CYP3A4 and eliminated by MDR1 and MRP2 biliary pumps. Simple tests exist to measure these processes in individuals (genotype and/or phenotype)
3. A dose grid is developed using a range of fixed doses and to take into account the two most important determinant of drug exposure. Four categories were selected for drug elimination and three for body size to reflect their relative contribution to drug disposition

Drug elimination capability				
Body size	Very poor	Poor	Efficient	Ultraefficient
Small	30	50	80	100
Medium	50	80	100	120
Large	80	100	120	150

Dose grid for drug Z in mg or percent of base dose

Note there are 6 fixed doses and the range of dose is 5-fold which approximated the known variation in drug exposure for drug X

4. This dose grid is tested in a phase 2 study. It is found that variability in drug exposure falls from 4- to 6-fold to 1.5-fold. This is considered clinically significant and the dose grid is accepted for use for drug Z as a single agent

capability. The starting dose can then be selected from a range of fixed doses according to low, normal, or high drug elimination/disposition type. If body size is found in phase 1/2 studies to be important in determining variability of drug exposure, then this can also be accounted for also. Fine-tuning of doses can be based on the presence or absence of toxicity or some other parameter that measures biological effect or by therapeutic drug monitoring. An example of development of such a method for dose calculation of a theoretical new drug is summarized in Table 6.

In summary, body size should be only one of a number of key parameters that are considered when determining chemotherapy dose for a new drug. For some drugs the effect of body size on drug disposition will be insignificant. For others, body size may contribute up to 30 % of interpatient variability. Body size may theoretically affect peak plasma concentrations for drugs with a low volume of distribution and care should be exercised when examining these drugs in phase 1 studies.

It should not be assumed that body size affects drug disposition of a new drug. This parameter should be examined in phase 1 studies along with other parameters after a fixed dose is given. For this reason, individuals with extremes of body size should be excluded from initial phase 1 studies. Drug disposition in individuals with extremes of body size should be examined in separate studies if appropriate, as occurs with other factors such as renal and hepatic function. Special attention should be applied to factors that are probably more important in determining variability such as measures of drug elimination phenotype and genotype. These should not be confined to drug metabolism alone but also include transmembrane influx and efflux pumps and key regulatory nuclear receptors.

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Phase 0 Trials in Oncology

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Abstract First-in-human clinical studies (phase I) of new anticancer agents have traditionally focused on determining the maximum tolerated dose and dose-limiting toxicities in patients with cancer. Subsequent phase II and III trials evaluate whether the new agent has potential efficacy. This process is time consuming, expensive, involves potentially hundreds of patients, and has a high rate of failure. To address some of these limitations and facilitate the development and approval of new drugs, the FDA allows phase 0 first-in-human trials to establish whether the investigational agent achieves the desired concentrations and/or modulates its target at clinically achievable concentrations. These trials administer subtherapeutic doses of drug, which are not anticipated to cause toxicity, to a small number of patients for the conduct of pharmacodynamic, pharmacokinetic, or imaging studies. If the agent demonstrates a desirable pharmacokinetic/pharmacodynamic profile, traditional phase I safety and tolerability studies are conducted; otherwise, further clinical development of the agent is unlikely to be justified. This chapter summarizes the key differences between phase 0 and phase I clinical trials.

Keywords Pre-phase 1 trials • Pharmacodynamically driven trials • Exploratory IND studies

1 Introduction

The current drug development paradigm of sequentially evaluating safety and toxicity, maximum tolerated dose, and potential efficacy in different phases of clinical trials is a time-consuming and resource-intensive process with a high rate of failure. Potentially hundreds of patients may be treated in phase I, II, and III trials before determining that the agent may not be effective (e.g., matrix

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Table 1 Summary of key differences between phase 0 and phase I clinical trials

Variable	Phase I trials	Phase 0 trials
Primary endpoint	Establish the maximum tolerated dose	Target modulation, achieving target plasma concentrations, or ability to image the target of interest
Dose escalation	Determine safety and toxicities; starting dose is low but then escalated to therapeutic and potentially toxic doses	Subtherapeutic, nontoxic doses; dose escalation performed to achieve desired systemic exposure or target modulation, enabling dose selection for future studies
Preclinical biomarker studies	Not consistently performed before the trial	Required to have plasma drug (pharmacokinetic) and preclinical pharmacodynamic assay development and qualification before the initiation of the clinical trial
Correlative studies for pharmacodynamic effect	Not performed consistently, most phase I trials do not emphasize pharmacodynamic markers	Pharmacodynamic assays and/or imaging studies are integrated to establish the mechanism of action
Number of patients	Usually >20	10–15
Dosing	Multiple	Limited
Therapeutic benefit	May occur; tumor response is evaluated to enable continued dosing in case evidence of clinical benefit is found	None; no assessment of response
Tumor biopsies	Optional	Serial tumor biopsies required to evaluate the effect of the drug on its target(s) in pharmacodynamic-driven phase 0 studies
Pharmacokinetic/pharmacodynamic analysis	Samples are usually batched and analyzed at a later time	Real time

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metalloproteinase inhibitors [10, 19]). Estimates of the proportion of oncologic agents entering clinical trials that receive FDA approval range from 5 % to approximately 25 % [4, 14], and less than 60 % of oncology drugs evaluated in phase III trials are approved [4]. This is at an estimated cost of upwards of 1 billion dollars from discovery through approval for each drug approved [22]. These statistics, in part, prompted the FDA to review its investigational drug evaluation process for limited first-in-human studies; one outcome of this review was the Exploratory Investigational New Drug (IND) Guidance, which changed the emphasis of first-in-human clinical trial design from assessing safety and tolerability to achieving target drug concentrations and drug-target effects [3, 23]. Clinical studies conducted under an exploratory IND (e.g., phase 0 trials) aim to establish whether an investigational agent achieves the desired concentrations and/or shows drug effect on target in patients at subtherapeutic doses not anticipated to cause toxicity. Only if the agent is deemed to show a desirable pharmacokinetic/pharmacodynamic profile in these studies are traditional phase I safety and tolerability studies conducted; if the pharmacokinetic/pharmacodynamic profile is not favorable, further clinical development of the agent is unlikely to be justified [15]. The key differences between phase 0 and phase I clinical trials are summarized in Table 1 and described in greater detail throughout this chapter.

2 Study Designs: Pharmacokinetic and Pharmacodynamic Endpoints

New investigational agents are suitable for PD-driven phase 0 clinical evaluation if they modulate a specific molecular target at low doses that are not associated with toxicity. Another consideration is that a robust assay can be developed to reliably measure drug effect on target, because demonstration of target modulation is the study endpoint. The FDA's Exploratory IND Guidance provides general examples of phase 0 trial designs, which include comparison of analogs to select a lead agent for further evaluation, assessment for molecular target modulation in a tumor, measurement of agent pharmacokinetics, and imaging studies [23]. These examples, as well as the different study objectives and dosing criteria, are summarized in Table 2. Whole-body imaging to assess tissue distribution and target-binding affinity is discussed further in Sect. 5 of this chapter.

Focusing a clinical study around pharmacokinetic or pharmacodynamic endpoints introduces critical questions about the underlying biology of the target. For example, does the heterogeneity of the patient population affect quantification of effect, especially given that phase 0 trials accrue typically no more than 12–15 patients? Phase 0 trials require new statistical methods to account for patient heterogeneity in such a limited patient population [21]. Another challenge is validating the PD assay using clinically relevant conditions and procedures. For example, the type of biopsy procedure, associated trauma to tissue, and the administration of anesthetic may have a substantial impact on the marker of interest (e.g., Akt phosphorylation is markedly changed by biopsy conditions [2]). Practical considerations, such as sample handling in a clinical situation, may also affect the assay readout. Therefore, well-trained staff available for tissue acquisition, handling, and processing is essential to stabilize the sample and ensure that reliable conclusions can be drawn from assay results; this requires close collaboration between bench scientists, clinicians, the interventional radiology staff collecting biopsy samples, and laboratory personnel. Additionally, the assay itself must be sufficiently sensitive, accurate, and precise such that any drug effect on the target is not obscured by the imprecision of the assay [13]. Establishing standard operating procedures for sample handling and analytically validating methods are essential to ensure reproducibility of an assay between different clinical samples and sites.

3 Drug Suitability for Phase 0 Trials

The decision to conduct a first-in-human trial as a phase 0 or phase I study is predicated on a number of factors (Fig. 1). Pharmacokinetic-driven phase 0 trials can be considered for agents that have a wide therapeutic index, when a pharmacokinetic assay has been validated to measure low levels of the drug in human plasma and when the clinical development of the agent is based on whether target plasma

Table 2 Examples of phase 0 studies supported by the Exploratory IND Guidance

Type of study	Objectives	Dose
Pharmacokinetics or imaging	Evaluate biodistribution and target binding	1/100th of the pharmacologically active dose (up to a maximum of 100 μg or 30 nmol for protein products)
Pharmacologic endpoint	Compare pharmacokinetics and/or pharmacodynamics (bioavailability) of analogs to select lead agent	1/50th of the NOAEL determined in 2-week rodent toxicology studies
Pharmacodynamic endpoint	Measure modulation of target	Less than 1/4 of the rat NOAEL, or dose at which the total exposure measured in human blood samples is 1/2 of that determined in the most sensitive species, whichever is lower

NOAEL no observed adverse effect level

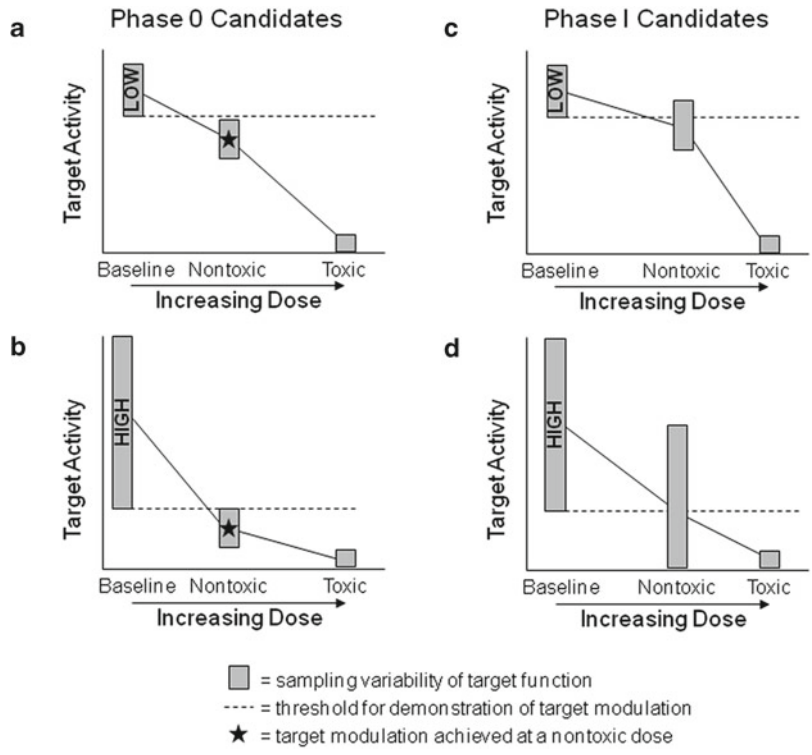

Fig. 1 Targets suitable for evaluation in phase 0 trials can have either low (**a**) or high (**b**) amounts of baseline variability because significant modulation of the target is measured after administration of sub-toxic doses of the drug. If target modulation is never significantly different from baseline (**c**), or if significant modulation is only achieved at potentially toxic doses, then the targets are not suitable for phase 0 evaluation (**d**). Reprinted from Doroshow JH, Parchment RE (2008) Oncologic phase 0 trials incorporating clinical pharmacodynamics: from concept to patient. Clin Cancer Res 14:3658–3663, with permission from AACR

concentrations can be achieved (e.g., determining oral bioavailability). Pharmacodynamic-driven phase 0 trials are considered for agents with a wide therapeutic index and known putative mechanism of action, when preclinical results support target modulation at nontoxic doses and when a pharmacodynamic assay can be validated to reliably measure drug effect [6, 16]. Because the important decision for further clinical development of the agent will be based on the results of the phase 0 trial with a small sample size, it is essential that pharmacokinetic/pharmacodynamic relationships be established in preclinical models and the pharmacodynamic effect shown to be associated with the observed antitumor effect prior to initiation of the phase 0 trial. A drug that modulates the target but only at doses associated with preclinical toxicity is more appropriate for phase I evaluation (Fig. 2).

4 Exploratory IND Requirements

One point emphasized in the FDA's Exploratory IND Guidance is the flexibility of information relating to preclinical toxicology studies and chemistry, manufacturing, and control information required in the application: "Because exploratory IND studies present fewer potential risks than do traditional Phase 1 studies that look for dose-limiting toxicities, such limited exploratory IND investigations in humans can be initiated with less, or different, preclinical support than is required for traditional IND

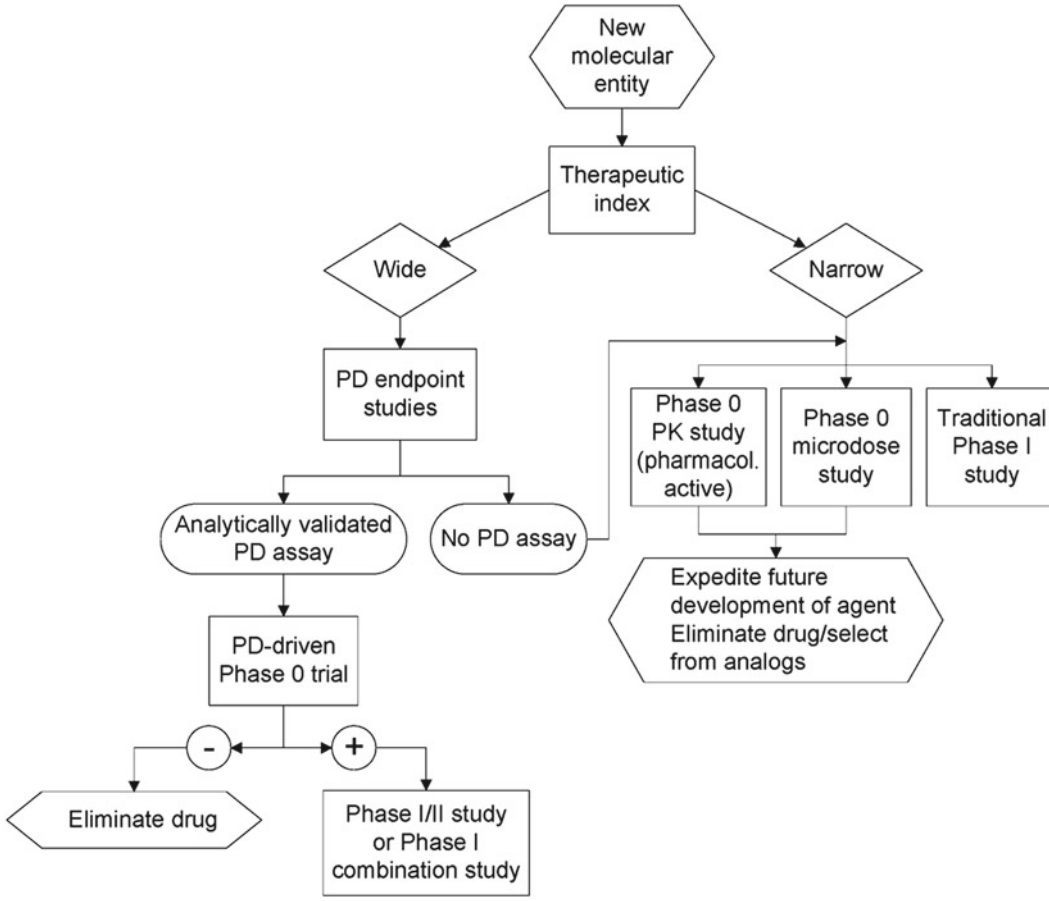

Fig. 2 Phase 0/phase I decision chart for clinical evaluation of a new molecular entity. The decision to proceed with a phase I or phase 0 study design depends on the characteristics of the agent as well as development objectives. Reprinted from Kummar S, Doroshow JH, Tomaszewski JE et al Phase 0 clinical trials: recommendations from the task force on methodology for the development of innovative cancer therapies. Eur J Cancer 45:741–746, copyright (2009), with permission from Elsevier

studies" [23]. A direct consequence of this flexibility is more readily attainable requirements for drug manufacture and animal toxicology studies and therefore shorter time to first-in-human trials; this facilitates the expeditious evaluation of new investigational agents (Fig. 3). Protecting human subjects remains the priority and purpose of preclinical work—both traditional and exploratory INDs require single-dose (i.e., acute) toxicology studies in two mammalian species to calculate a safe clinical starting dose, but the design of these studies varies depending on IND type [12]. Full pharmacokinetic and pharmacology repeat-dose studies with histopathology and clinical sign evaluation may not be required for an exploratory IND. The IND-enabling toxicology studies required before initiation of phase 0 trials support the clinical schedule to be evaluated, which is single dose or limited dosing. Therefore, they are more limited in scope; however, the studies must still be conducted in two species with histopathology and adverse event evaluation to ensure adequate safety assessment. Only if the results from the phase 0 study indicate that the drug is worth pursuing in phase I studies would complete pharmacology and toxicology studies be conducted in support of a traditional IND, in which case the exploratory IND would be closed. Limited human exposure also means that smaller quantities of drug must be produced to initiate the trial. An FDA Guidance document released in conjunction

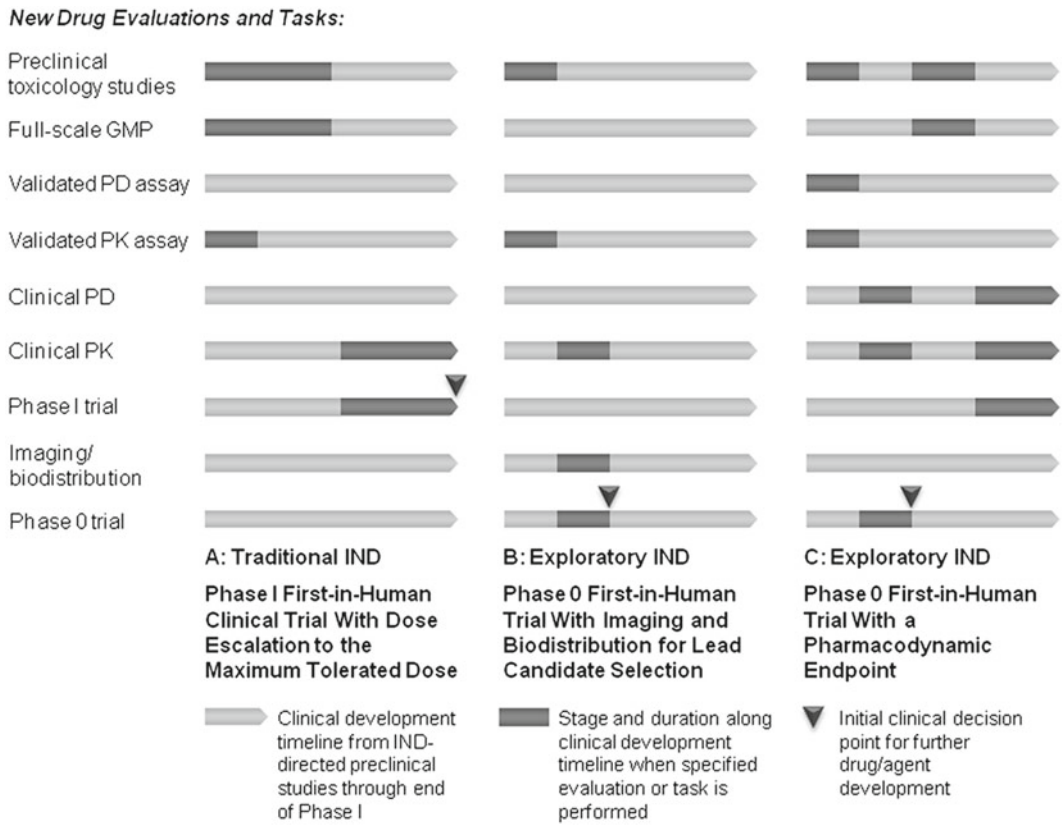

Fig. 3 The preclinical support required for clinical trials conducted under an exploratory IND differs from that required for a traditional IND because limited dosing is anticipated to present a lower risk to study participants. Key differences include that the exploratory IND requires less extensive preclinical toxicology studies and "laboratory-scale" CGMP drug production; complete preclinical toxicology studies and full-scale CGMP are needed before phase I evaluation. The decision for whether or not to continue clinical development of an agent under a traditional IND can be made once the phase 0 study proof of principle is met. Unlike phase I trials, phase 0 trials with a PD endpoint must have a validated PD assay prior to clinical trial accrual. Reprinted from Kummar S, Rubinstein L, Kinders R et al (2008) Phase 0 clinical trials: conceptions and misconceptions. Cancer J 14(3):133–137, with permission from Lippincott, Williams & Wilkins

with the Exploratory IND Guidance on complying with current good manufacturing practice (CGMP) regulations [24] describes an incremental (i.e., "laboratory" scale) rather than full-scale approach to the manufacture of investigational drugs for early-phase clinical trials. As with a traditional IND application, these would include appropriate quality control procedures for manufacturing, labeling, and documentation.

5 Imaging Studies

One avenue of molecularly targeted drug evaluation allowed by phase 0 studies is noninvasive whole-body imaging to assess tissue distribution and target binding affinity [3, 11]. For clinical imaging studies, the Exploratory IND Guidance supports administration of "microdoses" of drug (a dose less than 1/100th of that required to have a pharmacologic effect in preclinical studies, up to a maximum of 100 μg for imaging agents or 30 nmol for biologics) [23]. Radiolabeled agents can be followed over

time to collect invaluable information on dosimetry, biodistribution, and metabolism that would be impossible to obtain from blood or tumor biopsy samples. Drugs that have previously undergone clinical investigation can be labeled and then administered at sub-pharmacologic doses after Radioactive Drug Research Committee approval, an avenue not open for first-in-human trials.

6 Ethics of Clinical Trials That Lack Therapeutic Intent

Because phase 0 trials lack therapeutic intent, there has been considerable interest in evaluating potential risks to subjects associated with the need for research biopsies and the possible exclusion of patients from trials with therapeutic intent. Each of these issues is of valid ethical concern. The design of phase 0 trials is based on drugs that have a high therapeutic index and are administered at subtherapeutic doses. The administration of a single or limited number of doses provides additional safety for drugs that will eventually be developed for chronic administration, such as a number of oral targeted agents. Even though the risk of side effects is minimized, participants in phase 0 trials are carefully monitored for any evidence of toxicity.

Another consideration is the informed consent process. Care must be taken during the informed consent process to ensure that participants understand that there is no prospect of direct medical benefit from participation in the study and that participation entails donation of tumor biopsy samples [8]. Patients should be given ample time to review and discuss the consent form and should be asked to verbalize their understanding prior to signing it. Phase 0 trials should only be offered to patients who do not require immediate palliative care. While considering the potential options for the patient, physicians must confirm that participation in a phase 0 trial will not unduly delay or affect the eligibility of the patient for subsequent trials that may offer potential therapeutic benefit. This can be achieved through cooperation from other investigators in reducing the washout period (e.g., from 4 to 2 weeks) after administration of an experimental agent in a phase 0 trial because toxicities are not expected. A further consideration is that clinicians revise the eligibility criteria for later-stage trials to ensure that patients who receive an agent on a phase 0 trial are not excluded from participation in a definitive study with that agent or that class of agents [17].

Regarding the ethical considerations surrounding obtaining tumor biopsies for research purposes, it should be emphasized that tumor biopsies obtained during early-phase clinical trials are always done in support of investigative endpoints and do not provide benefit to the patient. Given the intent of the trial, patients who agree to participate in a PD-driven phase 0 trial should be willing to consider donating biopsy samples for PD analysis if the biopsy procedure is considered medically safe by the study team. Obtaining sequential pre- and posttreatment biopsies does confer more than minimal risk in that invasive procedures are performed with the possibility of complications [7]. However, patients are generally amenable to donating biopsies for research purposes [1]. As mentioned earlier in this chapter, the proper handling and analysis of all patient samples is of major concern. It may also be considered an ethical obligation to optimize the quality of pharmacodynamic and pharmacokinetic data obtained, because mishandled samples can compromise the results from and indeed the value of the clinical trial [9].

7 Phase 0 Experience and Future Directions

For its first phase 0 trial in oncology, the NCI selected ABT-888, an inhibitor of the DNA repair enzyme poly (ADP-ribose) polymerase (PARP), because it had good oral bioavailability as well as activity in tumor xenograft models in combination with DNA-damaging chemotherapeutic agents [5].

The trial was designed to administer a single dose of study drug with multiple blood sampling time points for pharmacokinetic and pharmacodynamic analysis and paired tumor biopsy sampling before and after drug administration [18]. Successive cohorts of patients received increasing doses of study drug to determine the dose range that inhibited the activity of PARP in tumor biopsy samples and peripheral blood mononuclear cells. The performance of pharmacokinetic and pharmacodynamic studies and communication of data within 48 h of sampling highlights the team-science approach critical to the conduct of phase 0 studies [18]. These data formed the basis for several combination trials of ABT-888 with DNA-damaging agents.

The pharmaceutical industry is also conducting exploratory IND studies, especially studies comparing the pharmacokinetic profile of different analogs to select lead compounds for further development [20]. The NCI has started accrual to its first phase 0 imaging trial and is currently designing a study with both imaging and pharmacodynamic endpoints to measure uptake of a nucleoside analog into tumor DNA as a potential radiosensitizer.

8 Conclusions

Conducting clinical trials of new anticancer drugs is an expensive and time-consuming process with a high rate of failure as measured by FDA approval rates for new drugs. Phase 0 trials represent a welcome and timely opportunity to improve and expedite the development of new molecularly targeted drugs for patients with cancer. Trials conducted under an exploratory IND have the potential to focus valuable development resources that allow proof of mechanism to be demonstrated for drugs with targeted activity, rather than on those that will fail to be approved because of lack of activity. There are no guarantees that a drug evaluated in a phase 0 clinical trial will be more likely to move forward to eventual FDA approval than a drug evaluated in a phase I safety and tolerability trial. However, if the drug does not modulate its target, it is less likely to have clinical efficacy and has a lower priority for further development.

Further advantages of the exploratory IND are the limited numbers of patients and small quantities of drug required, both of which, along with a reduced preclinical toxicology package, lower the barrier for investigators contemplating conducting an early-stage clinical trial. The flexibility allowed for clinical trial designs conducted under an exploratory IND is also a major innovation. This should not detract from the fact that a great deal is asked of patients who agree to participate in a phase 0 trial, not least the lack of therapeutic intent and the risks associated with the research biopsies that assess target modulation. Patients have shown themselves willing to participate, a consideration that must be reflected in the proper handling and analysis of patient samples by clinical team members. The outcome of phase 0 trials is data that can categorically demonstrate whether the investigational agent achieves the desired concentrations and/or modulates its target in humans at the earliest possible stage, allowing rational decisions to be made about allocation of development resources and potentially shortening the clinical development timeline of the most promising new anticancer drugs.

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Phase I Trials in Oncology: Design and Endpoints

Hilary Glen and Jim Cassidy

Abstract Early clinical development of anticancer drugs is beset with obstacles unique to this type of therapy. Typical healthy volunteer studies are seldom possible, and patients tend to have end-stage malignant processes, with many underlying symptoms and often organ dysfunction. This chapter will focus on the design of traditional early phase I clinical trials of anticancer therapies, including selection of patients, starting dose selection, dose-escalation approaches, and endpoints. It will go on to examine the limitations of the current, widely accepted approaches and some of the problems facing investigators. Finally, it will also discuss how early anticancer drug development now faces a paradigm shift due to the advent of novel, molecularly targeted anticancer drugs.

Keywords Phase I • Dose escalation • Maximum tolerated dose • Dose-limiting toxicity • Biomarker • Methodology for the Development of Innovative Cancer Therapies

1 Introduction

Development of anticancer drugs has a number of complications not relevant to drug development in other nonmalignant diseases. Cancer is perceived by patients as an immediate life-threatening event. In many cases this perception is correct and therefore there is a sense of urgency to initiate therapy and an understandable reluctance to take part in trials that involve a placebo of any kind. It is thus rare to conduct the "gold standard" double-blinded randomized controlled trial that is common in other disease entities. Moreover, owing to the side-effect profile and teratogenic effects of most traditional cytotoxic therapies, it is almost impossible to employ normal volunteer studies, which again are the mainstay of noncancer early drug trials. Thus, we are almost always trying to develop drugs in patients with cancer. It is clear that few, if any, patients will volunteer for an experimental therapy when standard care is available. The problem is then compounded by the need to use end-stage patients, for whom no other standard treatment is available, for our phase I studies in cancer.

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Often comparatively little is known about many of the fundamental issues of mechanism of action, schedule dependency, toxicity, pharmacodynamics, and pharmacokinetics when a new anticancer drug is first administered to humans. So we are forced to develop safety-conscious clinical plans but also one that will allow the therapeutic goals to be achieved as quickly and efficiently as possible. It is clear that these competing tensions result in a decision-making process that is far from ideal.

The paradigms that have been used for drug development in oncology have been designed to cope with traditional cytotoxic drugs, and these are not likely to be applicable to drugs that are cytostatic or act on a particular aspect of the malignant phenotype such as angiogenesis, invasion, or metastatic capacity.

The normal volunteer study used in traditional pharmacology has more than one endpoint. It is usual to measure the expected effect in volunteers (e.g., blood pressure in response to an antihypertensive drug). It is also usual that toxicity will not be observed and thus placebo controls are commonly used to exclude nonspecific effects such as nausea or headache. In cancer the commonly applied endpoint for a phase I study is the observation of a dose-limiting toxicity (DLT) side effect, which is then used to define a maximum tolerated dose (MTD). We monitor tumor response but realistically do not expect to see any such response in most cases. Cancer phase I studies therefore should be more appropriately thought of as toxicological investigation in humans. The traditional aim of a phase I study is to define a safe dose and schedule to be taken into phases II and III, with the aim of determining activity in these later trials. It is self-evident that declaring an inappropriate dose after phase I will have serious consequences—usually lack of activity if MTD is set too low or, conversely, too much toxicity if MTD is set too high.

This chapter outlines the usual methodology for "cytotoxic" drugs but will also discuss some of the problems facing drug development of novel "noncytotoxic" agents and highlights the important limitations of these approaches. In many instances we have not yet found the ideal way of developing certain classes of agents, and continued methodological developments are required to improve efficiency in this area of therapeutics [1, 9].

2 Selection of Patients

As previously mentioned, it would be very difficult to perform phase I trials of novel anticancer agents in healthy volunteers, mainly because of potential toxicity. Therefore, we are limited to working with cancer patients who have either failed standard therapy or for whom no standard therapy exists. It is self-evident that such patients tend to have widespread metastases, limited life expectancy, and numerous manifestations of the underlying cancer. These can be nonspecific such as malaise, nausea, anorexia, lethargy, or cachexia. Alternatively, they can be organ specific, such as neuropathy, renal dysfunction, diarrhea, or hepatic dysfunction.

This has important ramifications for drug testing in this group. It can be difficult to tease out drug-related effects from the clinical manifestations of the disease; intercurrent co-medication is the rule, with all of the potential for drug interactions, and the handling of the drug may be altered by organ dysfunction.

To attempt to limit such problems, we select patients within very careful entry criteria. They usually should have at least a 3-month life expectancy to allow time to observe any side effects. They should have critical organ function (hepatic and renal) that is normal or near normal. This will help limit variable pharmacokinetics between patients and allow for some comparison to be made between animal pharmacokinetics (done with normal organ function) and the human experience [10]. Unfortunately, this leads to a high degree of patient selection, as most people with advanced intractable cancer will have deranged organ function.

3 Starting Dose Selection

At the start of a phase I trial of an anticancer drug, we will have some (limited) data on dosing in animal model systems. Usually this will have been derived from toxicity (lethality) experiments and will have been performed across a limited dose range [4]. In the case of standard cytotoxics with marrow toxicity, there is fairly good correlation with human toxicology (reviewed in [1]). The convention with such drugs is to employ as a starting dose 1/10th of the lethal dose in 10 % of animals (LD10)—in the most sensitive animal species—and this has been shown to be generally safe, if somewhat conservative [11].

It is quite unlikely that such a correlation will exist for agents with alternative mechanisms of action. More subjective side effects such as malaise, nausea, headache, and myalgia cannot be observed in animals, yet it is these effects that are emerging in many instances as dose limiting for drugs aimed at "new targets." The real dilemma is selecting a starting dose that will be safe, but not so low that the duration of the trial is too long, and the patients on the early dose cohorts have no chance of responding to therapy.

4 Schedule Selection

Preclinical knowledge of schedule dependency with a new agent is usually sketchy at best. At most one will have some idea of an appropriate route of administration and a concept of whether the drug needs to be given often or as a single dose with time allowed for normal tissue recovery. The dilemma is then how often to give the new drug in early-phase studies? Considerations of mechanism of action, expected toxicities, and convenience will all have an influence here. Often sponsors and investigators try to avoid this issue by setting up studies with a variety of schedules; this does not usually solve the dilemma, but simply delays the decision-making process until the phase II plans are made.

5 Dose Escalation

The same dilemma applies in the case of dose-escalation schema. If the most efficient phase I is that which reaches the MTD as quickly as possible, the temptation is to be aggressive with dose escalation [14]. Two important questions govern the speed of dose escalation:

(a) In the absence of toxicity at the previous dose, how much of an increase should be made for the next dose level?
(b) How many patients should be treated at each dose level?

To address the first question, a variety of fairly arbitrary methods are utilized to try to overcome this dilemma. Traditionally dose escalation has been performed using a "modified" Fibonacci scheme; if level 1 is the starting dose X1, level 2 is X1 + 100 %, level 3 is X2 + 67 %, level 4 is X3 + 50 %, and level 5 and above Xn + 30–35 %. This method was introduced in the early 1970s with nitrosourea and epipodophyllotoxin. It has a few inherent problems. The "modified" part is usually a preset number of drug dose doublings that will be allowed before the more conservative part is commenced. This is too often decided in an arbitrary fashion but can have a profound effect on the trial. Too many doublings might lead to excessive toxicity and too few leads to a trial that lasts longer than it should and exposes too many patients to subtherapeutic drug doses. A widely practiced method to avoid this pitfall is to

maintain drug dose doubling until the first drug-related adverse events are observed and then to employ the Fibonacci element. This also has limitations—because of the amount of nondrug-related problems that such patients encounter, it is sometimes difficult to determine accurately the relationship to the study drug. The tendency is to err on the side of caution, enter the Fibonacci phase, and then find that these "toxicities" are absent in subsequent dose cohorts.

To address how many patients should be treated at each dose level, this is usually arbitrarily set at three patients per dose cohort in the absence of toxicity that would mandate expansion of the cohort. However, many investigators have switched to single-patient cohorts at least for the very early low doses to limit exposure of patients to doses that are too low to have a realistic expectation of efficacy. It is worth noting that although this aim may be achieved, the use of single-patient cohorts will not necessarily result in more rapid escalation through the doses.

Dose escalation usually takes place with each new cohort. Intra-patient dose escalation is less common, but at times the same patient has been reentered at a later (higher-dose) cohort. The argument against it is that if cumulative toxicities occur, it will be more complex to attribute them correctly if intra-patient escalation is performed. However, if an adequate washout period is allowed, it may be reasonable to allow patients to have a higher dose with more expectation of the therapeutic benefit.

This method is considered by many to be overconservative [14] and, as a result, alternatives have been sought based on pharmacokinetics [6] and, more recently, Bayesian approaches [12, 13]. As yet none has reached as widespread acceptance as the "modified" Fibonacci. One particularly intriguing possibility is to allow patients to select their own doses using a linear analog scale that ranges from "low dose—low toxicity with less chance of a response" up to "high dose—toxicity likely with more chance of a response."

6 Endpoints

The accepted dogma in oncology is the higher the dose, the better the antitumor effect. This can be verified for some cytotoxic drugs up to a threshold value, beyond which toxicity becomes limiting or even lethal. Conversely, we do not often have an identifiable lower-dose threshold for activity with a cytotoxic agent. In fact, most drugs we use have an apparently very narrow therapeutic index. The primary endpoint for a cytotoxic phase I study is determination of a recommended dose and schedule for phase II study. Important secondary goals include a description of toxicity, pharmacokinetic and pharmacodynamic effects, and description of any objective evidence of antitumor activity.

Objective measures of blood parameters can be simply applied to predefined acceptable levels of toxicity. Subjective toxicity causes much more of a problem. A lethargy that one person might consider intolerable may be of little significance to a more stoical individual. Even the apparently simple objective measures such as blood count parameters are under question now. The discovery and widespread use of hematological growth factors to support blood counts means that we could define MTD with and without such support (or even a cocktail of such "support" molecules). This has some merit in that we commonly define MTD in terms of nausea and vomiting despite maximal antiemetic support. Conceptually similar as these situations are, it is not yet widely accepted to perform initial phase I trials of drug plus growth factor.

It is also possible to influence such endpoints by patient selection. Prior exposure to cytotoxics or extensive radiotherapy with fields encompassing marrow primes patients to experience myelosuppression. It is necessary to take account of this, usually by including a cohort of "good risks" patients at the end of the trial to ensure that the MTD has not been set too low.

7 Limitations

The generic design of phase I drug development outlined in this chapter has several limitations. Patients in phase I trials are selected for good organ function, for reasons outlined above, and are based mainly on safety considerations. However, because those same considerations apply in phases II and III, we end up with a population that is not representative of the average patient with advanced cancer. This has two important long-term consequences:

(a) Response rates in phase II (and even some phase III) trials are higher than one might expect in a less select group of patients. This is often the basis for press reports of "wonder drugs" that can immeasurably damage the psychological well-being of cancer patients.
(b) The response rates from phase II are used to set the parameters for statistical considerations in the ensuing phase III trials—which then turn out to be insufficiently powered to reveal the smaller (but still clinically significant) advantage that one might realistically expect.

Moreover, the small numbers of patients enrolled in phase I trials are not sufficient to define fully the toxicity pattern of a drug. For this reason, not only should response rates be viewed with some suspicion but also reports of little or no toxicity should be treated with caution.

8 Novel, Molecularly Targeted Anticancer Drugs

Traditionally, the majority of new anticancer drugs undergoing early clinical trials were cytotoxics that targeted DNA or mechanics of cell division. Anticancer drug discovery often followed an empirical approach, characterized by random screening of a variety of natural and synthetic compounds using high-throughput cell-based cytotoxic assays [7]. In recent years, there has been a significant shift towards a more rational and mechanistic, target-based approach, with a goal of improving the efficacy and selectivity of cancer treatment by developing agents that specifically target a research-validated pathogenic mechanism, known to cause or drive the malignant process. Examples are drugs that inhibit angiogenesis [8] and invasion (matrix metalloproteinase inhibitors [MMPIs]) [5] and those that interfere with growth regulatory signals within cells, such as epidermal growth factor receptor (EGFR), Ras farnesylation inhibitors, and inhibitors of the mitogen-activated protein kinase pathway.

There is much anticipation for these novel "targeted" agents, and rightly so. In addition to exciting evidence of efficacy (often in tumor types typically resistant to traditional cytotoxics), by their very nature, targeted agents tend to have much less in the way of side effects, simply because they exert minimal, if any, effects on normal (nontarget) tissues.

However, these targeted agents tend to have unique toxicity profiles and often lack the usual "cytotoxic" effects, such as bone marrow damage. In addition, many of these agents tend to have mechanisms of action which do not necessarily result in tumor volume shrinkage. Thus, we have effectively lost our familiar markers of both toxicity and activity. Moreover, targeted agents tend to be better tolerated and can often be taken for a much longer duration, but the majority of toxicity data collected in phase I trials are from short-term use. Chronic dosing is almost impossible to achieve in the standard phase I patient population. Therefore, while a reasonable perception of the likely acute toxicities exists after completion of the phase I trial, often little is known about chronic toxicity which may only become apparent when these drugs become more widely used.

A further difficulty caused by the presence of often far fewer side effects is identifying the dose in phase I trials to take forward to future phases II and III clinical trials. We currently tend to adopt a similar dose-escalation scheme as for cytotoxics and continue until we reach the MTD. However, for

specific targeted agents where our aim is to "switch off" a specific cellular event (e.g., signaling through a specific growth factor receptor), once that event is inhibited, there is no merit in further dosing increases, and indeed worse than simply not adding any extra efficacy, inappropriately increasing the dose may introduce unnecessary toxicity which could be avoided. The aim therefore is to identify an "optimal biological dose," where maximum efficacy is achieved without causing needless side effects.

The real challenge is finding ways of identifying this optimal biological dose. Ideally, one would assay the activity of the target enzymes in the target tissues (tumor and normal tissue to define selectivity). However, it is uncommon to be able to attain serial tumor biopsies in the context of a clinical trial and almost impossible in the general clinical setting. Therefore, much research is underway to identify suitable "surrogate" markers (or biomarkers) of targeted drug activity.

9 Surrogate Endpoints/Biomarkers

An ideal biomarker would be able to define rapidly and efficiently that the target had been affected in the appropriate tissue in the manner and extent that one would predict. Blood-borne biomarkers (including peripheral blood mononuclear cells (PBMCs), circulating endothelial and tumor cells, and serum proteins) would therefore be the most preferred surrogate markers of drug activity, but other approaches include utilizing skin biopsies, hair follicles, and pharmacodynamic parameters (e.g., blood pressure changes or development of characteristic skin rashes). The complexities of developing such a marker are huge. However, when compared with the time and expense of developing a drug that fails to reach the clinic, the expenditure to develop worthwhile clinically applicable and validated surrogate endpoints can be viewed as good value. Increasingly, biomarker studies are now included in early phase I trials and beyond.

To address the problem of assessing efficacy of a new drug whose mechanism of action is unlikely to lead to significant tumor volume reduction, modern imaging modalities are employed to measure more directly the functional effect of the drug rather than just volume change in the tumor mass. The simplest example is the measure of blood flow in the tumor under the influence of drugs that purport to alter this, for example, angiogenesis inhibitors. More information is also available from positron emission tomography (PET) and magnetic resonance imaging (MRI), but these methodologies are still in development and will require prospective validation before drug development "stop–go" decisions would be possible.

A further approach taken by many investigators in this field is to combine the new cytostatic agent with a known cytotoxic, thereby allowing our original paradigm to be used [2].

10 Methodology for the Development of Innovative Cancer Therapies Task Force

Recently, the New Drug Development Office (NDDO) Research Foundation established the "Methodology for the Development of Innovative Cancer Therapies" (MDICT) task force as a forum for the discussion of methodological issues in contemporary oncology drug development. The mission of this independent international task force is to develop practical guidance on the optimal development of anticancer targeted agents. To date, they have focused on two topics: (1) the usefulness of MTD as an endpoint in phase I studies of targeted agents and (2) the use of biomarkers in phase I trials.

Currently, they have suggested that MTD and indeed pharmacokinetic data appear to be reasonable endpoints to establish the dosing range for novel compounds. However, they also point out that when molecular proof of principle is deemed important for subsequent development decisions, investigators

should consider expansion of one of more cohorts after the conclusion of the escalation phase or design a separate study to confirm that the doses identified on the basis of toxicity are able to affect the molecular target [3].

They also recommend that in the absence of toxicity, one could consider biomarker measurement (tissue-based or imaging) or pharmacokinetic measurements to establish a suitable dose range. However, they also caution that a clear distinction should be made between the observation of the desired molecular effect of drug (i.e., proof of concept) and the impact of the drug treatment on clinical measures such as tumor shrinkage or delay in progression (i.e., clinical benefit).

A full set of MDICT task force recommendations and an algorithm for the design, implementation, analysis, and output of first-in-man phase I trials of targeted agents is published [3].

11 Conclusions

Fairly sound methodology has been developed over the last 30 years for the development of cytotoxic agents. However, many elements in the overall plan are reliant on empirical decision-making. This may not have been so crucial when developing drugs with "standard" antiproliferative effects. It is very likely that this same plan will not apply to cytostatic-type agents. Further scientific protocols for the clinical development of such agents are urgently needed. It seems highly likely that time and energy spent developing surrogate markers of activity will pay dividends in the long run.

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Quantitative Analytical Methods: Development and Clinical Considerations

Erin R. Gardner

Abstract Accurate and precise data on drug concentrations is critical for anticancer drug development. The ability to measure anticancer agents is essential throughout the drug development process, and numerous analytical assays are typically developed—from initial purification and assessment of impurities, to in vitro transport and metabolism studies, through large-scale pharmacokinetic studies to therapeutic drug monitoring. To ensure that data generated from collected samples is representative of the actual drug concentration, it is crucial that method optimization begins early in the process and that a number of clinical considerations, including selection of analytes and matrices, sample collection volumes, and processing requirements, be evaluated prior to the start of sample collection. This chapter will focus primarily on the development process and clinical considerations relevant to quantitative analytical assays for small molecule anticancer agents with HPLC, LC-MS, or LC-MS-MS, though some considerations are applicable to all molecules under development.

Keywords Quantitative • Analytical • Method development • Assay • Sample • Specimen • Processing • Storage • HPLC • LC-MS-MS

1 Introduction

Accurate and precise data on drug concentrations is critical for anticancer drug development. The ability to measure anticancer agents is essential throughout the drug development process, and numerous analytical assays are typically developed—from initial purification and assessment of impurities, to in vitro transport and metabolism studies, through large-scale pharmacokinetic studies to therapeutic drug monitoring. The wide array of chemicals currently approved for use in anticancer therapy necessitates the use of a number of very different analytical techniques. These range from the widely available high-performance liquid chromatography with ultraviolet (HPLC-UV) detection to sensitive and specific tandem mass spectrometry (MS-MS) for many small molecules, to atomic absorption spectroscopy (AAS) for platinum-containing agents, and biological assays such as enzyme-linked immunosorbent assays (ELISA) for monoclonal antibodies.

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This chapter will focus primarily on the development of quantitative analytical assays for small molecule anticancer agents, though some considerations are applicable to all molecules under development.

2 Clinical, Pharmacological, and Chemical Considerations

A number of questions that may not seem relevant to the actual development of an analytical method but are in fact critical to the generation of accurate concentration data must be addressed prior to initiation of a clinical trial, including the following:

2.1 What Drug/Compounds Should We Be Evaluating?

The first major decision that must be made is which compound or compounds to quantitate. Though this may be simple in the case of drugs that are eliminated primarily as unchanged parent drug, it becomes more complicated in the case of prodrugs, active metabolites, or metabolites suspected to be responsible for toxicity. For example, following administration of irinotecan, the parent drug is esterified to the active metabolite SN-38 as well as oxidized to several inactive metabolites. SN-38 is subsequently glucuronidated to SN-38G, which is inactive. Reduced clearance of SN-38 has been associated with increased toxicity, specifically neutropenia [1]. As such, assays for irinotecan typically measure the parent drug, along with both SN-38 and SN-38G [2]. In the case of agents administered as prodrugs, it must be decided whether to measure both the prodrug and the active moiety or simply the latter. This is especially relevant to the numerous conjugated drug products being designed to increase activity or improve the physiochemical properties of existing anticancer agents.

2.2 Availability of Reference Standards

In order to accurately quantitate the analytes of interest, pure reference standards are required for method development, validation, and subsequent sample analysis. In the case of many older anticancer drugs, reference standards can be easily obtained from commercial chemical suppliers. For newer agents, the only source may be the drug company manufacturing it for research studies. Analysis of metabolites is often limited by the lack of availability of pure reference standards. For phase I metabolites, this necessitates chemical synthesis and purification of the metabolite, while for phase II metabolites transformation of the metabolite of interest into a compound for which a reference standard is available. Due to the unavailability of a reference standard for flavopiridol glucuronide, Innocenti et al. analyzed samples once to quantitate flavopiridol, then repeated the analysis after incubating plasma samples with β-glucuronidase enzyme, which cleaves the glucuronide group [3]. The difference in concentration between the two analyses represents the concentration of flavopiridol glucuronide present under the assumption of complete conversion.

2.3 What Matrix or Matrices Should We Be Evaluating?

In the development of agents for treatment of solid tumors, it is typically critical that the drug penetrates the tumor. Ideally, we would like to fully elucidate the pharmacokinetics in the tumor itself, but this is often unfeasible in humans, where invasive serial biopsies would be required.

Typically, human drug concentrations are evaluated in plasma, employing systemic exposure as a surrogate for tumor exposure. However, depending on the properties of the analyte and the anticipated routes of excretion, it may be desired or essential to collect other specimens. Some drugs will rapidly partition into red blood cells, resulting in significant differences in pharmacokinetics measured in plasma and whole blood [4, 5]. Hence, plasma may not always be the best surrogate for actual drug exposure. Urine specimens are often collected to assess urinary excretion of parent drug or metabolites. This can take the form of either spot (single collection) or continuous (e.g., cumulative 24 h) samples. For pharmacokinetic analysis, continuous urine collections aid in elucidating the rate of excretion and are often much more informative than single collections. Furthermore, unlike blood collection, urine collection is noninvasive, so it can typically be added to clinical protocols with relative ease. Saliva has also been collected as a surrogate for unbound drug concentrations and has been shown to be a noninvasive, indirect measurement of exposure, for example, with topotecan and UCN-01 [6, 7].

In earlier, nonclinical studies, especially those performed with rodents, many tissues can be collected to assess drug distribution. This can range from organs involved in elimination (liver, kidneys) to the brain or other tissues of interest. Though collection is relatively simple, additional method development and validation is often required for each tissue.

More recently, microdialysis has also been used to assess tumor exposure in both rodent xenograft models and patients with accessible tumors [8]. This technique has the potential to greatly improve understanding of tumor pharmacokinetics but adds numerous additional clinical and analytical considerations [9].

An additional consideration when deciding upon matrices to be evaluated is the availability of blank, or untreated, material. A source of blank material must be available or collected to be used for calibration and quality control samples. In the case of human blood, this is often available from institutional blood banks. However, for other matrices, it may be necessary to either purchase tissue from commercial sources or obtain additional animals for the purpose of harvesting untreated tissues. For human specimens other than plasma, the procurement of untreated tissue can be challenging and should be carefully considered prior to collection in treated patients. When blank tissue is unavailable, the dilution of tissue homogenate into plasma provides one alternative. Standards and analytes must all be prepared in this manner, and the process should be fully validated to ensure that any bias introduced is acceptable [10].

2.4 Are Special Processing Techniques Required?

Often times, immediate processing of clinical pharmacokinetic samples is requested. Though this is regularly performed for animal pharmacokinetic studies, it is perhaps less practical when considering patient studies that may have extensive blood collections. This is often a reflection of the lack of extended stability data in the matrix of interest as opposed to actual knowledge of drug degradation. Development of an analytical method early in the drug development process allows for stability of the analyte to be assessed and processing times and techniques to be scientifically guided, as opposed to arbitrarily fixed. It is important that the chemistry or degradation characteristics of the drug and any metabolites of interest be evaluated. When handling carboplatin, a widely used platinum chemotherapeutic agent, samples must be processed via and frozen at −80 °C immediately to avoid artifactual concentration changes. In plasma samples stored at −20 °C, Erkman et al. observed a steady decrease in free platinum concentrations [11]. Gemcitabine, an antimetabolite chemotherapeutic agent, is rapidly deaminated in plasma by cytidine deaminase to form the inactive compound 2′,2′-difluorodeoxyuridine (dFdU). Therefore, pre-addition of tetrahydrouridine to blood collection tubes prevents further degradation from occurring, allowing for accurate assessment of in vivo concentrations [12].

Another aspect that may need to be considered is whether specialty collection or storage tubes are required. Doxorubicin is known to adsorb onto glass, with polypropylene tubes considered the least reactive [13]. Similarly, it has been shown that recovery of docetaxel in microdialysis collections is very low, due to nonspecific binding to the catheters employed [14].

2.5 How Long Can Samples Be Stored After Processing?

Long-term storage stability testing is typically performed as a component of the method validation process. However, if method development is not initiated prior to the start of the clinical trial when sample collection and storage begins, the time frame for which how long samples can be stored will remain unknown and investigators risk sample degradation.

Some anticancer agents, such as paclitaxel, have been shown to be stable in plasma for over 24 h when refrigerated at 4 °C and stable for over 2 years at −20 °C [15]. Others such as melphalan degrade rapidly at room or refrigerated temperatures and must be placed on ice immediately after collection, followed by storage at temperatures lower that −20 °C [16]. If utilizing published stability data, caution should be exercised to ensure that the testing was performed in the same matrix, since analytes may have very different stability in different matrices.

2.6 What Sample Volume Can or Should Be Obtained?

In the case of invasive sampling, such as blood collection, it is highly desirable to minimize the sample volume collected from the patient. In some cases, such as those studies in which a large number of serial collections are planned or in which very limited volumes can be collected as is the case with pediatric studies, minimizing sample volume collected per specimen is vital. However, the sensitivity levels of different analytical methodologies will dictate to a large extent the minimum sample volume required for accurate and sensitive measurement. If method development is initiated prior to the sample collection and the lower limit of quantitation is determined, it enables rational calculation of minimum sample collection volumes. It is typically preferred to collect whole blood volume of at least 7× the volume of plasma required for analysis. After centrifugation, this should provide enough plasma to allow for triplicate analysis.

3 Method Development

For new anticancer agents, many of the initial questions proposed above cannot be answered without the development of an analytical method. Therefore, the process will become an iterative one. A graphical representation of the entire process is shown in Fig. 1.

3.1 Instrumentation

Selecting the best technique for quantitative analysis is based on a number of factors, including the analyte, the sensitivity required, and the availability of instrumentation. For most small molecule anticancer drugs, LC-MS-MS (high-pressure or ultra-high-pressure liquid chromatography coupled

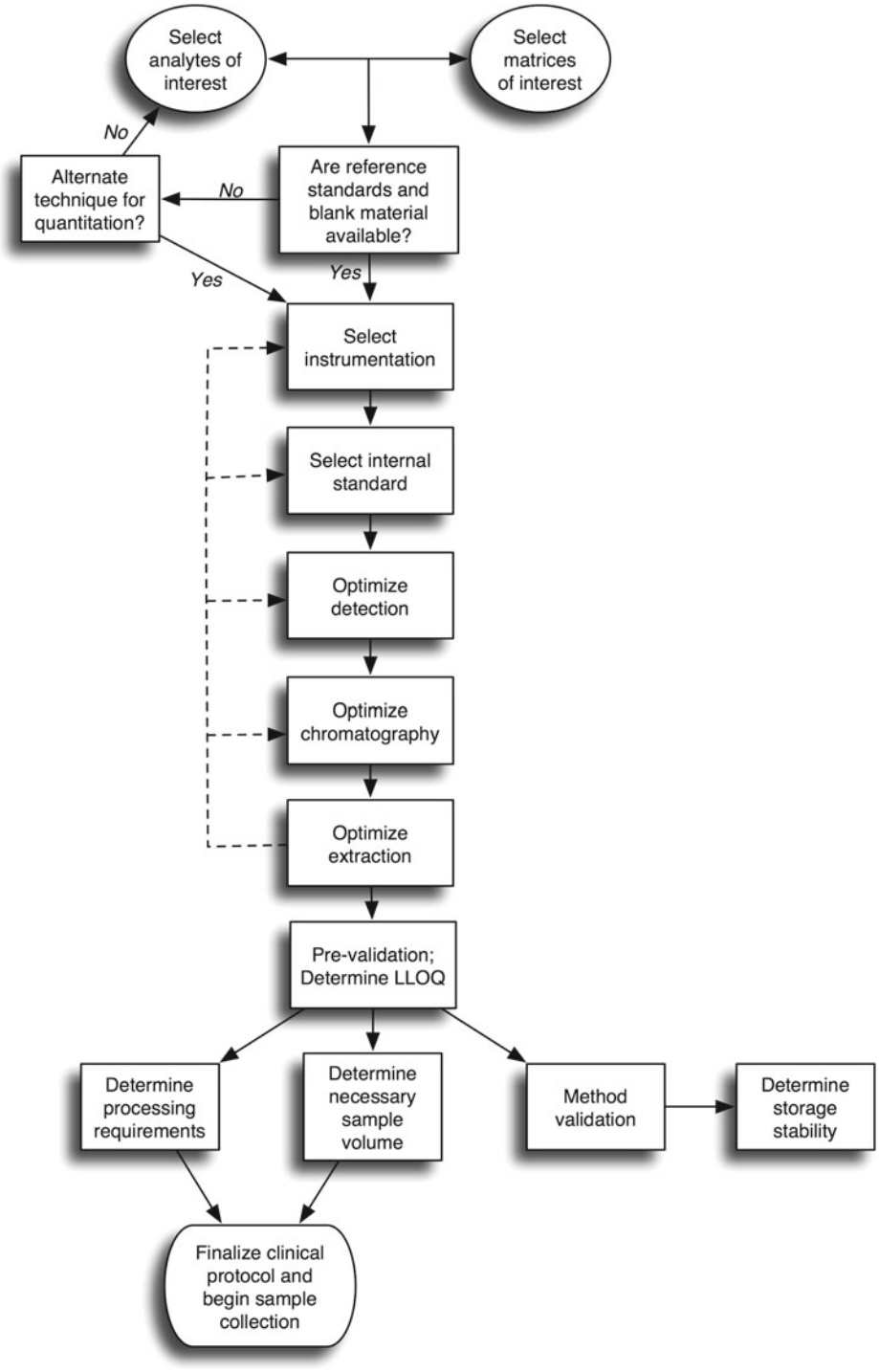

Fig. 1 Sample workflow for development of a quantitative analytical method for anticancer drug development, including clinical considerations. As shown, performing significant method development work prior to initiation of sample collection can greatly aid in decision making and ensure that concentration data generated from collected samples accurately represents the in vivo concentration

with a tandem mass spectrometric detector) provides the most sensitivity and selectivity, allowing for small sample volumes and low limits of detection. Selectivity is defined as the extent to which an assay method can determine an analyte in a complex matrix without interferences. However, due to the relative high cost of LC-MS-MS, this technology may not be available in many hospitals or academic labs. Access to high-pressure liquid chromatography with ultraviolet detection (HPLC with UV) is more widely available, but may not provide the sensitivity required, due to often limited selectivity.

The sensitivity required and hence analytical technique needed is highly dependent on the anticipated drug concentration in the samples to be collected. For a new small molecule anticancer agent being administered in a dose-escalation study, LC-MS-MS will often be chosen since it likely provides the best sensitivity, and therefore, ability to detect drug concentrations following low doses or in samples collected after significant time has elapsed since administration for accurate determination of pharmacokinetic parameters. Conversely, if there is a need to assess steady-state concentrations of an oral anticancer agent administered once or twice daily, HPLC-UV may provide ample sensitivity. If a molecule contains a fluorophore, HPLC with fluorescence (HPLC-FL) detection may also be a suitable option. In the case of irinotecan, published methodology employing HPLC-FL has reported comparable sensitivity to methodology using LC-MS-MS [17, 18]. However, there may be significant differences in sensitivity between techniques. Atomic absorption spectroscopy (AAS) is considered to be the standard method of analysis for platinum-containing agents such as cisplatin and carboplatin; however, newer instruments such as inductively coupled plasma mass spectrometry (ICP-MS) can provide approximately 4,000-fold greater sensitivity when analyzing tissue, with similar findings in plasma [19].

Comparison of selectivity and sensitivity can be illustrated using the example of imatinib. A simple method using HPLC-UV for estimation of plasma concentrations in patients receiving imatinib for treatment of chronic myeloid leukemia has a lower limit of quantitation (LLOQ) of 50 ng/mL using 100 μL of plasma [20]. This is based on detection at 265 nm. At concentrations less than 50 ng/mL, a peak cannot be quantified accurately, likely due to UV absorption by other concomitant medications, endogenous compounds, or simply components of the plasma matrix at the same wavelength. A subsequent assay achieved a lower limit of quantitation of 30 ng/mL [21], utilizing LC-MS, though this method required 200 μL of plasma. Employing LC-MS-MS, another assay was able to achieve a LLOQ of 10 ng/mL using 200 μL of plasma, threefold lower than the LC-MS assay described above [22]. With LC-MS-MS, selectivity is increased further by limiting detection to only those ions with the same molecular weight that also form the same daughter ion fragments as imatinib after controlled collision. Though these increasingly lower limits of detection are essential when evaluating terminal phase pharmacokinetics, such as elimination half-life, HPLC with UV detection has been shown to be accurate and sufficiently sensitive for monitoring of steady-state trough concentrations of imatinib [23].

3.2 Internal Standard Selection

Quantitative analytical methods typically employ an internal standard which can be spiked at a known concentration into all samples during processing, prior to analysis. The use of an internal standard can significantly improve accuracy and precision by minimizing any effects of the matrix, a shift in conditions during the analysis, or imprecise injection. However, it is essential that the internal standard be chosen rationally. The ideal internal standard (IS) is a compound which has very similar, but not exact, chemical properties. For methods employing chromatographic separation prior to detection, this ensures that the internal standard and the analyte(s) of interest will display similar:

- Extraction from the matrix
- Chromatographic retention
- Stability in the extraction/injection solvent

In the case of LC-MS-MS, this is often accomplished with the use of stable isotopes. d_5-paclitaxel or ^{13}C-paclitaxel can be used as an internal standard when analyzing paclitaxel. Both exhibit the same extraction, retention, and stability as unlabeled paclitaxel but are differentiated based on the difference in molecular mass of both the parent and daughter ions.

If a stable-labeled version of the compound is unavailable or a technique other than LC-MS-MS is being employed, it is often necessary to find a commercially available compound that meets the above criteria for an internal standard. The selected compound should not be something that occurs endogenously or could be coadministered to patients, in order to avoid interferences.

3.3 Optimization

The process of developing an analytical method typically occurs in the reverse order from the steps performed in the actual analysis of samples or standards. In the case of developing a method employing any of the "hyphenated" chromatographic techniques such as HPLC-UV or LC-MS-MS, development is often best achieved in the following order.

3.3.1 Detection

The mass spectrometer or other detection method is optimized for signal of compounds of interest. This step may involve testing multiple methods of detection to evaluate which provides the best sensitivity. This may include testing UV, FL, and MS-MS or may be limited to a single technology. In the case of MS or MS-MS, it is often useful to evaluate more than one ionization mode, for example, atmospheric-pressure chemical ionization (APCI) and electrospray ionization (ESI), in both positive and negative mode. It may be possible to limit the necessary testing based on rational evaluation of the chemical structures of the analytes. Software included with many modern MS instruments can aid in optimization, changing a series of parameters in a stepwise manner to determine the best conditions for maximum sensitivity and specificity. Though optimization of the internal standard is also performed at this point, it is secondary to analyte optimization. Optimization of detector conditions for UV detection is often much simpler, relying on identification of the maximal UV absorption wavelength for each compound and ensuring that mobile phase solvents will not interfere at the selected wavelength(s).

Optimization of conditions for MS-MS typically involves significantly more parameter tuning than optimization of simpler detection techniques such as UV. However, the specificity of MS-MS typically results in much simpler method development overall, with shorter run times and less optimization of chromatography and sample preparation required.

3.3.2 Chromatography

All of the hyphenated techniques mentioned thus far rely on reversed-phase liquid chromatography (RPLC) for separation of the individual analytes. Method development using RPLC becomes increasingly challenging as the number of compounds in the sample mixture increases for environmental samples or drug screening. In anticancer drug development, the number of analytes is typically very manageable—parent drug, several metabolites, and an internal standard. The required resolution, or degree of separation between the analyte peaks, is greater with less specific techniques. The selectivity of MS-MS allows for accurate detection of both parent drug and a stable-labeled internal standard

which elute at the exact same retention time. As such, in progressing from HPLC-UV to LC-MS to LC-MS-MS and UPLC-MS-MS, shorter, narrower columns can be used, with significantly decreased solvent usage and run time.

Column Selection

Reversed-phase liquid chromatography requires a nonpolar stationary phase to be used with aqueous, polar mobile phase. The vast majority of published assays for anticancer drugs employ columns packed with silica modified with alkyl groups (C_{18} or C_8). Additional options are also available, including silica with bound phenyl or cyano groups. Rational column selection based on the chemical structures of the analytes of interest and the expected interaction with each bound functional group can significantly improve resolution and decrease time spent on optimization of chromatography. Some instruments include multicolumn compartments with column-switching valves. These allow for semiautomated testing of a range of columns with static or changing conditions to quickly identify the best column for the application.

Mobile Phase Selection

Selection of the aqueous mobile phase must occur simultaneously with column selection, since the interaction of the analytes with both the mobile and stationary phases will determine the retention. The mobile phase is comprised of an organic component (typically methanol or acetonitrile), along with an aqueous component (often an acidic buffer). Optimization of mobile phase selection is focused on maximizing signal and resolution of the analytes while minimizing run time. Elution can be performed with constant proportions of aqueous and organic solvents (isocratic) or with a gradient, where the proportion of organic solvent increases over time.

3.3.3 Sample Preparation

Following optimization of detection and chromatography, testing of sample preparation often begins. Sample preparation is highly dependent on the matrix of interest as well as the analytical technique being employed. It is performed in order to remove numerous endogenous compounds that are contained within the matrix. Common sample preparation techniques for liquid matrices include the following:

- Dilution: Often described as “dilute-and-shoot,” for use in urine analyses.
- Protein precipitation: Often described as “crash-and-shoot,” using acetonitrile or methanol to denature and precipitate the plasma proteins. This is followed by centrifugation or filtration to remove the solids.
- Liquid–liquid extraction (LLE): A solvent or solvent mixture is chosen in which the drug is soluble and added to the specimen. Following mixing, the drug will partition into the solvent layer, leaving many impurities in the aqueous layer. The solvent layer is then evaporated, and the sample reconstituted prior to injection.
- Solid-phase extraction (SPE): Sample is loaded onto a preconditioned extraction cartridge which contains a chromatographic sorbent. This is followed by several washes to remove unwanted components. SPE can be expensive, time consuming without automation, and result in decreased recovery; however, SPE can also result in the cleanest sample, with lowest background.

Sample preparation for tissues involves homogenization, followed by one of the techniques listed above.

In general, the more specific the detection method, the less sample preparation is inherently required; however, more extensive sample preparation can improve detection limits. As such, one must carefully consider sensitivity requirements against cost and workload involved in sample preparation, based on available technology in the laboratory. Large analytical laboratories often have fully automated liquid-handling robots, which can substantially decrease processing time and increase accuracy of SPE.

Typically, all method development work prior to sample preparation has been performed with pure drug diluted into mobile phase. However, the introduction of the matrix with associated matrix effects and endogenous compounds may require reevaluation of the chromatography to optimize selectivity and sensitivity.

3.3.4 Pre-validation Studies and Method Revision

Following optimization of sample preparation, chromatography, and detection, initial calibrator samples (reference standard spiked into blank matrix) can be prepared. These should encompass a wider range of concentrations than is necessary in the final method, in order to determine the linear range of the assay, the limits of detection, and the limits of quantitation. All of this work is considered to be pre-validation, and it is still likely that additional revision of the method will need to take place once samples across a range of concentrations are tested and associated problems are identified. Method validation is discussed in chapter "Validation and Control of Bioanalytical Methods" by Karnes and Shah.

4 Conclusion

Development of quantitative analytical methods can be a complex process but is greatly simplified by rational, stepwise optimization. To ensure that the resulting data can be relied upon for scientific and clinical decision making, it is essential that analytes, matrices, stability, processing, and collection volumes be considered prior to initiating a study. Close collaboration between analytical chemists, pharmacokineticists, and clinicians provides the ideal setting for these decisions to be made.

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Validation and Control of Bioanalytical Methods

H. Thomas Karnes and Kumar A. Shah

Abstract The results of toxicokinetic, pharmacokinetic, and bioequivalence studies are used to make critical decisions regarding the safety and efficacy of anticancer drug substances. Therefore, measurement of anticancer drug concentrations in biological matrices is an important aspect in the development of these products. Such data are required by regulating agencies to support new drug applications as well as for line extensions and generic products of these drugs. It is therefore most essential to adequately characterize and fully validate the applied bioanalytical methods used in the determination of this class of compounds to ensure that they function in the manner in which they are intended. Since the release of the FDA prescribed Guidance for Industry in Bioanalytical Method Validation in May 2001, it is much clearer what is required for method validation. There are however a number of areas that are still not well developed in the FDA guidance, and the recently proposed draft European Medical Agency guidance addresses some of these. Apart from discussing acceptance criteria on the primary matrices required to determine bioanalytical assay suitability such as accuracy, precision, and selectivity, the draft guidance proposes additional criteria for other important aspects such stability tests, matrix effects, cross validation, and incurred sample reanalysis. The current chapter provides an overview of the current scientific approaches based on the literature while considering them in the context of these guidances in this highly regulated area.

Keywords Bioanalysis • Method validation • Acceptance criteria • Quality control

1 Introduction

Validation and control of bioanalytical methods as practiced in United States Food and Drug Administration (FDA) regulated drug development studies is the approach most often used for anticancer drugs. The discipline has progressed from one which was in its infancy a decade ago to a

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largely mature endeavor more recently. Validation and control procedures in other areas of bioanalysis such as clinical chemistry and forensic toxicology have been largely consistent for a number of decades. The primary difference between the drug development discipline and other areas of bioanalysis is the fact that drug development requires application of consistent standards for analytical methods that are investigational than routine. Validation and control attempts in drug development studies carried out prior to 1990 were the result of individual policies that varied a great deal from company to company. The importance of consistent procedures for validation and control in drug development was first outlined by Shah in 1987 [1] and specific procedures were proposed by Karnes et al. in 1991 [2]. Since these two works on the subject, there have been a number of reviews and research articles published along with several conferences that have led to the establishment of a "Guidance for Industry" on Bioanalytical Method Validation [3]. The first conference was held in 1990 with the results published in 1992 [4]. A draft guidance was also published as a result of this conference in 1999 [5]. Following an acknowledgement that small molecules should be treated differently than large molecules, two more conferences were held in 2000 and published in 2000 and 2001 for small and large molecules, respectively [6, 7]. All of this activity resulted in the final guidance which was approved by the Center for Drug Evaluation and Research (CDER) of FDA in cooperation with the Center for Veterinary Medicine (CVM) and published in May of 2001 [3]. The guidance has regulatory implications for a variety of biological matrices analyzed in human and animal clinical and preclinical studies. The document applies to chromatographic, spectrometric, immunological, and microbiological procedures and was intended as a nonbinding general recommendation which can be adjusted depending on circumstances. The document outlines fundamental parameters for bioanalytical method validation which include accuracy, precision, selectivity, sensitivity, reproducibility, and stability. The document addresses situations in which a bioanalytical method may be modified and suggests different levels of validation to ensure that validity is maintained. The document also includes a glossary of terms. Although this document represents the current thinking of the FDA and is based on the conferences held, the procedures and criteria were primarily negotiated. They were based on an amalgam of procedures that existed within the industry prior to the conferences and are not necessarily based on the best scientific approach. Since the FDA guidance has been issued, there have been several publications which challenge the validity of some of the approaches used and additional conferences have been held to address issues that were not addressed in the guidance. As a result of this and in an effort to extend and clarify the FDA guidance, the European Medical Agency (EMA), Committee for Medicinal Products for Human Use (CHMP) recently issued a draft guideline on validation of bioanalytical methods that is currently posted for comment [8]. This draft guidance goes further than the FDA guidance in terms of specific recommendations while implying more flexibility. The numerical acceptance criteria in the draft guidance are the same as the FDA guidance for selectivity, calibration standards, accuracy, precision, and analytical batch acceptance. There are additional criteria proposed for stability tests, matrix effects, cross validation, and incurred sample reanalysis. The EMA draft guidance has been discussed at a number of conferences and is certain to be a popular topic in the coming year. It has sparked a renewed interest, with note on the part of FDA, in international harmonization of such guidances. This chapter will present scientific approaches based on the literature while considering them in the context of these guidances, particularly the FDA guidance since it is finally approved.

There are two major divisions in the endeavor to ensure the quality of analytical results. These two divisions consist of method validation and method quality control. These are referred to as prestudy validation and during-study validation, respectively, in the draft FDA guidance [5] but no nominal distinction is made in the final FDA guidance [3]. Another division that is used often in describing validation and control processes is a method development or establishment phase in which the method is not yet complete but some validation results may be collected in an effort to establish optimal conditions. As such, the method development or establishment phase should be free from regulatory scrutiny for the most part since the method is dynamic at this point. The validation phase represents the stage

at which a method is complete but has not yet been used for analysis of "real samples." The question to be addressed at this phase is whether or not the method is good enough for an intended purpose. It could be argued that the criteria used here should be flexible so that methods used for critical purposes such as therapeutic monitoring of a narrow therapeutic index drug would require strict and tight guidelines, whereas other situations may not require such rigorous criteria. The approach of the FDA recommendations has been to apply a "one size fits all" approach without built-in flexibility for a large variety of drug types and for a large number of different applications. The FDA guidance makes no distinction between the method development/establishment and validation phases. The quality control phase represents the period in which data is collected from quality control samples and exists to ensure the quality of "real sample" results. The question to be addressed in this phase is no longer related to how good a method is but to determine whether the method is performing according to specifications set during method validation. The procedures used for these three phases should reflect the goals to be achieved. The FDA guidance does this for the most part but fails to address some valid scientific issues related to these goals in some cases. The following sections will present approaches suggested in the guidance along with scientific justifications when appropriate. Other approaches will be presented as alternatives to the guidance that may have more scientific validity or better address the individual goals of method development, validation, and quality control.

2 Method Development

Two important factors in achieving good performance of bioanalytical methods in the method development phase are selective recovery from sample processing and calibration with appropriate primary standards. Selective recovery for a bioanalytical method refers to the provision of an analytical response for the entire amount of analyte contained in a sample without residual interferences or matrix effects from other sample components [2]. Although selectivity must be dealt with in method development from the standpoint of achievement of selectivity, this is largely a validation parameter and will be dealt with in that section. Recovery of a bioanalytical method most appropriately refers to analyte extraction efficiency and is termed absolute recovery. Absolute recovery may be measured in a number of ways and is calculated using the general formula below:

$$\frac{Extracted\ response}{Unextracted\ response} \times 100 = \%\ Recovery.$$

The extracted response is the quantitative instrumental measurement from a sample, spiked at a known concentration, into a blank matrix sample that is processed and measured. The unextracted sample may be represented by a number of response values depending on the particular situation. The simplest experiment is to measure the unextracted response from a nonmatrix solvent solution spiked at the same concentration. This provides absolute recovery although the value may not be representative due to residual matrix effects in the extracted sample or poor reproducibility of the instrument response. Matrix effects can be compensated for by adding an appropriate amount of analyte to an extracted blank matrix then measuring the unextracted response in the presence of the blank extract. Instrument response variability can be lessened by addition of an internal standard to both the extracted sample following the extraction process and at the same concentration to the unextracted sample. The measured response then becomes the response ratio of the analyte to that of the internal standard. Absolute recovery can also be easily estimated if radioactive analogues of the drug are available. In this experiment, radioactivity counts prior to extraction provide the unextracted response whereas the radioactivity counts following extraction from the same spiked sample provide the extracted response. This procedure eliminates intersample variability and the possibility of a matrix effect with

an isotopically labeled analogue is remote. Sufficient replication needs to be employed to provide sufficient confidence in the calculated recovery and the more variable measurements (typically the extracted samples) require greater replication then the less variable measurements (typically the unextracted samples).

There are a number of experiments that have been referred to as recovery experiments that do not provide absolute recovery or an estimate of sample processing efficiency. They include experiments evaluating the measured response ratio of a sample extracted from the intended matrix to that extracted from a nonmatrix solution. This experiment provides information on the effect of components of the matrix on the measured signal and is an important experiment to evaluate method selectivity but should not be confused with an experiment to measure absolute recovery. Another experiment that has been reported as a recovery experiment is the ratio of the assayed concentration to that of the prepared concentration. This is an accuracy experiment and again does not address recovery as is intended in the FDA guidance. One last example of an experiment that may be reported as recovery but does not address processing efficiency is the ratio of the internal standard compensated response which has been extracted to the corresponding response unextracted, provided the internal standard is added prior to processing. This experiment will evaluate how well the internal standard is functioning but again provides no information on sample processing efficiency. The FDA guidance defines recovery as specifically pertaining to absolute recovery experiments that indicate extraction efficiency. The guidance suggests that recovery experiments should be conducted but that recovery need not be 100 % [3].

For chromatographic methods, another question to be addressed pertains to the use of an internal standard. As mentioned above the use of an internal standard involves adding a structural or isotopic analogue to a sample prior to processing so that errors in sample processing can be corrected for by including a ratio of the response of the analyte to that of the analogue. It has been noted by a few authors that the use of an internal standard is not necessary in many cases [9] or can actually lead to a degradation of analytical results in the absence of systematic errors [10]. Method degradation from the use of an internal standard will occur if the following is true:

$$RSDb > r\,RSDa,$$

where RSDb and RSDa are the relative standard deviations of the internal standard and analyte responses, respectively, and r represents the correlation coefficient for the responses of the analyte versus the internal standard. This relationship was derived mathematically and proven with experimental data by Haefelfinger [10]. Even though there are good arguments for not using an internal standard for chromatographic procedures, they are based on random and not systematic error. It is well accepted that internal standards are essential for correcting technical systematic errors such as loss of sample due to variable phase transfers or dilutions and allow for many volume transfers to be nonquantitative, thus increasing sample throughput. Correction of errors or shifts related to partition, chemical reactivity, and detector stability will depend on the characteristics of the internal standard relative to the analyte, and the closer the chemical and physical properties of the analyte and internal standard are, the greater the probability that these errors will be accurately corrected for. Internal standards that are isotopes of the analyte have become popular for this reason although a mass detector is required to discriminate between responses. The FDA guidance does not specifically require the use of an internal standard but it is generally expected for chromatographic procedures. Care must be taken, however, not to use an internal standard that is chemically inappropriate simply to address this expectation or the quality of results could suffer.

Calibration of an analytical method is an important consideration in method development. The concentration range for calibration must be established and an appropriate model applied to the data which will allow accurate calculation of unknown sample concentrations. The lower limit of calibration is usually established through a consideration of the lower limit of quantitation (LLOQ) and the point at which the data no longer fits the calibration model determines the upper limit. Practical considerations

such as the concentration range expected for samples are also employed in setting up the calibration range. The choice of a calibration model should be determined by experimental concentration versus response data and the model that best fits the data should be used. The FDA guidance suggests that the simplest model that adequately describes the relationship be used, thus indicating a bias toward the linear model, but use of nonlinear functions is not prohibited. Determination of the appropriate range for calibration and application of the most appropriate model requires a consideration of the quality of fit of the experimental data and is intimately related to method validation which is covered in the following section.

3 Method Validation

3.1 Calibration

Method calibration is the crossover point between method development and method validation since it involves both setting up procedures and also showing that they work well enough for a stated purpose. The quality of fit of the data to the selected calibration model will determine the allowable upper limit of calibration. This will be the highest concentration that will consistently provide an acceptable fit throughout the entire range of calibration. In order to establish the range and model for calibration, it is most helpful to evaluate residual errors and to use the model which provides the lowest residual error. For example, residual error for the linear model can be calculated as follows:

$$e_i = y_i - a - bx_i,$$

where e_i represents the residual error at a given concentration, y_i and x_i are the dependent and independent variables, respectively, and a and b are the best fit intercept and slope form of a linear regression of the entire calibration range. These residuals are often expressed as a concentration by "backfitting" individual calibration data. Although the FDA guidance has a bias in favor of the simplest calibration model (linear), it does not prevent use of nonlinear calibration models that may provide a better fit to the data and allow more accurate calculation of unknown values over a wider concentration range. If residual values are plotted versus concentration, a pattern as shown in Fig. 1a will result for homoscedastic data and the use of a nonweighted linear calibration model can be considered appropriate. If the residuals demonstrate heteroscedastic data in which there is a proportional increase in the

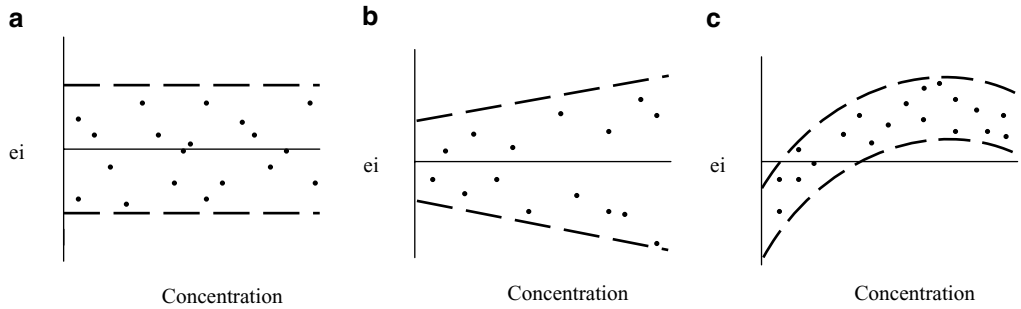

Fig. 1 Schematic representation of calibration residuals versus concentration. Homoscedastic data are represented in (**a**) whereas heteroscedastic data are represented in (**b**) and (**c**). Part (**b**) shows a proportional increase in the residuals that may be corrected by weighting. Part (**c**) represents a residuals pattern that is indicative of nonlinear data for which an alternate nonlinear calibration model should be used (*reproduced from* [11])

Table 1 Back-calculated standards (range and goodness of fit)

Mean deviation ($n=5$ %)			
Conc. (ng/ml)	Power fit	Weighted 1/conc.	Unweighted
10	5.15	5.98	18.55
20	3.40	3.40	6.06
50	1.78	1.82	1.70
100	2.23	4.04	3.26
250	0.99	0.34	0.23
500	3.26	4.31	3.32

Reproduced from [11]

residuals as concentration increases, represented in Fig. 1b, a weighted linear calibration is most appropriate. Fig. 1c is representative of a residuals pattern that indicates the data to be nonlinear and the linear model is therefore inappropriate. Weighted linear calibration is carried out using a normal linear regression modified to include a weighting factor as a multiplier when calculating the sum of squared residuals [12]. The most appropriate weighting factor is the inverse of the variance at each concentration. However, since this variance has been shown to be proportional to concentration, the inverse of concentration squared or simply the inverse of concentration can be used. These weighting factors can be used on a trial basis to determine which factor provides the lowest residual error throughout the concentration range. If a pattern of residuals emerges which is similar to the heteroscedastic pattern shown in Fig. 1c, then a systematic departure from the model is indicated and an alternative to the linear model such as a power or a polynomial fit should be investigated. Caution should be used in attempts to force truly nonlinear data to a linear calibration model in response to the FDA bias. Table 1 shows concentration residual data for linear, weighted linear, and a nonlinear power fit of real bioanalytical data. It can be seen from Table 1 that the linear calibration provides unacceptably high residuals at low concentration. These residuals are improved significantly by use of the weighted linear model. The function of the weighting factor is to increase the influence of the low-concentration data on the best fit regression slope, and therefore, the low-concentration residuals are improved whereas the high-concentration residuals are made worse. This occurs because forcing the line closer at the low concentrations acts as a fulcrum to force the inflexible linear calibration line away from the data at high concentrations. The solution to this problem is to allow some flex in the calibration curve and to use a nonlinear calibration model which will provide a better fit at both extremes of calibration for such data.

There are many approaches to assessment of the quality of fit for analytical calibration data in addition to an evaluation of residuals. These include but are not limited to correlation coefficients, sensitivity plots, polynomial fits, log–log plots, and the *F*-test for lack of fit [13]. Sensitivity plots, polynomial fits, and log–log plots are limited to evaluation of the linear model and are not widely used in bioanalysis so they will not be addressed in this chapter. Log–log plots have been shown to provide comparable results to the *F*-test for lack of fit and residuals analysis whereas the polynomial fit approach was found to be more conservative [13]. For linear analytical data, calculation of the correlation coefficient involves the false statistical assumption that the independent variable in regression analysis (concentration) is errorless. The correlation coefficient is essentially a measure of the amount of variation in the dependent variable (analytical response) that is accounted for by the independent variable (concentration). It does not distinguish random from systematic error well. Also, with regard to testing the linear model, correlation coefficients have been shown to produce good correlation for data, which does not conform to the linear model [14], and have been shown to be more liberal criteria than other approaches [13]. For these reasons, the correlation coefficient has been de-emphasized as a method of evaluation for goodness of fit and is not mentioned in the final FDA guidance.

The *F*-test for lack of fit is a statistical test of whether or not the sum of the variances due to lack of fit (the differences between mean and fitted values for the analytical response at each calibrator

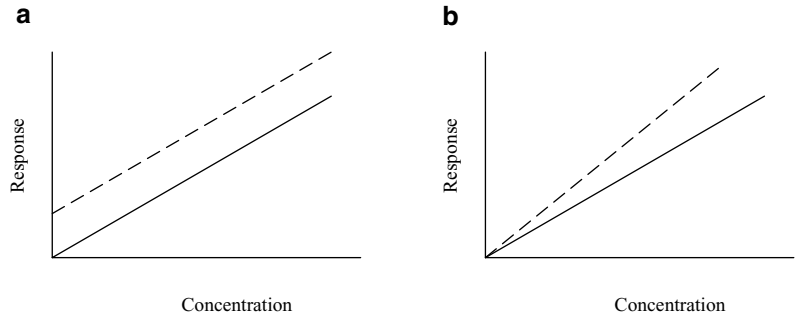

Fig. 2 Errors in selectivity caused by a predeterminant and constant shift in the calibration are referred to as interferences and are represented in (**a**). Matrix effects are proportional errors of slope as shown in (**b**). The *dotted lines* represent calibration with the error present and the *solid line* represents the unaffected calibration curve (*reproduced from* [11])

concentration) is significantly different from the sum of the variances due to pure error (the differences of individual calibrators from the mean at a given concentration). The *F*-test for lack of fit has been shown to provide comparable results to residuals analysis and log–log plots. Although the *F*-test for lack of fit is the most appropriate test of goodness of fit statistically and can be used to evaluate both linear and nonlinear models, replication is required to obtain statistical significance and the test may not be easily understood at all levels of bioanalytical practice. The only specific criteria for calibration goodness of fit to the model offered by the FDA guidance is a criteria applied to concentration residuals [3]. This criterion states that all concentration residuals must be within 20 % of the nominal value for the lower limit of quantitation and within 15 % at all other concentrations. This criterion is easy to understand and does not require deviation from a set protocol in order to achieve statistical significance. A criticism of this criterion is that it is based on consensus opinion and does not possess statistical foundation.

3.2 Selectivity

There has been confusion over the terms selectivity and specificity, and they are often used interchangeably. Specificity may be used appropriately to refer to an analytical method that provides a response for only a single analyte. The term has also been used appropriately to describe the absolute condition of selectivity. Selectivity is the more appropriate term for analytical purposes, as few if any analytical systems can be said to respond to only a single species without being affected by components of the matrix. In an analytical method in which concentration is determined as a function of response, the degree to which the response is unaffected by contributions from the matrix is referred to as the selectivity of the method. There are two independent components to selectivity referred to as matrix effects and interferences. Interferences are predeterminate errors caused most frequently in bioanalysis by a component of the matrix producing a measurable response. This causes an error in the intercept of the calibration curve, which is represented in Fig. 2a where the dotted line represents a calibration with the interference and the solid line represents the unaffected calibration curve. Interferences are best evaluated by analysis of the baseline from a blank measurement if a suitable blank exists. Interferences must be differentiated from contamination (a response from the intended analyte in the blank) by qualitative means when contamination is suspected. In biopharmaceutical analysis interferences are relatively easy to evaluate because the analyte is normally a xenobiotic and a blank is readily available for each biological source as a predose sample. Analysis of this predose

sample by the method demonstrating a lack of significant response indicates good selectivity. The FDA guidance states that a lack of interferences needs to be demonstrated in six independent sources of blank matrix and that there should be evidence that the substance being quantified is the intended analyte. The guidance defines a lack of interference as any response less than 20 % of the limit of quantitation response. However, it does not specify what evidence is needed for demonstration of qualitative identification of the analyte. Commonly used criteria for chromatographic procedures include a lack of interference of <5 % or 20 % of the limit of quantitation and a retention time match with a primary standard for qualitative identification.

The situations in which the predose blank matrix approach to interference evaluation does not prove adequate include instances in which interferences may appear over time due to lack of stability of the matrix or analyte, situations in which interferences are caused by metabolites of the drug that are not present in the predosed blank, and situations in which the analyte is an endogenous compound for which there is no predose sample. Stability issues are a separate concern and include a consideration of more than just the maintenance of the selectivity of the method with time under storage conditions and will be discussed later in this chapter. Interference from metabolites is a concern especially when using mass spectrometry (MS) as an analytical method since the source of ions for MS is a reaction chamber where metabolites and other analogues of the analyte, most notably internal standards, can be fragmented into ions that are the same as those that originate from the analyte [15]. This phenomenon is referred to as "cross-talk." For this reason, it is necessary for evaluation of interferences by use of primary standards of the individual metabolites or by modification of chromatographic conditions to effect a separation of the analogue form the analyte. Once the modified chromatographic conditions have been shown to produce no differences in measured response, the original chromatographic conditions may be used. This special precaution, which is important for MS, is less of a concern for other methods, as complete separation from components of the matrix is routinely carried out for these methods. No prevision for this extra concern regarding interferences with MS methods is included in the FDA guidance. It is recommended practice, however, to evaluate "cross-talk" at high levels for potentially interfering internal standards and metabolites along with conducting experiments at altered retention times in dosed (ex vivo) samples.

Matrix effects are proportional errors of slope as shown in Fig. 2b, where the dotted line represents a calibration with the matrix effect and the solid line represents the unaffected calibration curve. Unlike interferences that are caused most often by components from the matrix that yield a response, matrix effects are generally caused by some interaction, either chemical or physical, of the analyte with some component of the matrix. An example of this is shown below for charge transfer and proton transfer reactions that occur in atmospheric pressure ionization (API) MS:

$$\textit{Charge transfer} \quad \mathrm{A}^+ + \mathrm{M} \rightarrow \mathrm{M}^+ + \mathrm{A}$$

$$\textit{Proton transfer} \quad \mathit{AH}^+ + \mathrm{M} \rightarrow \mathit{MH}^+ + \mathrm{A},$$

where A^+ and AH^+ represent charged and protonated analyte molecules, respectively, and M^+ and MH^+ represent the corresponding species for a matrix component. A and M represent the uncharged, unprotonated forms and negative charges may be involved as well as the positive charges pictured. These reactions both lead to ion suppression matrix effects which result in a decreased response for the analyte as compared to nonmatrix analysis. The proportional nature of the matrix effect error is a result of this type of interaction because the effect is mediated through an interaction constant and is proportional to concentration in the simplest case. If not compensated for or avoided, matrix effects may compromise the integrity of bioanalytical methods.

Matrix effects can often be compensated for by duplication of the sample matrix in calibration standards. This will adjust the slope in calibration standards to match that of the sample matrix. The assumption involved is that the blank matrix used for calibration standards is sufficiently similar to that of the samples to yield accurate results. This assumption is generally valid for most bioanalytical

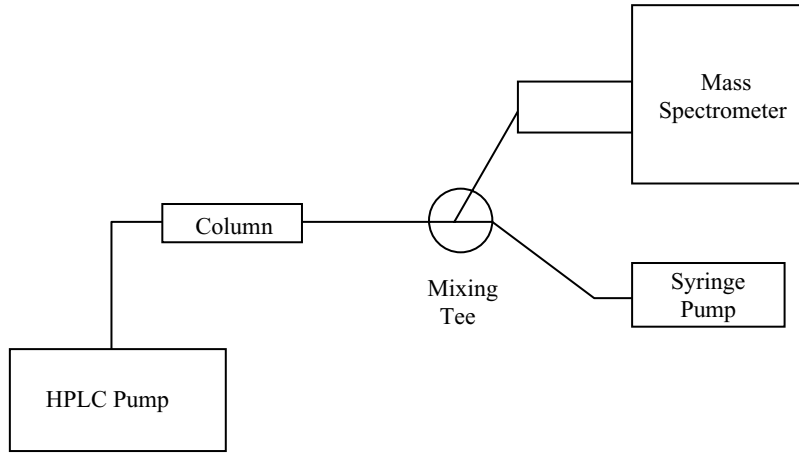

Fig. 3 A schematic representation of a post-column infusion hardware setup in which a steady infusion of the analyte is pumped into the column eluent. Blank matrix can be injected pre-column in order to monitor ion suppression or enhancement (*reproduced from* [11])

methods that involve an extraction and chromatographic separation, as the factors that may affect extractability and separation such as pH and protein content are relatively constant in biological samples from the same origin. MS methods again require special attention because quantitation is often carried out without complete extraction and separation of the analyte from matrix components. Although the FDA guidance does not specify any test of matrix effects, it is recommended practice for MS methods. It is advisable to use an isotopically labeled internal standard if possible to increase chromatographic retention times as a test to see if results change and to evaluate instrument response in a variety of sources of biological matrix. A very useful experiment for validation of the lack of ion suppression or enhancement in MS is the post-column infusion experiment in which a steady infusion of the analyte is pumped into the system post-column generating a steady response from the analyte (Fig. 3). In this configuration, blank matrix is injected pre-column and the ion suppression or enhancement appears as a negative or positive deflection of the baseline response. This negative or positive deflection (Fig. 4b) in the baseline (Fig. 4a) can be compared to where the analyte elutes if injected pre-column (Fig. 4c). The chromatography can then be modified so that the elution time of the analyte is not coincident with suppression or enhancement peaks. Another common approach to assess matrix effects is the post-extraction addition method. In contrast to the post-column infusion experiment that is a qualitative assessment of matrix effects, the post-extraction addition method provides a quantitative assessment of matrix effects by comparing the response of an analyte in a neat solution to the response of the analyte spiked into an extracted blank matrix sample. The ratio of the response in the presence of matrix to the response in the absence of matrix is often referred to as the matrix factor (MF) and the EMA draft guidance has proposed the MF for the analyte and the internal standard be no more than 15 % different. This proposed criterion is a relative measure of the effectiveness of the internal standard in correcting for matrix effects but does not measure the matrix effect alone. It is also important to look at the uncorrected matrix factor to determine whether or not acceptable signal to noise has been achieved.

Phospholipids are a prominent source of matrix effects in biological samples analyzed by MS methods. Monitoring of these matrix components during analysis may be advantageous in revealing their presence after sample extraction. Likewise, excipients such as polyethylene glycol or polysorbates contained in dosage formulations may also contribute to matrix effects and should also be evaluated in this respect. Assessment of relative matrix effects between different lots of biological samples is also essential. Although there is no current guidance set forth by the FDA to evaluate the impact that matrix effects may have, it may be a good practice to investigate matrix effects when using mass

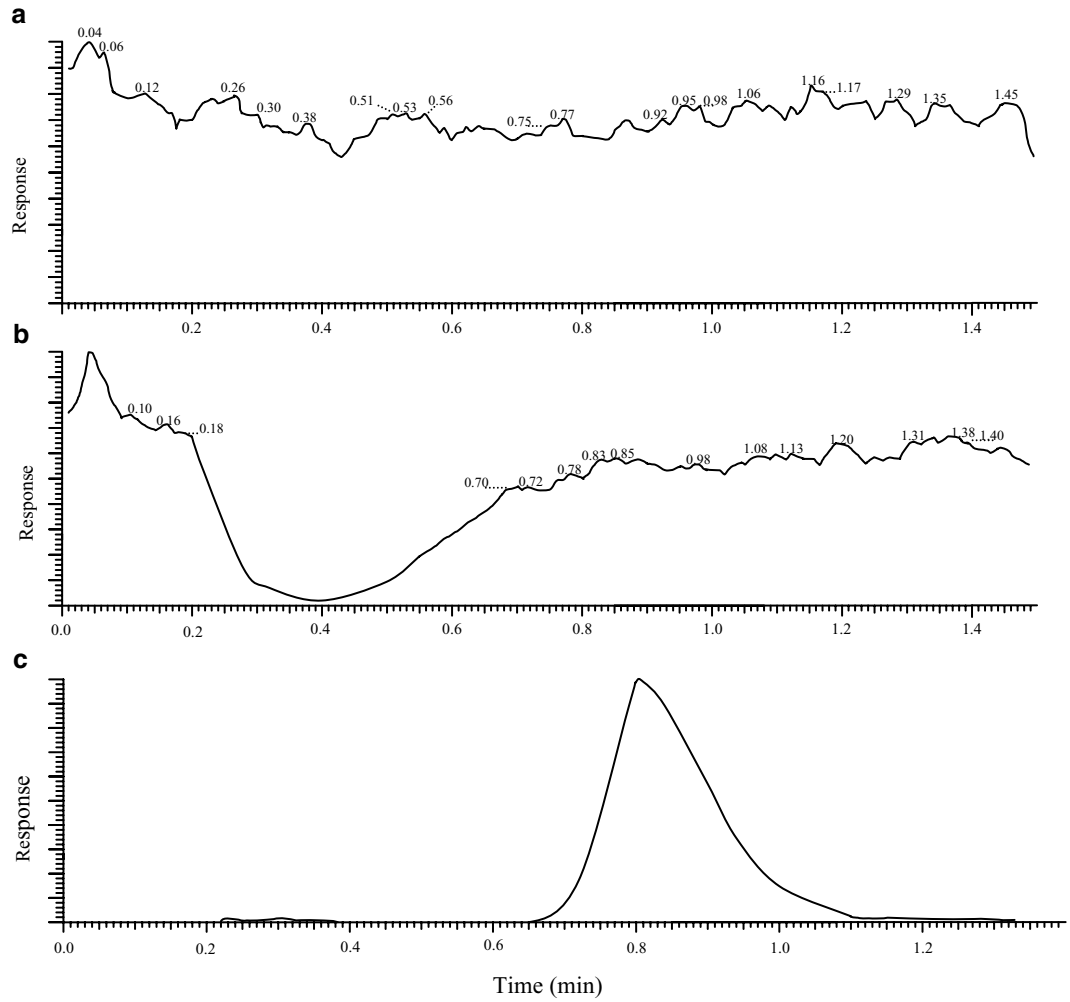

Fig. 4 The typical appearance of matrix effects in a post-column infusion experiment. Ion suppression or enhancement appears as negative (**b**) or positive (**c**) deflections in the baseline (**a**), respectively (*reproduced from* [11])

spectrometric methods, using at least six different lots of matrix just as in the case of interference testing. Individual lots may be fortified with the target analyte at or near the LLOQ level and internal standard at the level of use. In the case of MS methods, stable isotope-labeled internal standards minimize the influence of matrix effects most effectively since the matrix effects observed for stable isotope-labeled internal standard are generally similar to those observed for the matching analyte and should be used whenever possible and practical. Hemolyzed, hyperlipidemic, and sample matrices from special populations should also be included in matrix effect investigation if the method is to be used for these populations [16].

3.3 Detectability

Detectability has been one of the most broadly interpreted parameters of validation. The term detectability is used here because it more accurately reflects the parameters used for validation of the lowest

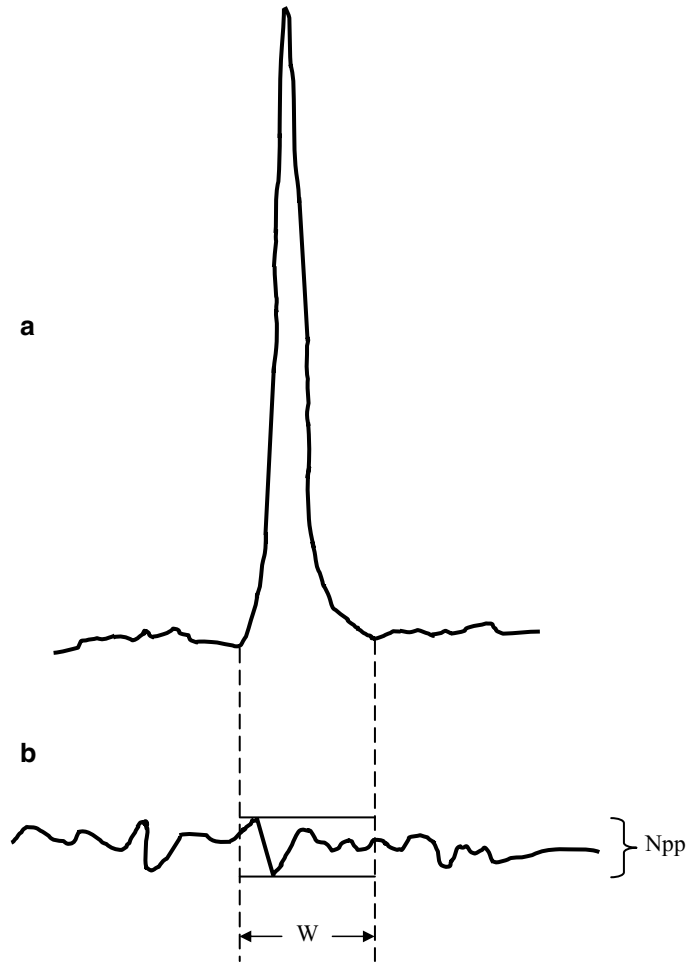

Fig. 5 A common way to calculate signal-to-noise ratios. Part (**a**) represents a chromatogram of standard material and (**b**) represents the baseline from a blank injection. Npp represents peak-to-peak noise and "w" indicates the elution window of the peak (*reproduced from* [11])

concentrations to be measured. It is often used for this purpose. The term sensitivity is used in the literature to indicate the slope of the analytical calibration curve, however, and is not defined in the FDA glossary of terms so it would seem inappropriate to use it to refer to detectability except in the most general sense. There are a number of different mathematical definitions for detectability that will yield different results and many of them are referred to by the same terminology [12]. Most often, detectability has been defined based on blank noise measurements. Valid statistical approaches have also been based on confidence limits associated with a calibration curve [17]. Discussion of detectability will be limited to the blank noise approach here because this approach is more accepted in FDA-regulated drug development. Blank noise can be defined a number of ways. For chromatographic methods this consists of measuring the biological analytical signal over the elution window of the peak of interest in a matrix blank sample. This is shown in Fig. 5, where Fig. 5a represents a chromatogram of standard material and Fig. 5b represents the baseline from a blank injection. The blank signal should be an average from a number of blank matrices. The most conservative estimates will be yielded by use of the peak-to-peak noise signal rather than the peak noise signal although both have been used. Root mean square (RMS) noise has also been used, although use of this noise estimate would provide a very liberal estimate of detectability relative to the others.

The blank noise approach involves multiplication of some factor (K) times the standard deviation of the blank noise to yield a confidence interval. The confidence interval then allows prediction of an

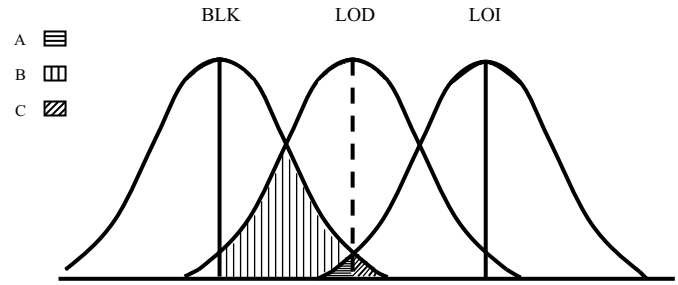

Fig. 6 A graphical representation of the probabilities related to various detectability parameters. Probability A represents the low probability of a measurement made at the limit of identification (LOI) actually being blank (BLK). Probability B represents the probability that a measurement made at the limit of detection (LOD) will be indistinguishable from a measurement made from a blank. Probability C represents the probability of a blank measurement yielding a response above the limit of detection (*reproduced from* [11])

error probability associated with making an incorrect decision of detection for the analyte [18]. This value is then divided by the slope of the calibration curve to yield results in concentration units as shown below:

$$\frac{KS_{\mathrm{b}}}{m} = X_{\mathrm{LLOD}},$$

where S_{b} represents the standard deviation of the blank noise measurement, m is the slope of the calibration curve near the limit, and X_{LLOD} is the lower limit of detection. This limit represents the concentration that can be distinguished as nonzero with great probability or with low probability of actually being a blank (probability A in Fig. 6). The K factor used determines width of the confidence interval and thus the probability of an incorrect decision, that is, the sample being measured as above the lower limit of detection (LLOD) but one that is truly blank. Typically used values for K are 2 and 3 representing error probabilities of 95.4 % and 99.7 %, respectively, 3 being the most conservative and most commonly used. The LLOD calculated in this way is not useful for the purpose of setting a parameter for quantification and should be used only to compare absolute detectability potentials of analytical systems. This is the case because an analytical measurement made at the LLOD would yield a high probability of being indistinguishable from a measurement made at zero. This is shown in Fig. 6 as the sum of probabilities A + B + C. To solve this problem, a new limit is defined as the lower limit of identification (LLOI) which uses a value for the K factor of 6 and defines the concentration at which there is a low probability (area C in Fig. 6) of being less than the defined LLOD. The LLOI has a practical meaning as the threshold for presence of an analyte in a sample. It can be said with a defined level of confidence that if the measured concentration is above the LLOI, then the analyte is present in a sample. The most useful application of this is in purity testing of chemical substances and dosage forms, but the parameter has little application in quantitative bioanalysis except for situations in which a method selectivity argument is made.

The most useful limit in quantitative bioanalysis is the LLOQ. The LLOQ represents the concentration above which accurate and precise quantification can be carried out. This limit can be traditionally estimated using the blank noise approach with a K factor of 10. This factor for K is intended to target a coefficient of variation (CV) of 10 %, in the absence of systematic errors. An appropriate K factor for a desired level of precision can be arrived at through the following expression:

$$K = \frac{1}{CV},$$

where CV is the coefficient of variation expressed as a decimal fraction. The expression above is derived through a combination of the general LLOD equation and the expression for CV. A *K* factor of 5 as recommended in the FDA guidance would therefore predict a CV of 20 % which is the precision limit at the LLOQ allowable in the FDA guidance [3]. The FDA guidance is therefore consistent with accepted theory although the approach does not account for the fact that this value is lower than the LLOI and is technically not present in the sample with great probability (99.7 %). A slightly more conservative approach would be to use a *K* factor of 6 or 10 as an estimate of the LLOQ.

LLOQ may be estimated with the blank noise approach but is established through actual testing of concentrations prepared at that level to ensure that precision and accuracy limits are met. The estimates provided by the blank noise approach should be used as a guide as to where to set concentration for evaluation but should not be used as evidence for validation. The best approach to establishment of the LLOQ is to prepare several concentrations near the LLOQ estimate and to measure them in replicate. The lowest concentration to yield the desired acceptable level of accuracy and precision would therefore represent the LLOQ and should be established as the lowest concentration in the calibration curve. The greater the number of concentrations tested, the better will be the estimate for LLOQ. In practice, it is inefficient to measure a large number of low concentrations with sufficient replication to establish the LLOQ, rigorously. Typically, the blank noise estimated LLOQ is used as a guide and a single LLOQ is tested. If the results of this test yield acceptable accuracy and precision (20 % systematic error and 20 % CV in the FDA guidance), then this single concentration is established as the LLOQ and used as the lowest calibrator. The question of whether or not there is a lower concentration that would yield acceptable results is often not addressed.

3.4 Accuracy and Precision

The accuracy and precision of an analytical method is by far the most important determinant of analytical method quality. The impact of these two parameters has already been discussed in establishment of the LLOQ and the acceptability of residuals in the evaluation of the quality of fit for calibration curves. They will be considered together here, as they are often lumped together to represent the total error of a measurement, and it is important to distinguish these three parameters. Accuracy and precision are interdependent in assessment of the acceptability of an analytical method. The FDA guidance defines accuracy as "the degree of closeness of the determined value to the nominal or known true value under prescribed conditions." The accuracy component of total error can be represented by the following:

$$\frac{u-\overline{x}}{u}\times 100 = \%\ accuracy\ deviation,$$

where u is the "true" or "nominal" value and $\overline{x}$ is the average of measured values. It is important to make sure that the average has been calculated from enough measurements to allow for an adequate reduction in the random (imprecision) error so that only the systematic (inaccuracy) error is represented. It is therefore incorrect to represent accuracy for an individual measurement. This concept is illustrated in Fig. 7, where the Gaussian distribution shown represents a distribution around a measured average which determines an analytical method's precision. The difference between this average and the true value is the accuracy as described mathematically above, and the sum of the two errors is referred to as total error or bias. Both accuracy and precision are traditionally evaluated through control samples prepared at various concentrations to reflect the range of expected values and should be measured independent of the calibration standards. The "true value" for these control samples can be established either through comparison of results to a reference method or through assigning a known concentration from spiking of weighed standards into blank matrix.

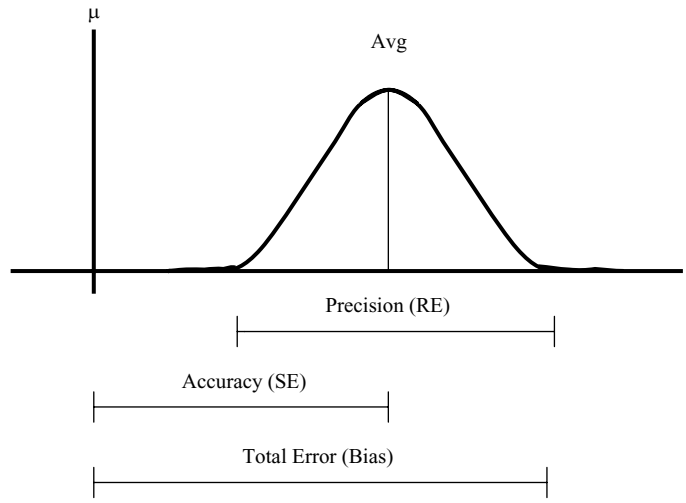

Fig. 7 The concept of total error. Imprecision or random error (RE) is related to the standard deviation of the set of measurements. The accuracy or systematic error (SE) of the measurements is shown as the difference between the measured average (AVG) and the true value (μ). Total error of bias is shown as the sum of systematic and random errors (*reproduced from* [11])

The imprecision or random error component of total error can be estimated from calculation of the percent RSD, which is also referred to as the CV. This value is calculated as follows:

$$\frac{sd}{\overline{x}} \times 100 = \%\, RSD,$$

where sd represents the standard deviation of a group of multiple measurements of the same sample and $\overline{x}$ again represents the calculated average from the same group. Precision is often confused with reproducibility and repeatability, although these terms have distinctly different meanings. Reproducibility is the closeness of results measured under different conditions, such as different laboratories, and repeatability refers to the closeness of results from successive measurements of the same sample [2]. Precision is normally assessed on a within-batch and a between-batch basis. The within-batch assessment being considered an estimate of the precision under optimal conditions without the variability associated with batch-to-batch results. It is for this reason that it is advisable to run unknown samples generated from the same subject but different legs of a clinical study in the same batch if possible. The between-batch assessment is a more realistic estimate of the precision of a method because it normally is subjected to a greater number of sources of variability.

The total error, or "bias" of an analytical measurement, is appropriately represented for individual measurements, and it is not appropriate, therefore, to apply an accuracy criterion to a single measurement [2, 19]. Any criterion for individual measurements should be referred to as a total error criterion. The criterion should be broad enough to include both the random and systematic error components of the total error. Total error for an individual measurement can be calculated as follows:

$$|x - u| + 2.58sd = E,$$

where x represents the individual measured value and E represents the total error or bias of that individual measurement. The random error component for the example above has been considered to be the width of a confidence interval specified at 99 %. The random error component of an analytical measurement is a characteristic of the method and the primary measure of the method's performance. Systematic errors are theoretically correctable errors and are related more to calibration and the purity of primary standards. It may be desirable to determine if the observed error is due to random error alone so that systematic errors can be corrected if they exist. This is accomplished through application

of a *t*-test to determine whether or not the average value of a set of measurements differs significantly from that of the true or nominal value [19]. If the difference is significant, there is some correctable systematic error.

The FDA lists accuracy and precision criteria for acceptance of a method for the purpose of bioanalytical data submissions [3]. It is recommended that four control concentrations spanning the range of calibration should be employed, one of which is no more than three times the LLOQ and one prepared at the LLOQ. The percent accuracy deviation and percent RSD must be within 15 % for all controls except for the LLOQ, which is expected to be within 20 % RSD and accuracy. This absolute approach taken by the FDA guidance is generally appropriate for validation of analytical methods, as the question addressed in validation is whether or not the method is good enough for the intended purpose. The fixed acceptance criterion for bioanalytical methods suggests that all data be at least as good as a minimal threshold value and that all methods should conform to this threshold. It could be argued that some methods that are very precise by nature or that are measuring relatively high concentrations should be held to a higher standard or conversely that very challenging methods should be given more flexibility. It also follows that certain applications such as very narrow therapeutic window drugs might require tighter control whereas other applications may not. These situations may be compensated for in application of the guidelines although deviations from the fixed recommendations will no doubt require rigorous scientific justification. If it is simply not possible for a given analytical method at a needed concentration to provide results within the fixed guidelines, the most straightforward solution to the problem is to conduct sample analysis and validation in replicate. The RSD of a method can be effectively reduced by a factor of $1/\sqrt{n}$ where n represents the number of replicates assayed. In this way, the variability of both the sample measurement and the validation data will be lowered through use of an average value rather than the single individual measurement. This approach is recommended in the FDA guidance. In cases where inaccuracy exceeds the threshold value, use of the average of replicate measurements may provide a better estimate of the true value but will not necessarily reduce inaccuracy. The systematic error may also be corrected through better calibration or standardization of the method or by limiting calibration to a smaller concentration range.

3.5 Stability Testing

Stability of an analyte in the matrix in question is an important part of the validation process. The stability of an analyte is not only a function of the chemical nature of the substance itself but of the matrix and container in which it is stored. Instability can result from both chemical and physical processes and the most accepted way to show stability is to monitor the concentration of the analyte in question over a time period and under conditions set to reflect the handling of unknown samples. Although the FDA guidance suggests that stability should be evaluated during the sample collection process, there are few analytical laboratories that have control over this process. Stability studies that are normally carried out by analytical laboratories include:

1. Freeze/thaw stability testing in which three control samples are frozen at the storage temperature and thawed and refrozen a total of three times. The sample is then analyzed after the third cycle and the results are compared to results measured prior to the freezing cycles.
2. Short-term stability in which controls are stored at room temperature from 4 to 24 h (based on the expected time samples will be kept at room temperature) and then analyzed and compared to results from samples not left at room temperature.
3. Long-term storage stability in which samples are stored under long-term storage conditions and analyzed after a time expected to be the longest storage time for samples. Results measured from freshly prepared controls are compared to the results from the stored samples to indicate storage stability or instability.

4. Stock solution stability in which standard solutions in the appropriate solvent and container are analyzed before and after a minimum of 6 h of storage or the longest time expected for stock solution storage. The storage conditions should replicate that which is employed for normal conditions. The instrument responses of these samples should then be compared to those of freshly prepared solutions.
5. Prepared sample stability in which processed samples stored under the conditions needed for analysis such as on an autosampler tray are analyzed and compared to results obtained from samples not stored for the indicated period of time.

All of these stability studies involve comparison of results from stored samples to those freshly prepared or unstored. The guidance suggests that samples from dosed subjects may also be investigated for stability but stop short of requiring that this type of sample be tested. There is no acceptance criterion established in the FDA guidance although it is suggested that a statistical approach based on confidence limits may be employed. The FDA guidance does not prevent employment of combined stability studies and these may make sense in terms of preserving laboratory efficiency. For example, samples could be analyzed that not only have been frozen and thawed three times but have also been kept at room temperature and left prepared on an autosampler for a designated amount of time. In this way three stability studies are combined into one, and if stability is indicated, nothing further needs to be done. If instability is indicated, however, individual studies should be carried out to determine the source of the instability.

In contrast to procedures for determination of accuracy and precision, the FDA guidance offers little information as to how to conduct stability studies. General principles for stability tests of drug products have been established and would apply to bioanalytical studies as well [20]. These include the following:

1. The method used for stability testing must be shown to be stability indicating. For bioanalytical methods, this should consist of making sure that degradation products, either known or created through forced degradation, do not interfere with quantitation of the analyte.
2. The time zero reference should be a measured (not nominal) value which has been rigorously established with sufficient replication.
3. Sufficient replication of the timed stability samples needs to be carried out to provide a reliable mean measured result.
4. The entire concentration range in question should be investigated since significant differences in rated of degradation can occur at different concentrations.
5. Blank matrix samples should be run in conjunction with stability samples to ensure the absence of interferences that may appear with time.
6. Freshly prepared and matrix-matched samples should be used.

The decision as to whether or not to conclude stability or instability should be based on the precision of the method and the acceptance criterion for validation. The EMA draft guidance proposes a 15 % acceptance criterion for stability studies based on nominal concentrations in biological matrices. This criterion is too small in comparison to the accuracy criterion, which is also 15 %, and it is incorrect to use nominal concentrations for comparison here. The assayed mean concentrations should be used. Otherwise if a method is inaccurate by −10 %, then the stability criterion on the minus side becomes 5 % and on the plus side 25 %, which is not a desirable situation. This problem could be solved in part by use of the assayed mean concentrations, but with the 15 % acceptance criterion, the true stability criterion is 0 % since there is no allowance over and above the accuracy acceptance criterion. As the FDA guidance states that a method should be accurate to within 15 %, it would follow that an acceptance criterion for stability should be of the same magnitude. This would result in a threshold value that would be consistent with the accuracy criterion. Although as stated above, stability studies are more appropriately based on measured mean reference values rather than nominal

values. If a criterion of 15 % is to be applied to stability testing in matrix, then it should be measured relative to the mean time zero reference. A more statistically valid approach would be to compare the measured stability values via a confidence interval approach to allow for variability. This may involve the upper limit of the confidence interval of stability measurements being not less than some lower acceptable limit (usually 90 % of the reference) or that the lower limit of the confidence interval be not greater than some higher acceptable limit (usually 110 % of the reference). This approach ensures that a method does not fail the stability test unless there is a high level of confidence that the sample mean is outside the acceptance range. The level of confidence or probability that the sample is actually unstable when you have concluded that it is not is determined by the size of the confidence interval chosen. Confidence intervals of 90 or 95 % have both been used for this purpose.

4 Quality Control

The goal of a good quality control program is to determine whether or not a method is performing up to specifications during the process of analyzing unknown samples. This is in contrast to the goal of validation, in which it is desired to show that a method is good enough for an intended purpose. For this reason, it is necessary to view the quality control process as more of a relative criterion than an absolute one. Relative assessment of quality control data from a sample run can be efficiently carried out employing the use of quality control charts [21]. The control chart concept involves setting up an acceptance criterion based on the mean of quality control measurements at a given concentration plus and minus some factor, related to the desired level of confidence, times the standard deviation of the quality control measurements. For example, an acceptance range of 12.06–18.34 would result from a mean of 15.2 with a standard deviation of 1.22 if the level of confidence chosen were 99 % (a factor of 2.58). The mean and standard deviation are established with the control results themselves as collected or based on validation data collected prior to the sample run. They can be updated with new data as quality control runs are carried out. The precision of the method itself therefore determines the acceptance criterion and more precise methods would generate a narrower acceptance range whereas less precise methods would generate a broader acceptance range. This is appropriate if the established goal is to monitor whether or not a method is performing as well as it should be expected to perform. A fixed criterion applied to the same data set would not allow this kind of flexibility and the fixed criteria would inherently be too wide to be effective for precise methods and would be sufficiently narrow such that less precise methods would fail at a high rate, even though the method is performing as well as expected based on validation data [2].

The control chart approach is the standard in areas such as forensic science, clinical chemistry, and general manufacturing. There are a large number of scientific and statistical investigations and procedures to draw from which utilize the general control chart approach. These allow decision making such as trend and shift analysis [12]. Westgard's rules, which are based on control charts, have been shown to be optimal in terms of maximizing error detection while minimizing false rejection of data [22]. Although control charts do a good job of monitoring method precision and consistency, they do not alone address accuracy of control data during the time samples are analyzed, and an additional accuracy criterion should be employed to make sure the mean value for the control data is accurate to within an established reasonable limit.

Questions to be addressed when setting up an analytical run incorporating quality control samples are the number and sequence of quality control samples and the way in which acceptance criteria will be applied. It is generally accepted that control samples should be prepared at three concentrations that are representative of the concentration range of the method. The number of replicates of these three concentrations will of course determine the total number of quality control samples run, which in turn will affect the error detection and false rejection probabilities as well as the sample throughput

efficiency. The number of replicates is therefore an important consideration, and this number should be established as a percentage of the total number of samples in the run to preserve consistency in these critical parameters from run to run. It is also important to keep the number of replicates at each of the three concentrations consistent to preserve a balanced statistical design and to ensure the same decision-making power at each concentration level.

Quality control samples should be sequenced within the analytical run to maximize the degree of concentration coverage over the entire run and to minimize the number of samples run between each control. The best decision-making power would be derived from a run sequence that involves all three controls being run before and after each sample. In this way each sample would be controlled at each concentration just prior to and after it is run, minimizing any time delay before a control sample is run. Few industries can allow such inefficient sample throughput, however, and a good compromise would be to alternate high-, medium-, and low-concentration controls each separated by an equal number of samples. Acceptance of the sample data can then be done according to criteria applied to the entire run or the run can be subdivided into "brackets." Samples that are contained between each control would constitute a bracket and whether or not the samples are acceptable depends on the acceptability of only the controls that bracket the samples. The advantages of the brackets approach are that acceptable samples are taken only from between acceptable controls and portions of a run may be preserved even if a significant portion of the run is out of specification. The brackets approach does a much better job of controlling for transient errors that may appear and disappear during the course of a run. In contrast, a criterion based on rejection of an entire run would result in data being rejected even though the problem had disappeared if rejection was due to a transient problem. The advantage of a criterion applied to the entire run is that the process is simpler and easier to manage.

The FDA guidance [3] favors a criterion applied to the entire run. The guidance further states that quality control samples should be run at three concentrations in duplicate but further stipulates that the number of quality control samples (run in multiples of the three concentrations to provide a balanced design) should be dependent on the number of samples in a run. The minimum percentage of quality controls to samples is specified as 5 %. The FDA guidance offers no stipulation on the sequence of samples, calibration standards, and quality controls within an analytical run.

The primary acceptance criterion for the entire run is that at least four of six quality control samples must be within 15 % of their respective nominal value although the two allowed outside this range cannot be the same concentration. This so-called 4/6/15 rule was modified from a draft version of the guidance which stipulated a 4/6/20 rule. The FDA guidance also states that a confidence interval approach yielding comparable accuracy and precision is an appropriate alternative to the 4/6/15 rule but does not specify the level of confidence to be applied. The 4/6/15 rule is loosely based on a quality control procedure proposed by Causey et al., which used a 67 % (1S) confidence interval to establish acceptance limits although their limit was 10 % rather than 15 or 20 % [23]. The FDA guidance contains further stipulations on the acceptance of concentration residuals from the calibration curve and implies that validation-type criteria are applied to intrarun quality control data [3].

The acceptance criterion proposed by Causey et al. is consistent with the criterion of 10 % they proposed for validation of precision and is statistically valid. The 4/6/10 rule with a 10 % acceptance criterion for the RSD (also based on 1S) is statistically valid, if applied relative to a mean measured value, since a 67 % confidence interval around a mean would be predicted statistically to yield 67 % of measurements within this interval. This is provided that the method demonstrated a precision of 1S (10 % in this case) and there were no method errors beyond the level of error demonstrated during validation. These method errors that inflate the level of error beyond what has been determined to be acceptable during validation are what a quality control program is supposed to detect. The FDA guidance criterion of 4/6/15 would also be consistent with their validation criterion of 15 % RSD if it were based on a mean measured value. The guidance states however that the criterion is to be applied to a "nominal" value which is most often taken as the target value the quality control sample was prepared to be. This means that the FDA guidance criterion encompasses both random and systematic error.

The validation criteria in the guidance for both random and systematic error (accuracy and precision) are 15 % and since these errors are additive, the criterion is actually a criterion for total error. The statistically valid criterion for quality control based on a nominal value would therefore be 30 %, which is derived from the sum of the allowable accuracy and precision errors. This concept is again illustrated by Fig. 7. The allowable random error is determined in this case by the 1S interval (67 % or four of six) and is 15 % according to the guidance on precision validation. The allowable systematic error is 15 % also as determined by the accuracy criterion and the criteria for bias should therefore be 30 % to be statistically valid. The FDA guidance could also be made statistically valid by application of the 4/6/15 rule to the mean of quality control values and with a separate accuracy criterion of 15 %. The FDA guidance approach has been compared using real bioanalytical data for ten analytical methods and found to be in disagreement with three statistically derived approaches involving confidence intervals, Westgard's Rules, and a range chart approach [24]. The statistically valid approaches were all relatively consistent with one another. The danger that exists for the bioanalytical scientist is that methods that pass the FDA guidance validation criteria but are borderline in terms of accuracy and precision can be expected to incur a large number of quality control failures in routine analysis. In order to avoid this, a reasonable practice would be proceeded with routine analysis of samples only when methods demonstrate a total error (inaccuracy plus imprecision) of no more than 15 %, even though the acceptance limit for total error in validation is 30 % according to the FDA guidance. It is also important to be aware that the FDA criterion is not statistically valid and, although it is the standard of practice in drug development, can be challenged successfully on a scientific basis.

5 Method Transfers and Comparisons

Bioanalytical methods are also routinely transferred and redeveloped for a number of reasons including a change in analytical technology (e.g., changes in detection system), change in the relevant concentration range (e.g., need for improved sensitivity), transfer to new laboratories, or the addition of metabolites, new species, or matrices. Assays may also be redeveloped with an aim to provide better characteristics than an assay of reference (higher throughput, economic benefits, etc.). This constitutes a situation where the new test method is compared to the reference assay. In each case, the objective should be to demonstrate that the test bioanalytical assay, or the bioanalytical assay in the new settings, generates results that are comparable to those obtained using the reference bioassay. Cross-validation of bioanalytical methods is also an area that requires consideration of method transfers concepts. These concepts include (a) an ability to demonstrate that two laboratories or methods are capable of producing equivalent results through appropriate experimental design, (b) to identify the source of any differences, and (c) to resolve the differences. At present, however, several approaches have been used for method comparisons and there is no clear consensus on the most appropriate acceptance criteria or study design in such bioanalytical method data comparisons.

Cross-validation and/or transfer of bioanalytical methods encompasses comparison of control data for two or more bioanalytical circumstances used to generate data within the same studies or across different studies as described by the FDA [3]. Interlaboratory and cross-validation studies are generally evaluated using spiked matrix controls. One of the most important questions that need to be addressed when transferring a method is whether or not one can assure comparability of data. There is generally a trade-off between the following two situations, when acceptance criteria are not too restrictive or too narrow. Acceptance criteria that are not very restrictive might fail to adequately demonstrate the equivalence of two methods. In this case, the acceptance criteria are too wide and can lead to acceptance of nonequivalent results from the two participating laboratories (also referred to as β-error or false negative error). Conversely, a criterion that is too narrow might lead to generation of unnecessary data and may lead to rejection of equivalent results from the two participating

laboratories (also referred to as α-error or false positive error). The goal of a bioanalytical method transfer should be to limit the number of false positive as well as false negative errors and ensure that the test method performs equivalently to the reference method. Currently, the predominant approaches used for evaluation of control data for bioanalytical method transfer include (1) the independent validation approach, (2) statistical difference testing using a Student's *t*-test and (3) statistical equivalence testing, and (4) the total error-based approach.

According to the independent validation approach, both the reference and the test method must be shown to meet the validation criteria for accuracy (±15 % of the nominal concentration) and precision (≤15 % coefficient of variation) as prescribed by the FDA [3]. The means of the two methods (e.g., x_1 and x_2, respectively) are compared against the true reference value (μ) which is taken as the nominal spiked concentration. The school of thought with this approach is that if both methods are valid to within FDA criteria using the same control, then the methods are also comparable within FDA-established criteria. However, it has been shown that the nonstatistical approach of simply comparing the observed bias and precision between two laboratories to preset acceptance limits can result in both rejection of results that are truly equivalent and acceptance of results that are truly nonequivalent [25]. When using the Student's *t*-test, this approach controls the false positive error as the level of significance is fixed by performing the test. However, the false negative error is controlled only if the number of results is sufficiently high. In addition, it has been demonstrated that the higher the number of experiments, the smaller is the probability of accepting the transfer. The statistical equivalence test is based on reversing the null (H_0) and the alternate (H_1) hypotheses as follows:

$$H_0 : \left|\mu_T - \mu_R\right| \geq \delta \; and \; H_1 : \left|\mu_T - \mu_R\right| < \delta,$$

where μ_T and μ_R are the mean results of the test and reference methods and δ is the prespecified acceptance limit. The statistical equivalence test is very rigorous, and in contrast to the *t*-test, it controls the false negative error as the level of significance is fixed by performing the test. However, the false positive error is controlled only if the number of results is sufficiently high. Moreover, in all the three approaches described above, the trueness criterion is separately evaluated from the precision criterion. Failure to fulfill either criterion may result in rejection of the cross validation and/or transfer of analytical methods. In this context, such criteria do not allow acceptance in situations where there is no bias but a random variation higher than the acceptance limit. In such a case, the absence of systematic error may compensate for the imprecision and it can still guarantee that the results will be close enough to their true value. Conversely, when a systematic difference is not accepted by the trueness criterion, methods may still be comparable because the random variation is small.

In order to avoid limitations observed with the above-described approaches, it has been proposed to combine the estimates of systematic and random errors into one single decision criterion—the total error defined as the sum of the systematic and random errors. As described earlier, the concept of total error is shown in Fig. 7. The total error approach simultaneously controls the risk of both false positive and false negative errors and is statistically the most correct approach. Dewé et al. have recently suggested a decision criterion using total error for method transfers [26]. This is a sophisticated statistical approach that is based on a full risk analysis and is very robust. However, such an approach may not always be easily applicable within laboratories lacking statisticians. Bioanalysts have been historically more prepared to accept criteria which are fixed and easy to use. The 4/6/15 rule is one such example in method validation. It would be desirable to employ approaches based on statistical considerations to establish fixed criteria rather than using fixed criteria based on a consensus opinion without statistical considerations. Such an approach using fixed criterion may not be entirely statistically correct but can have advantages over existing approaches. In this context, if μ_T is defined as the test method mean result, and μ_R as the reference method mean result, the acceptance criterion may be more accurately represented as the sum of the FDA guidance accuracy limit of ±15 % and the standard error of the mean of μ_T, the ratio of the standard deviation "*s*" of the test method to the square root of

the number of replicate measurements "*n*." The standard error of the mean provides a gauge for how variable the mean can be expected to be when performing *n* replicate analyses. Considering the FDA guidance limit of precision is set at 15 % CV, fixed acceptance limits for bioanalytical method transfer can be set as $\pm(15\ \% + 15\ \%/\sqrt{n})$. This provides a tool to determine a reasonable fixed criterion for bioanalytical method transfer that changes with experimental design. Such a tool would depend on the number of samples, the number of times the samples are run, number of quality control samples, etc.

6 Incurred Sample Reanalysis

For the purpose of validation of bioanalytical assays, calibration standards and quality control samples are prepared by spiking the same pool of blank biological matrix with the analyte of interest at fixed concentrations. The use of these calibration standards and quality control samples might not necessarily mimic actual study samples drawn from subjects who have received the drug. There is a reasonable possibility that the inter- and intra-patient matrix variability may not be accounted for. Several factors can affect the precision and accuracy of the results obtained from the analysis of study samples, such as protein binding, metabolite–parent interconversion, presence of unidentified metabolites, matrix effects, sample inhomogeneity, analyte stability, and concomitant medications. While it is not practical to prepare calibration standards and quality controls for each individual source of matrix, some assessment of patient variability should be undertaken. During the third American Association of Pharmaceutical Scientists (AAPS)/FDA Bioanalytical Workshop [27], it was recommended that in addition to the usual prestudy validation, reproducibility in the analysis of incurred samples should also be evaluated for both clinical and nonclinical studies. Incurred sample reanalysis can sometimes yield dramatically different results, even when using a validated assay. In order to identify these cases, it was suggested that the reanalysis of a limited number of incurred samples be systematically verified and that this should be part of assay validation. It is also necessary to distinguish between reproducibility and stability when reanalyzing incurred samples. A report summarizing the recommendations for the implementation of incurred sample reanalysis has been published [28].

Incurred sample reanalysis should be performed in support of PK data interpretation studies. In the case of nonclinical studies, animal populations are often quite homogenous. Dietary conditions and other factors are also relatively constant among animals. Incurred sample reanalysis may be included as a component of validation to gauge any reproducibility issues as early in the study as possible. For clinical studies, incurred sample reanalysis assessment should be included for all bioequivalence studies, with other study types verified as appropriate (e.g., healthy volunteers, drug–drug interactions, patient populations, etc.). First-in-human oncology studies have slow and sporadic enrollment of patients. Often these patients are on a multitude of medications and undergo variations in metabolism as well as endogenous compounds. In such a situation, incurred sample reanalysis becomes a challenging proposition, where the timing of analysis and sample stability issues need to be cautiously assessed in order to make a reasonable conclusion.

The report suggests that individual samples, rather than matrix pools, should be selected for incurred sample reanalysis, since these would provide the appropriate conditions that will test the reproducibility of the assay. Pooled samples should be used for stability analysis of incurred samples. Samples should be selected near the time of maximal drug concentration and during the elimination phase, in order to obtain results from samples that would potentially contain metabolites. It is also preferable to select a few samples from several subjects, rather than full subject profiles, in order to better identify inconsistent samples. Sample size considerations are critical for incurred sample reanalysis. The number of samples repeated for reanalysis should be representative of the study conducted. Generally 5–10 % of the total sample size repeated for analyses has been recommended. While different proposals for incurred sample reanalysis acceptance criteria based on molecule class

or the technological platform employed can be suggested, the published report suggests utilizing a criteria similar to the 4/6/15-20 rule as used for quality control samples. For small molecules, 67 % of all the repeat samples should agree within 20 %, and for ligand-binding assays, 67 % of the repeat samples should agree within 30 % of the original result. A failed incurred sample reanalysis assessment does not necessarily invalidate an entire study. Rather, an examination should be performed to assess the reasons for failure and the assay should be thoroughly investigated. The EMA draft guidance [8] is consistent with the AAPS workshop report [28] for the most part.

7 Conclusion

A scientific and statistically valid approach to validation and quality control is important to be consistent with the standard of practice outside of the drug development discipline. The science of validation and quality control is generally well developed and easily understood for those with a background in statistics. Standard operating procedures should be developed with this in mind and a balance should be struck between what is perceived to be compliance and good science. It is inappropriate to sacrifice good science in order to comply with what is perceived to be regulatory preferences. This concept is strongly supported by the FDA guidance in its introduction, where it states that the guidance is intended to provide general recommendations for bioanalytical method validation and which can be adjusted or modified. There are a number of areas that are not well developed in the FDA guidance and the proposed draft EMA guidance addresses some of these. This would imply that the current FDA guidance is a work in progress and additional conferences will be held to further refine and expand the current guidelines. Because of the ever-expanding global marketplace, it is critical to be inclusive of all major regulatory bodies in this endeavor. This will hopefully achieve global harmonization of these regulatory practices and establishment of a single guidance document that is accepted worldwide.

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Anticancer Clinical Pharmacology Overview

Uday B. Dandamudi, Andrew Beelen, and Lionel D. Lewis

Abstract This chapter is an overview of the principles of clinical pharmacology with a focus on oncology therapeutics. It covers the basic pharmacologic principles of pharmacokinetics and pharmacodynamics which underpin the individualization of a patient's drug therapy and focuses on the utilization of these principles in anticancer drug therapy prescribing while addressing some of the most recent advances in the field. A unique aspect of this chapter is the intention to bridge the gap between clinical pharmacologists and subspecialty oncologists. This chapter showcases the rationale as to why the discipline of clinical pharmacology plays an increasingly significant role in clinical therapeutics (prescribing) in all specialties where drugs are used to treat disease.

Keywords Anti-cancer therapeutics principles • Pharmacokinetics • Pharmacodynamics

1 Introduction

Clinical pharmacology is the science of drug action and use in human beings. It spans an understanding of the basic pharmacology of a drug with a broad scope, from discovery of new targets to new molecules that hit the target to the safe usage of drugs in clinical practice. A comprehensive understanding of the principles of clinical pharmacology is essential for any clinician to deliver optimal therapeutics to individual patients. Over the last 50 years, the clinical pharmacology of many drugs has been elucidated with advances in sophisticated and accurate, analytical tools to determine plasma drug and/or metabolite concentrations in biologic fluids. This has permitted a better understanding of the relationship between the pharmacokinetics (derived from the Greek words *pharmakeus* [drug] and *kinēsis* [movement] and meaning drug over time) and the pharmacodynamics (derived from the Greek words *pharmakeus* and *dynameos tis* [power], meaning drug action or power) for many drugs (Fig. 1).

In oncology cytotoxic drug treatment demands close attention to pharmacologic principles because the therapeutic index of many such anticancer drugs is narrow, that is, the ratio of the $TD_{50}/ED_{50} \leq 2$ (see Fig. 2). To achieve the desired primary therapeutic end point (tumor cell death leading to tumor

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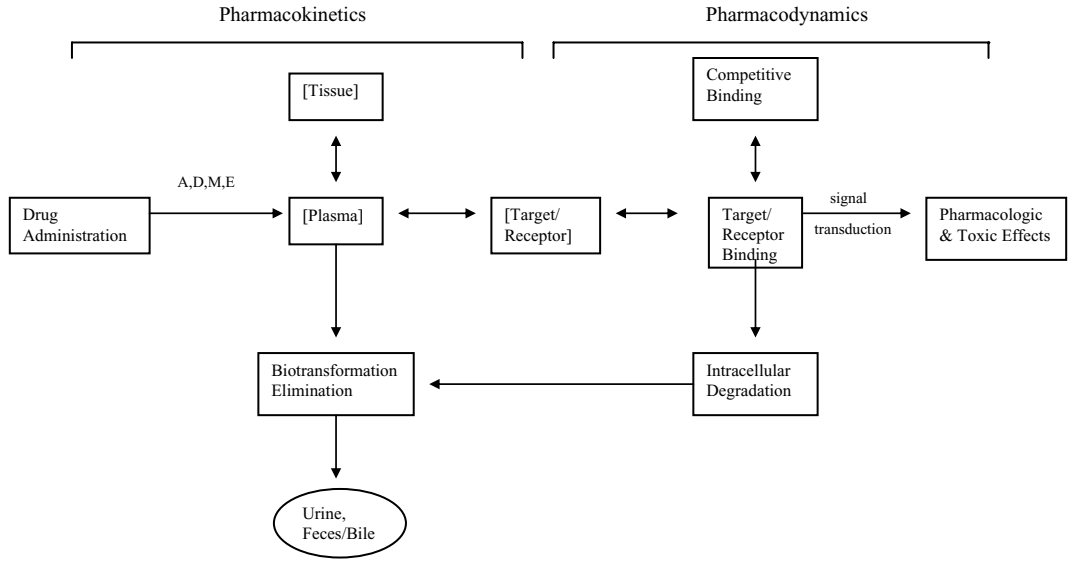

Fig. 1 Schematic representation of the processes determining drug disposition in the human body and the relationship of pharmacokinetics and pharmacodynamics to these processes (Reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004.)

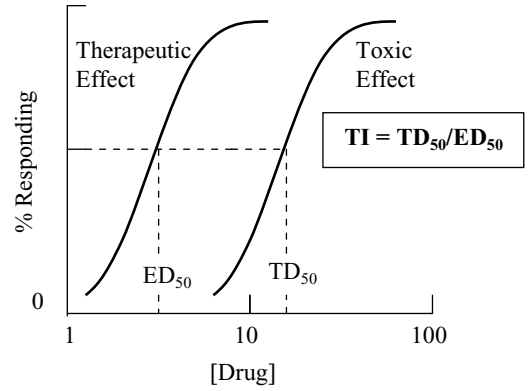

Fig. 2 *Therapeutic index* (*TI*): The ratio of the TD_{50} to the ED_{50} is an indicator of a drug's selectivity for producing a desired effect in relation to a toxic effect. The higher the ratio, the more selective the drug (Reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004.)

shrinkage), the limits of tolerable toxicity to normal tissues are often encroached with the use of many classical chemotherapeutic agents. Adverse events, both anticipated and unexpected, must be integrated into therapeutic decision-making to optimize patient outcome; thus ongoing assessment and reassessment of the cytotoxic drug effects on the tumor and normal tissues are required. Drug–drug, drug–herb, drug–food, and drug–comorbid disease interactions, if not considered and anticipated, can have dire consequences for cancer patients. Furthermore, the rapidly increasing knowledge of genetic polymorphisms in proteins involved in the primary mechanism of a drug action and/or the processes that determine drug pharmacokinetics (absorption, distribution, metabolism, and excretion) further increase the complexity of therapeutic decision-making and optimizing therapy in this era of personalized medicine. This chapter focuses on the principles of clinical pharmacology as applied to cytotoxic chemotherapy, illustrating how these principles can lead to an enhanced ability to optimize the efficacy/toxicity ratio for anticancer agents in individual patients.

2 Mechanisms of Drug Action (Pharmacodynamics)

The study of the effects of drugs on biologic, physiological, and molecular processes is termed pharmacodynamics. Most drug effects result from interactions with specific macromolecules or *targets* that induce a biochemical, physiological, or molecular change [1–3]. The target of the drug may be an enzyme found in plasma or located intracellularly; a cell membrane-located protein; an ion channel protein or a structural protein; or DNA, RNA, or other macromolecules (e.g., microtubules). The molecular site of action for many drugs is a *receptor* which normally binds an endogenous ligand (e.g., hormones, growth factors), the receptor function is modified on drug binding. Drugs that bind to receptors and mimic the function of an endogenous compound are termed *agonists* (e.g., opiates, granulocyte colony-stimulating factor [G-CSF], recombinant human erythropoietin [rhEPO]). When a drug binds to a receptor and blocks the effects of the endogenous ligand, the drug is termed an *antagonist* (e.g., bicalutamide, an androgen receptor antagonist; trastuzumab, a monoclonal antibody against HER-2/neu; and bevacizumab, a monoclonal antibody against several of the forms of vascular endothelial growth factor (VEGF)). Certain agents have both agonist and antagonist properties and are termed partial agonists (e.g., tamoxifen or raloxifene-mixed estrogen receptor agonist/antagonist, nalbuphine-mixed μ/κ/δ opiate receptor agonist/antagonist). Many established and novel anticancer agents inhibit the function of endogenous enzymes by binding directly to the enzyme and are thus termed *enzyme inhibitors* (e.g., dihydrofolate reductase inhibitors (methotrexate); DNA synthesis and folate metabolism at multiple target enzymes (pemetrexed); topoisomerase I inhibitors such as the camptothecins (irinotecan) ;aromatase inhibitors (anastrozole and letrozole) epidermal growth factor receptor [EGFR]-associated tyrosine kinase I inhibitors (erlotinib, gefitinib, and lapatinib); multi-tyrosine kinase inhibitors (sunitinib, sorafenib, and pazopanib); and histone deacetylase [HDAC] inhibitors (vorinostat and panobinostat)).

2.1 Drug Action

Binding of a drug to its target is often highly specific, dictated by the three-dimensional structure of both the ligand and the target molecule as well as electrostatic, dipole–dipole, ionic, van der Waals, hydrophobic, and hydrogen bonding forces. The greater the net sum of these forces, the higher the affinity of the drug to bind to its target [1–3]. In some cases, a drug will form irreversible covalent bonds with its target, for example, alkylation of 7-nitrogen and 6-oxygen atom in the guanine ring by ifosforamide mustard, the active metabolite of the pro-drug ifosfamide. The pharmacologic effects of any drug most often occur in a graded, effect site drug concentration-dependent manner [3–5]. In many cases, the plasma drug concentration is linearly related to the dose of the drug administered; the graphical representation of drug effect is thus referred to as a *dose–response curve*, although from a scientific purist's perspective, the term *concentration–response curve* would be preferred (Fig. 3a, b). Agonist drugs produce a graded concentration response up to a maximum effect value (termed E_{max}), above which increasing the drug concentration no longer produces any increase in effect. Antagonists produce no response and partial agonists have a reduced effect and reduced maximal effect–response (Fig. 4). Each drug has a specific shape to its concentration (dose)–response curve at its target site. In clinical prescribing, concentration–response curve importance lies in the titration of the dose of a drug to optimize the desired effect [6].

2.2 Receptor Pharmacology and Function

Molecular cloning techniques, along with advanced biochemical methods, have greatly enhanced our ability to discover and characterize physiological receptors, signal transduction pathways, and effector proteins. Receptors for endogenous ligands are classified into four "superfamilies" with distinct

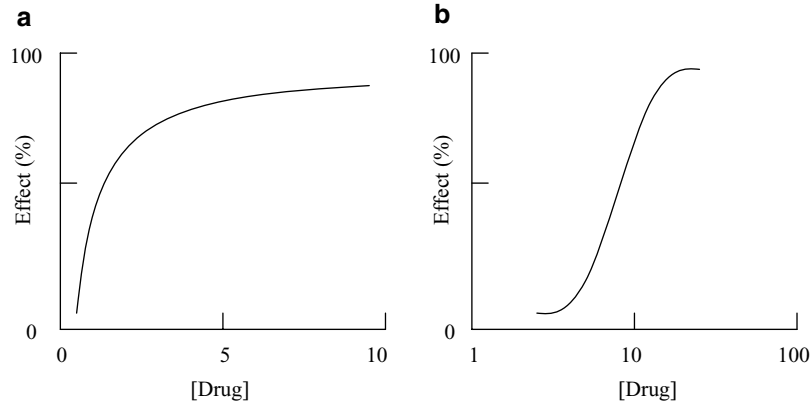

Fig. 3 Concentration (dose)–response curves plotted (**a**) arithmetically and (**b**) semilogarithmically (Reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004.)

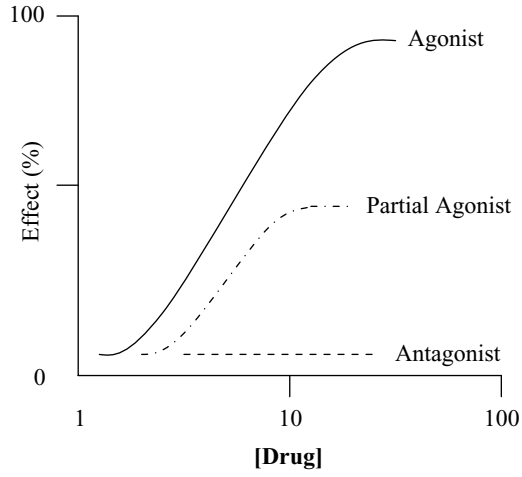

Fig. 4 Semilogarithmic plot of the percent maximal effect versus drug concentration for an agonist, partial agonist, and antagonist (Reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004.)

functional properties. Three families are localized to the cell membrane, ligand-gated ion channel receptors (e.g., glutamate, nicotinic acetylcholine, and γ-aminobutyric acid receptors), G-protein-coupled receptors (e.g., opiate receptors), and receptors with enzymatic activity (e.g., EGFR, VEGFR, and platelet-derived growth factor receptors [PDGFr]) [7, 8]. The fourth family of receptors is located within the cell and is known as nuclear transcription factor receptors (e.g., androgen and estrogen receptors, retinoic acid [RA] receptors and retinoid X receptors [RXR], and proliferating peroxisome gamma receptor [PPAR gamma]). Agonist binding to any one of these types of receptors, regardless of family, activates a signal transduction pathway such as the activation of a specific enzyme or cascade of enzymes, release of a second messenger(s), or transcription of a particular gene; it is this physiological/biochemical change that mediates the effect of a ligand stimulating the receptor.

2.2.1 Agonists

Agonists (e.g., morphine, erythropoietin, granulocyte colony-stimulating factor, gonadotropin-releasing hormone, [leuprolide]) produce an effect by interacting with and activating specific receptors

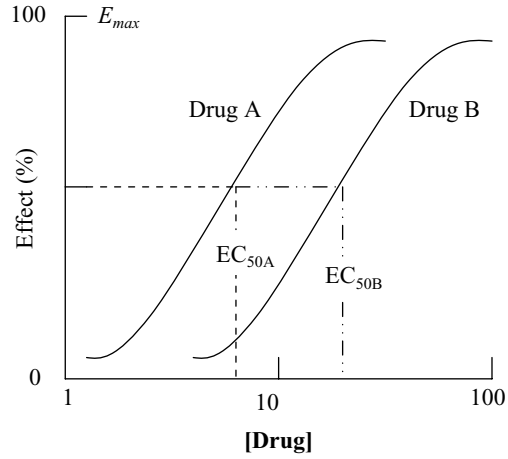

Fig. 5 *Relative potency*: Semilogarithmic plot of the percent maximal effect versus drug concentration for two drugs (A and B) with equal maximum pharmacologic effect (E_{max}). The relative potency can be estimated by the ratio of EC_{50B}:EC_{50A} (when measured in the same biologic system). EC_{50} = drug concentration at which a 50 % maximal response is observed (Reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004.)

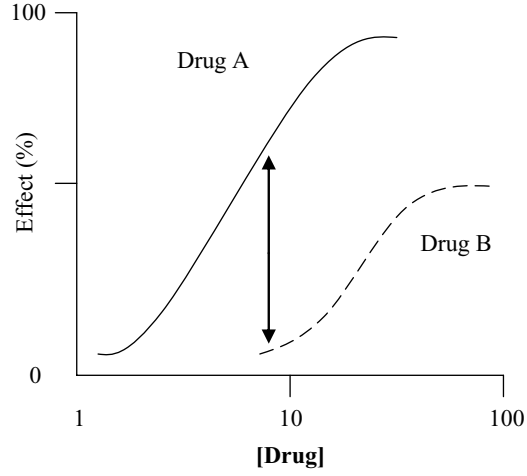

Fig. 6 *Relative efficacy*: Semilogarithmic plot of percent maximal effect versus drug concentration for two drugs (A and B) with differing maximal effects. Drug A elicits a greater pharmacologic effect and is therefore considered more potent (when measured in the same biologic system)

for endogenous ligands [1–3, 7]. The particular signal transduction pathway linked to a receptor determines the process of receptor activation. Drugs that bind directly to and inhibit the activity of enzymes are not considered agonists because they do not first interact with an endogenous receptor. A useful parameter to compare drugs with equal maximal effect is the parameter $EC_{50,}$ the concentration of drug at which a 50 % maximal effect (response) is produced. Agonist properties can be quantified in terms of potency and magnitude of effect. Potency depends on four factors: receptor density, efficiency of receptor signal transduction, drug affinity for the receptor, and the degree of signal transduction induced by the drug binding to the receptor (*efficacy*). The latter two are properties of the drug itself and can be quantitated by plotting the percentage maximal effect versus log drug concentration for two comparison drugs, which will give relative potency (Fig. 5) or relative efficacy (Fig. 6).

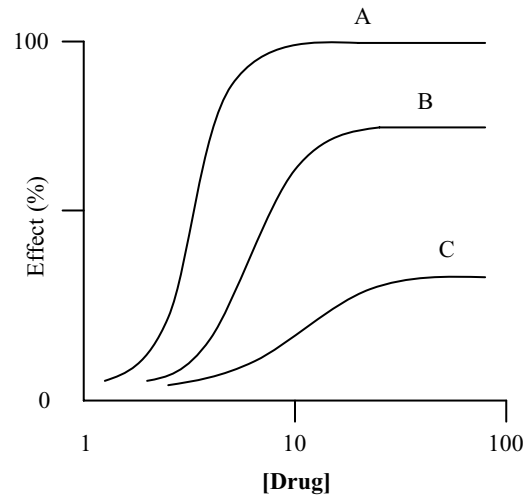

Fig. 7 *Noncompetitive antagonism*: The maximal effect of an agonist (line A) is attenuated by the addition of increasing concentrations of a noncompetitive antagonist (lines B and C) (Reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004.)

2.2.2 Competitive Antagonists

Competitive antagonists (e.g., alemtuzumab, trastuzumab, and rituximab) bind the same endogenous receptors as the agonist, but they fail to induce a response (i.e., there is no receptor-mediated signal transduction). Agonists in the presence of competitive antagonists simultaneously compete for the same receptors. The drug concentration in the effect compartment and receptor affinity determine the degree of receptor occupancy of each agent at any given moment in time. The effects of a competitive antagonist can be overcome by increasing the concentration of the agonist. Noncompetitive antagonists, on the other hand, in effect decrease the number of "effective" receptors and attenuate the maximal response to an agonist (Fig. 7). The effects of a noncompetitive antagonist cannot be overcome by increasing the agonist concentration [1, 2, 4, 6].

2.2.3 Enzyme Inhibition

Similar concepts can be applied to drugs that act as enzyme inhibitors (e.g., methotrexate, irinotecan, or topotecan; BCR-ABL kinase inhibitors (imatinib, dasatinib, nilotinib); EGFR tyrosine kinase inhibitors (lapatinib or multi-targeted kinase inhibitors (pazopanib, sorafenib, sunitinib)). Thus the drug and the endogenous substrate compete for the same binding site on the enzyme. When the drug is bound, the enzyme can no longer bind substrate and the rate of the enzymatic reaction is reduced. One of the most successful molecularly targeted agents that possessed such a mechanism is imatinib mesylate, which inhibits ATP binding to the tyrosine kinase of the proto-oncogene KIT, PDGFr, and BCR-ABL, inhibiting protein phosphorylation and signal transduction [9–11]. Newer ATP mimetic agents targeting these and other kinases include dasatinib and nilotinib. Alternatively, some drugs (e.g., chloroadenosine as its anabolite chlorodeoxy ATP) bind to enzymes at sites other than endogenous substrate-binding site and induce a conformational change in the enzyme structure [12]. This structural change modifies the three-dimensional shape of the endogenous substrate-binding site such that the endogenous substrate is no longer recognized and is unable to bind. These drugs are termed allosteric or noncompetitive enzyme inhibitors.

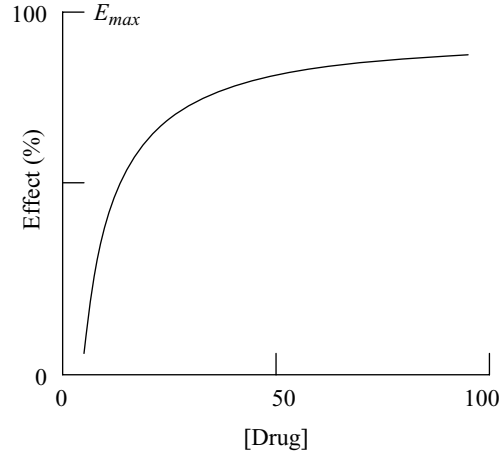

Fig. 8 E_{max} pharmacodynamic model (Reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004.)

2.2.4 Partial Agonists

Partial agonists (e.g., tamoxifen [a partial agonist at the estrogen receptor] [13], bryostatin [a partial agonist of protein kinase C] [14] and certain opiates [buprenorphine, nalbuphine]) stimulate endogenous receptors, but to a lesser degree than full agonists because of their intrinsically low efficacy. When an agonist is administered in the presence of a partial agonist, the maximal agonist effect is diminished due to some receptor occupancy by the less effective partial agonist, which implies that partial agonists are also partial antagonists (Fig. 4). The partial agonist activity can be overcome by increasing the concentration of pure agonist.

2.3 Non-receptor-Mediated Drug Actions

Some drugs exert their effects based solely on the physical or chemical nature of the drug. In oncology, examples of the drugs that work via this mechanism are the purine analogs (e.g., 6-mercaptopurine, thioguanine, chloroadenosine) and pyrimidine analogs (e.g., capecitabine, cytarabine [Ara-C], 5-fluorouracil, fludarabine, gemcitabine), which do not target specific endogenous receptors. Instead, after anabolic phosphorylation, they are incorporated into nucleic acids impairing DNA or RNA synthesis. This mechanism has been termed "counterfeit incorporation."

2.4 Pharmacodynamic Models

Pharmacodynamic models quantify the pharmacologic effect of a drug as it relates to the concentration of drug at its site of action (effect compartment concentration [1, 3, 7]). This theory states that the intensity of the drug effect is proportional to the number of receptors bound by the drug and that the maximum effect occurs when all receptors are occupied by the drug. The assumptions of receptor *occupancy theory* are as follows: (1) drug–receptor association/dissociation is rapid and at equilibrium, (2) each receptor binds only one drug molecule at a time, and (3) drug–receptor binding is reversible. The clinically most pertinent pharmacodynamic model is the E_{max} model, which is based on the hyperbolic relationship between pharmacologic effect and drug concentration (Fig. 8). The effect (E) can be quantitated by the following equation:

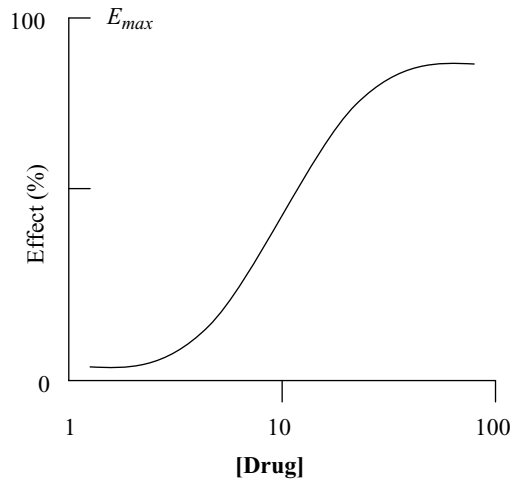

Fig. 9 Sigmoid E_{max} pharmacodynamic model (Reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004.)

$$E = \left(E_{\max} \times C_{\mathrm{p}}\right) / \left(EC_{50} + C_{\mathrm{p}}\right)$$

where E_{max} is the maximal effect, C_p is the plasma drug concentration, and EC_{50} is the concentration of drug at which a 50 % maximal effect (response) is observed. If a receptor can bind more than one drug molecule simultaneously (e.g., oxygen binding to hemoglobin), then the sigmoid E_{max} model is used and the equation relating drug concentration to effect becomes

$$E = \left(E_{\max} \times C^{\gamma}\right) / \left(EC^{\gamma}{}_{50} + C^{\gamma}\right)$$

where γ is the "Hill coefficient" and relates to the number of drug binding sites per receptor; it determines the slope of the curvilinear relationship (Fig. 9) [1–5].

3 Pharmacokinetics

The study of the time course of drug absorption, distribution, metabolism, and elimination by the human body is termed *clinical pharmacokinetics* [4, 6, 15, 16]. An adequate understanding of the basic principles of pharmacokinetics combined with the specific pharmacokinetic parameters for an individual drug enables the prescriber to choose the most appropriate route of administration, dose, and dosing frequency to obtain an optimal pharmacologic response, while minimizing toxicity (Fig. 1) [15, 16].

3.1 Absorption

Most drugs must enter the systemic circulation to reach specific sites of action (usually intracellular targets for cancer drugs), which are often distant from the site of administration. Drug absorption is a highly variable process dependent upon the physicochemical properties of the drug such as molecular size and shape, lipid solubility, degree of ionization at different tissue pH, and protein and tissue binding characteristics. Passive diffusion is by far the most important process by which drugs move across cell membranes. The thickness of the cell membrane and the presence or absence of drug efflux pumps [18] (e.g., ATP-binding cassette [ABC] transporters, e.g., ABCB1 also known as MDR-1 or P-glycoprotein

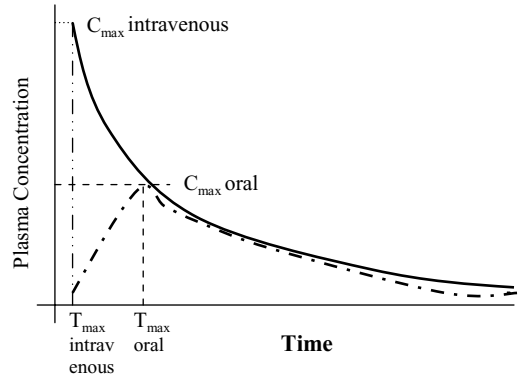

Fig. 10 Plasma drug concentration–time curves following intravenous (*solid line*) and oral (*dashed line*) administration of the same dose of a drug. C_{max} is the observed maximal plasma drug concentration and T_{max} is the time required to reach C_{max}. The oral bioavailability (F) is defined/calculated from AUC_{oral}/AUC_{iv}, where AUC is the area under the plasma concentration–time curve

(P-gp)) also determine the rate and extent of drug absorption. Oral (enteral) ingestion is the most common method of drug delivery because it is convenient, safe, and economical. The vast majority of older cytotoxic drugs are either poorly absorbed from the gastrointestinal tract or undergo significant metabolism or excretion by the liver prior to entering the systemic circulation. This process is known as the *first-pass effect*. Drugs with a high first-pass effect have low *bioavailability* (F), a term used to describe the fractional extent to which a dose of drug reaches the systemic circulation (Fig. 10). Examples of drugs with low oral bioavailability include morphine, many cytotoxics (e.g., paclitaxel, docetaxel, daunorubicin, and vincristine), and monoclonal antibodies which are proteins and therefore degraded by acid in the stomach (e.g., bevacizumab, cetuximab, and rituximab). Intravenous administration of drugs used in cancer chemotherapy circumvents the factors related to absorption and the hepatic first-pass effect and by definition provides 100 % bioavailability. However the newer targeted tyrosine kinase inhibitors (e.g., imatinib, erlotinib, gefitinib, lapatinib, sunitinib, sorafenib, pazopanib) or the histone deacetylase inhibitors (e.g., vorinostat) have sufficient bioavailability for chronic oral administration. Other routes of drug administration (e.g., subcutaneous, intramuscular, intra-arterial, intrathecal, and topical) are important in cancer therapeutics, but will not be discussed in detail in this chapter.

3.2 Distribution

Once a drug enters the systemic circulation, it begins to equilibrate (distribute) throughout the body. Many factors contribute to drug distribution including cardiac output, regional blood flow (specifically for anticancer therapeutics blood flow within a tumor), pH of the local environment, presence of drug efflux pumps (especially ABCB1[MDR-1/P-gp] and other ABC transporters that are present in many tumors [19]), and the physicochemical properties of the drug. Binding to plasma proteins (mainly albumin for acidic drugs e.g., topotecan and alpha-1 acid glycoprotein for basic drugs e.g. docetaxel) can limit the degree of drug distribution because only unbound (free) drug can passively diffuse through cell membranes. Some drugs accumulate in certain tissues preferentially, usually due to being highly lipophilic or secondary to tissue-specific binding (e.g., paclitaxel to beta-tubulin). Many chemotherapeutic agents have to enter tumor cells to produce a cytotoxic effect. Distribution into tumor cells can be facilitated by membrane transport proteins (carriers) and may be energy dependent (i.e., active transport). Active transport moves drugs against electrochemical and concentration gradients, which can significantly increase drug concentration in tumor cells. Examples of drugs that are actively transported into cells in addition to their passive diffusion include fludarabine, gemcitabine, and methotrexate [20–22].

3.3 Metabolism (Biotransformation)

Many drugs undergo enzymatic modification (metabolism), which most commonly reduces their pharmacologic activity (phase I metabolism) and enhances the body's ability to excrete (phase II metabolism) the drug. In some instances, the metabolite is more pharmacologically active than the parent drug (e.g., conversion of ifosfamide to ifosforamide mustard and the carboxylesterase mediated conversion of irinotecan to SN-38) or an active metabolite may be excreted more slowly than the parent compound (e.g., irinotecan metabolite SN-38 or the morphine metabolite morphine-6-glucuronide [23]). Drug metabolism can be categorized into two phases: phase I reactions, which involve metabolic modifications of the drug (often oxidation, reduction, or hydrolysis). and phase II reactions which are synthetic conjugation reactions involving the covalent linkage of a highly polar molecule (glucuronic acid, sulfate, amino acid, glutathione, acetate) to the parent drug or its metabolite. The products of phase II reactions have increased water solubility and are readily excreted in the urine (or bile).

The primary site of drug metabolism (both phase I and phase II reactions) is the liver, although the gastrointestinal tract, kidney, and lungs play important roles for some drugs. Within the liver, the cytochrome P450 (CYP450) monooxygenase system accounts for the vast majority of phase I drug metabolism. There are more than 70 known functionally active CYP450s in humans, with only eight isoforms accounting for more than 90 % of all drug metabolism. CYP3A4 and CYP3A5 (nearly identical isoforms also expressed in the intestinal epithelium) metabolize approximately 50 % of all drugs; CYP2D6accounts for approximately 20 % of drugs and CYP2C9/19 account for the metabolism of another 20 %–25 % of drugs, with the other major active isoforms (CYP1A1/2, CYP2B6, CYP2A6, CYP2E1) accounting for the remaining CYP450 metabolic activity [15–17]. Many drugs are substrates for (and thus metabolized by) more than a single member of the CYP450 enzyme family, having different affinities for binding to the different CYP450s. Drugs can be both substrates for the CYP450 enzymes and inducers or inhibitors of these enzymes.

The prolific recent identification of multiple genetic variations in the DNA sequence for many of the CYP450 enzymes has, in part, given us further insight into interindividual variability in drug metabolism. Currently the best example of this is the four different CYP2D6 phenotypes that yield poor, intermediate, extensive, and ultrapid metabolizers of drugs that are substrates of this enzyme and the evolving data that CYP2D6 poor metabolizer patients with breast cancer who receive tamoxifen do not generate as much active metabolite (endoxifen) and have a poorer survival than extensive CYP2D6 metbolizers [24, 25].

Phase II conjugation reactions also take place in the liver, the most important of which is glucuronidation. This involves the addition of a glucuronide group to the drug by over 15 isoforms of uridine diphosphate glucuronosyltransferase (UGT). As with the CYP450 system, hypofunctional polymorphisms have been identified in UGT1A1 (this catalyzes the glucuronidation of SN-38 to SN-38 glucuronide). The same holds true for *N*-acetyltransferase (NAT) and accounts for the "slow and fast acetylator" phenotypes, which affects the metabolism of amonafide to N-acetyl-amonafide (NAT2) and its toxicity profile (fast acetylators experience greater myelosuppression) [26].

Intracellular metabolism is another important mechanism of drug biotransformation. Many antimetabolite drugs are dependent upon intracellular metabolism to yield pharmacologically active moieties (e.g., 5-fluorouracil [5-FU], gemcitabine, and 6-mercaptopurine).

3.4 Excretion (Elimination)

Drugs can be eliminated from the body either in an unchanged form (parent drug) or as metabolites. Lipid soluble drugs generally need to be metabolized (as described in Sect. 3.3) to more polar compounds to facilitate their elimination from the body via the kidney. The kidneys are primarily

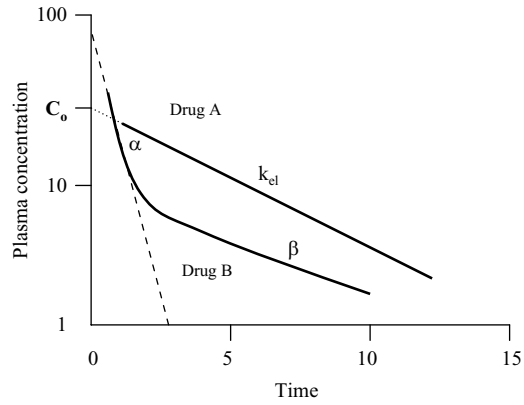

Fig. 11 One-compartment model (*straight line*) versus two-compartment model (*curvilinear line*) following rapid intravenous drug administration. Drug A exhibits a monoexponential decay (one-compartment model). C_o is the theoretical plasma concentration at time zero and k_{el} is the elimination rate constant. Drug B exhibits a biexponential decay representing a two-compartment model where **α** represents the redistribution phase rate constant and β represents the terminal elimination phase rate constant (Reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004.)

responsible for the excretion of drugs and their metabolites while biliary excretion plays an important role for certain drugs (e.g., taxanes and SN-38 glucuronide). Elimination of drugs via the urine is dependent upon three processes: glomerular filtration, active tubular secretion, and passive tubular reabsorption. The glomerular filtration rate is reduced in the elderly and in many disease states and dependent on cardiac output and intravascular volume. Drug molecules that are not protein bound ("free drug") can be filtered. Other physicochemical properties of drugs and metabolites that facilitate renal excretion include small molecular size (molecular weight <500 Da) and being unionized at physiological pH, which depends on the pK_a (the pH at which the molecule is 50 % ionized and 50 % unionized) of the molecule.

3.5 Pharmacokinetic Parameters

A simple plot of plasma drug concentration versus time offers the prescriber useful pharmacokinetic data (Fig. 10). C_{max} is defined as the maximal plasma concentration following a specific dose and T_{max} is the time at which C_{max} is observed. The area under the plasma drug concentration versus time curve (AUC) is a useful measure of the body's total exposure to drug.

3.5.1 Volume of Distribution [14]

The concept of volume of distribution can be best demonstrated by the theoretical administration of a drug as a rapid intravenous bolus injection with sampling and measurement of plasma concentrations at specified time intervals (pre- and postdrug administration). The resultant log plasma drug concentration versus time plot for a drug that rapidly distributes and equilibrates throughout the body (i.e., the one-compartment, well-stirred model with first-order elimination) will appear similar to that represented by drug A in Fig. 11. Extrapolation of the line back to time zero gives a theoretical plasma drug concentration (C_0) that would have occurred if drug equilibration were instantaneous.

This theoretical concentration results from the dilution of a known amount of drug (usually milligrams) into an unknown volume of the human body, which is known as the *apparent volume of distribution* or V_d. Dividing the dose (D) by C_0 gives the value for V_d (usually expressed in liters): $V_d = D/C_0$. Factors affecting a drugs volume of distribution include the physicochemical properties of the drug (see Sect. 3.2) and many patient-dependent factors such as body size, fat composition, water content, and plasma protein concentration and drug binding affinity and extent. The V_d is often referred to as the "apparent" volume of distribution because it does not represent a true physiological space or compartment within the human body, but rather a theoretical composite value for all the compartments to which the drug distributes. The one-compartment model is a convenient mathematical representation of drug distribution and elimination for many, but not all, drugs. More complex models are required for drugs that have protracted distribution times (e.g., paclitaxel, daunorubicin). In the two-compartment model represented by drug B in Fig. 11 (e.g., carboplatin, etoposide), the body is divided into two theoretical spaces, a smaller central compartment (blood volume plus the extracellular space of highly perfused tissues, heart, lung, liver, kidneys) and a larger peripheral compartment, which represents all other tissues. A semilogarithmic plot of plasma drug B concentration versus time reveals a biphasic decline in plasma drug concentration over time (Fig. 11). The first phase, known as the alpha phase, represents redistribution of the drug B out of the central (sampling) compartment and into the peripheral tissues. The beta phase, also known as the terminal elimination phase, occurs after the drug B has equilibrated between the two compartments and primarily represents drug elimination. Three-compartment models are necessary to describe some anticancer drugs (e.g., docetaxel, many anthracyclines) that have two distribution phases preceding the terminal elimination phase. The volume of distribution for drugs following a multi-compartment model is conceptually the same as for one-compartment modeling, but calculated in a slightly different way.

3.5.2 Clearance

Clearance represents the rate at which a drug is eliminated from the body and is expressed in terms of volume per unit time for first-order elimination. The volume term represents the theoretical volume of blood (or more often plasma) totally cleared of drug during a given time, for many drugs clearance remains constant and is independent of plasma drug concentration. The amount or mass of drug removed from the body per unit time, however, is constantly changing (depending on plasma drug concentration) during first-order elimination and is therefore not a convenient means to express clearance. When clearance mechanisms are saturated (i.e., operating at full capacity), zero-order elimination kinetics is followed and a constant amount (milligrams) of drug is cleared from the body per unit time regardless of the plasma drug concentration.

Most drug plasma concentration versus time profiles fit a one-compartment, first-order elimination kinetics model with an elimination rate constant (k_e) equal to the slope of the line for the log plasma drug concentration versus time plot (drug A in Fig. 11). The total body clearance, Cl_T (which is a summation of all clearance mechanisms—renal, hepatic, and other), of a drug is directly proportional to k_e and V_d: $Cl_T = k_e \times V_d$. Another useful equation to calculate Cl_T for first-order elimination is

$$Cl_T = F \times Dose / AUC$$

where F is the bioavailability and AUC is the area under the log plasma drug concentration versus time curve.

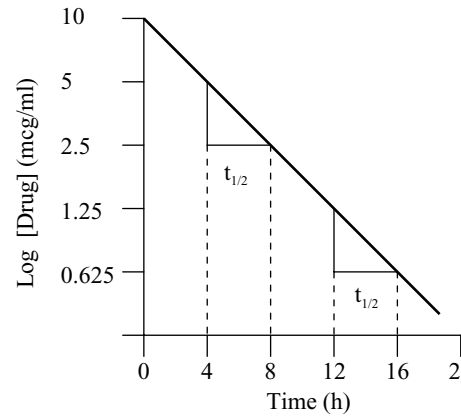

Fig. 12 Drug half-life: Log plasma drug concentration versus time plot for a drug following first-order elimination kinetics with a half-life of 4 h (Reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004.)

3.5.3 Elimination Half-Life ($t_{1/2}$)

The amount of time it takes for the plasma drug concentration to decline by 50 % is defined as the half-life ($t_{1/2}$; Fig. 12). Half-life is also related to the k_e: $t_{1/2} = 0.693/k_e$. Substitution of Cl_T/V_d for k_e yields the equation

$$t_{1/2} = 0.693 \times V_d / Cl_T$$

Thus, $t_{1/2}$ changes as a function of both V_d and Cl_T (under steady-state conditions). The half-life of a drug is useful in determining the dosing interval for many drugs that are dosed to a steady state and the time required to reach steady-state plasma concentrations (i.e., four half-lives to reach 94 % of steady state) as well as being useful for estimating the time for a specific percentage of administered drug to be removed from the body (i.e., upon cessation of drug therapy, the plasma concentration and the amount of drug in the body will decrease by 50 % for each $t_{1/2}$ time interval).

3.5.4 Noncompartmental Modeling

Noncompartmental modeling uses statistical moment theory to derive the same pharmacokinetic parameters and provides the additional parameters of AUMC or area under the first-moment curve (analogous to AUC) and the mean residence time (MRT). The primary advantage of noncompartmental modeling is the requirement for fewer mathematical model-specific assumptions but does assume dose- and time-dependent linearity.

3.5.5 Nonlinear "Dose-Dependent" Pharmacokinetics [26]

Clearance, for most drugs, remains constant (independent of plasma drug concentration over the therapeutic dose range), and as a result, first-order kinetics is obeyed. Occasionally, clearance mechanisms become overwhelmed (i.e., saturated) and there is no longer an exponential decline in plasma drug concentration over time (i.e., zero-order kinetics are followed). Under such circumstances in which clearance mechanisms are saturated (Michaelis–Menten kinetics apply), small increases in dose can dramatically increase plasma drug concentration or AUC (Fig. 13). In such cases (e.g., paclitaxel at doses >135 mg/m^2 administered over 3 h), the pharmacokinetics are considered "dose dependent" or "capacity limited." This is also termed Michaelis–Menten pharmacokinetics as the nonlinear

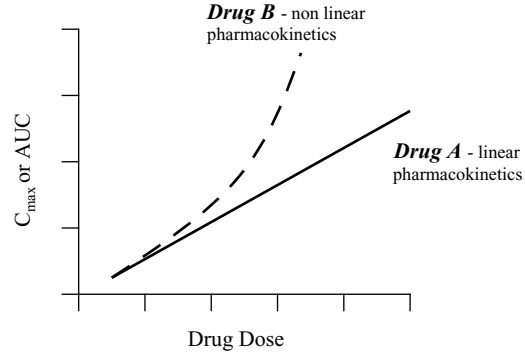

Fig. 13 Drug A has linear pharmacokinetics with dose proportional increases in C_{max} and AUC with increasing dose. Drug B exhibits nonlinear (dose-dependent) pharmacokinetics with nonlinear increases in C_{max} and AUC with increasing dose. Drug B is said to obey Michaelis–Menten (or saturation) pharmacokinetics (Reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004.)

relationship of concentration and dose can be fitted to the classical enzyme kinetic model. The processes of drug absorption (e.g., oral methotrexate, melphalan), distribution, and excretion can also become saturated, which in turn leads to a drug exhibiting nonlinear pharmacokinetics.

4 Population Pharmacokinetics

Pharmacokinetic parameters can vary widely from one patient to the next, which may lead to significant toxicity in some patients and therapeutic failure in others. Population pharmacokinetic modeling of pharmacokinetic data from many different patients can help quantify some of this variability [28]. This can be especially useful when the target population for the drug is heterogeneous or when the therapeutic window is narrow (i.e., effective plasma drug concentrations approach toxic concentrations). These models can simultaneously quantitate the effects of identifiable patient demographic variables (e.g., age, sex, weight, etc.), pathophysiological variables (e.g., renal or liver function, congestive heart failure, etc), and therapeutic variables such as concomitant drug therapy on drug disposition. Another advantage is that the residual variability (the variability not accounted for by the other specified covariates) is quantitated, which includes intraindividual variability, model misspecification, and measurement error (see the Pharmacometrics Chapter for further detail).

5 Pharmacokinetic–Pharmacodynamic Relationship

Pharmacokinetic modeling describes the change of plasma drug concentration over time and pharmacodynamic modeling relates drug concentration to pharmacologic effect (without regard to time). Pharmacokinetic–pharmacodynamic (PK–PD) modeling relates pharmacologic effect to the change of plasma drug concentration over time. The goal is to predict not only the magnitude but also the duration of pharmacologic effect based on the pharmacokinetic parameters of a particular drug. PK–PD models are predicated on the assumption that the concentration of drug in the plasma (accessible compartment) is proportional to the drug concentration at the receptor site (effect compartment).

There are some drugs in which there is no correlation between plasma concentration and pharmacologic effect; however, toxicity may be correlated to plasma concentration in some cases (e.g., methotrexate, docetaxel). Such models have perhaps been best used in oncology to predict drug toxicity rather than antitumor effect [28, 29].

6 Interpatient Variability

The pharmacokinetic parameters and the pharmacologic response from a specific dose of a drug may vary widely from patient to patient. There are multiple reasons for the observed interpatient variability in drug response that involves both pharmacokinetic [15, 30] and pharmacodynamics processes [29, 31, 32]. These include organ dysfunction (see the Organ Dysfunction Trials: Background, Historical Barriers, Progress in Overcoming Barriers, and Suggestions for Future Trials Chapter for further detail), disease state, concurrent medications, receptor and metabolic enzyme phenotype, age, sex, and other demographic characteristics. Drug oral bioavailability may vary from patient to patient secondary to increased or decreased expression or activity of intestinal proteins involved in drug metabolism or drug transport. Drug transporters such as ABCB1 (MDR-1/P-gp) also pump drugs out of the cells, thus lowering the intracellular drug concentration. In the gastrointestinal tract, ABCB1 leads to reduced bioavailability and its overexpression in tumor cells is a well-documented mechanism of tumor cell resistance. One of the primary causes of pharmacokinetic variability is interpatient differences in rate of drug clearance. In the case of a drug (or drugs with active metabolites) that is primary cleared by the kidney, decreased renal function will dictate the need for dose reduction to avoid toxicity (e.g., methotrexate, carboplatin). Drugs that undergo extensive hepatic biotransformation and/or biliary excretion may require dose modification in patients with severely compromised hepatic function (e.g., taxanes, anthracyclines, vinca alkaloids). Current evidence suggests that in end-stage renal disease patients with uremia, there is substantial downregulation of CYP450 enzymes in the liver [33], which would indicate the need for thoughtful dose modification (reduction) of drugs cleared by both the kidney and liver. Genetic polymorphisms in the CYP450 enzyme and other phase II hepatic enzyme systems (e.g., N-acetylation transferase-2, glutathione-*S*-transferase, and uridine diphosphate glucuronosyltransferase (UGT)) will also contribute to difference in an individual's ability to metabolize [30]. Another major reason for altered CYP450 activity is the use of concurrent medications/herbal supplements that either inhibit or induce one or more isoforms (e.g., St. John's wort increased irinotecan and imatinib clearance decreasing the systemic exposure to the drug and thus compromising drug efficacy [34, 35]). Up-to-date information on concurrent drug–drug interaction information can be obtained at http://drug-interactions.com. Variability in the volume of distribution of a drug can also account for some of the observed interpatient variability. Age is particularly important for volume of distribution. Infants have approximately 70–80 % total body water compared to 60 % for adults. Elderly patients have relatively more adipose tissue and less water content as well as decreased muscle mass. Disease-related alterations in plasma protein concentrations in cancer patients can affect the volume of distribution of drugs that are highly protein bound (e.g., docetaxel, anthracyclines, sorafenib, and imatinib), influencing free drug concentrations and thus potentially drug clearance.

Pharmacodynamic variability is produced not only by differences between patients in the concentration of drug at the effect site as result of pharmacokinetic variation (Fig. 1) but also by receptor/target polymorphisms. Examples of these polymorphisms include cases in which a receptor is more or less responsive to a certain drug concentration, as is the case for opioid receptors [36], or where paclitaxel resistance is linked to variants in the β-tubulin protein [37].

7 Conclusion

It is important for all physicians and nonphysician prescribers to understand the principles of clinical pharmacology in order to optimize drug dose and schedule for their patients, to prospectively be aware of the factors causing variability in drug response, and to minimize drug toxicity wherever possible.

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Pharmacokinetic Modeling

Jing Li and Michelle A. Rudek

Abstract Pharmacokinetic modeling is used to describe and predict concentration–time profile of a drug in the body. Common pharmacokinetic modeling approaches include noncompartmental analysis and compartmental modeling. Noncompartmental analysis is based on the statistical moment theory, while compartmental analysis is based on a mathematical model as a representation of the body to define model parameters by fitting the model to drug concentration–time data. This chapter serves to provide concepts and a set of guidelines for pharmacokinetic analysis using noncompartmental analysis and compartmental modeling approaches.

Keywords Pharmacokinetics • Non-compartmental analysis • Compartmental Analysis • Non-linear pharmacokinetics • Metabolite pharmacokinetics

1 Introduction

Pharmacokinetics is the study of absorption, distribution, metabolism, and excretion of a drug over a time course. Measurement of a drug in the body is usually limited to the blood or plasma. Pharmacokinetic data analysis consists of examining plasma concentration–time data and estimating pharmacokinetic parameters that describe drug disposition. Methods used for pharmacokinetic analysis include noncompartmental analysis and compartmental modeling. Noncompartmental analysis is based on the statistical moment theory, but does not depend on fitting mathematical models to the drug concentration data. Compartmental analysis is based on a mathematical model as a representation of the body to define model parameters by fitting the model to drug concentration data. This chapter serves to provide concepts and a set of guidelines for pharmacokinetic analysis using noncompartmental and compartmental modeling approaches.

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2 Noncompartmental Analysis

Noncompartmental analysis is based on application of the statistical theory called the moments of a random variable [1]. The time course of drug concentration in the plasma can be regarded as a statistical distribution curve. Noncompartmental analysis does not require the assumption of a specific compartmental model for drug disposition, which for some drugs can be a complex process, and thus this method is routinely performed to calculate pharmacokinetic parameters quickly and easily. An underlying assumption with noncompartmental analysis is pharmacokinetic linearity, where the pharmacokinetic parameter values do not vary with dose and/or time. This assumption applies to all parameters describing drug absorption, distribution, metabolism, and elimination.

In the noncompartmental analysis, the pharmacokinetic parameters are calculated from formulas using one or more of the following parameters, which are derived from the plasma concentration–time data:

- AUC: area under the plasma concentration–time curve, also referred to as the area under the zero moment curve.

$$AUC_0^{\infty} = \int_0^{\infty} C \cdot dt. \tag{1}$$

- AUMC: area under the curve of a plot of the product of concentration and time versus time, also referred to as the area under the first moment curve.

$$AUMC_0^{\infty} = \int_0^{\infty} tC \cdot dt. \tag{2}$$

- λ_z: terminal disposition rate constant, also depicted by k, is the rate of decline of the log–linear terminal portion of the plasma concentration–time curve.

2.1 Estimation of AUC

The area under the curve from 0 to time t_n is estimated by application of trapezoidal rule that depicts the curve as a series of straight lines, and thereby enabling the area under the curve to be divided into a number of trapezoids. The area can be calculated by means of either linear trapezoidal rule or by log–linear trapezoidal rule. The total area is measured by summing up a number of incremental areas from each trapezoid.

- Using linear trapezoidal rule, the area under the zero or first moment curve from 0 to time t_n is calculated as

$$AUC_0^{tn} = \sum_{i=1}^{n} \frac{C_i + C_{i+1}}{2} \cdot \left(t_{i+1} - t_i\right). \tag{3}$$

$$AUMC_0^{tn} = \sum_{i=1}^{n} \frac{t_i C_i + t_{i+1} C_{i+1}}{2} \cdot \left(t_{i+1} - t_i\right). \tag{4}$$

- Using log–linear trapezoidal rule, the area under the zero or first moment curve from 0 to time t_n is calculated as

$$\text{illegible} = \sum_{\text{illegible}}^{\text{illegible}} \frac{\text{illegible}}{\text{illegible}} \cdot \text{illegible} \cdot \tag{5}$$

$$AUMC_0^{tn} = \sum_{i=1}^{n} \frac{t_i C_i - t_{i+1} C_{i+1}}{\ln\left(C_i / C_{i+1}\right)} \cdot \left(t_{i+1} - t_i\right). \tag{6}$$

When there are large intervals between time points (e.g., wide trapezoids), linear trapezoidal rule may underestimate area during the ascending part of the curve and overestimate area during the descending phase. The log–linear trapezoidal method is better than the linear trapezoidal method for descending data (e.g., post-infusion, post-absorption), where the underlying assumption is that plasma concentrations decline mono-exponentially between two measured concentrations. Commonly, linear trapezoidal method is used for increasing or equal concentrations (e.g., before the peak or at a plateau), while log–linear trapezoidal method is used for decreasing concentrations (e.g., after the peak).

The extrapolated area under the zero (AUC_{extr}) or first ($AUMC_{extr}$) moment curve from the last sampling time point (t_{last}) to infinity is calculated as

$$AUC_{extr} = \frac{C_{last}}{\lambda_z}, \tag{7}$$

$$AUMC_{extr} = \frac{t_{last} C_{last}}{\lambda_z} + \frac{C_{last}}{\lambda_z^2}, \tag{8}$$

where C_{last} is the last measurable drug plasma concentration and λ_z is the terminal disposition rate constant that is obtained from the slope of the log–linear terminal portion of the plasma concentration–time curve. Therefore, the area from 0 to infinity is calculated as

$$AUC_0^{\infty} = AUC_0^{tn} + \frac{C_{last}}{\lambda_z}, \tag{9}$$

$$AUMC_0^{\infty} = AUMC_0^{tn} + \frac{t_{last} C_{last}}{\lambda_z} + \frac{C_{last}}{\lambda_z^2}. \tag{10}$$

2.2 Estimation of Other Parameters

Based on AUC, AUMC, and λ_z, other important pharmacokinetic parameters such as clearance (CL), mean residence time (MRT), steady-state volume of distribution (V_{ss}), volume of distribution during the terminal phase (V_z), and terminal half-life ($t_{1/2}$) can be calculated using the following formula.

After intravenous injection:

$$CL = \frac{Dose_{iv}}{AUC_0^{\infty}}, \tag{11}$$

$$MRT = \frac{AUMC_0^{\infty}}{AUC_0^{\infty}}, \tag{12}$$

$$V_{ss} = CL \times MRT = \frac{Dose_{iv} \times AUMC_0^{\infty}}{AUC_0^{\infty} \times AUC_0^{\infty}}, \tag{13}$$

$$V_z = \frac{CL}{\lambda_z} = \frac{Dose_{iv}}{AUC_0^{\infty} \times \lambda_z}, \tag{14}$$

$$t_{1/2} = \frac{0.693}{\lambda_z}. \tag{15}$$

After intravenous infusion:

$$MRT = \frac{AUMC_0^{\infty}}{AUC_0^{\infty}} - \frac{T_{\text{inf}}}{2}. \tag{16}$$

After oral administration:

$$CL / F = \frac{Dose_{po}}{AUC_0^{\infty}}, \tag{17}$$

$$MRT = \frac{AUMC_0^{\infty}}{AUC_0^{\infty}} - \frac{1}{k_a}, \tag{18}$$

where T_{inf} is the infusion time, F is the oral bioavailability, and k_a is the absorption rate constant.

2.3 Estimation of Parameters at Steady State After Multiple Dosing

The $AUC_{0-\infty}$ after a single dose is equivalent to the AUC during one dosing interval (AUC_τ) at steady state after multiple dosing with a dosing interval τ. The clearance can be calculated as

$$CL = \frac{Dose_{iv}}{AUC_\tau}. \tag{19}$$

The average drug plasma concentration at steady state ($C_{ss,ave}$) can be calculated as

$$C_{ss,ave} = \frac{AUC_\tau}{\tau}. \tag{20}$$

3 Compartmental Analysis

3.1 Compartmental Models

In compartmental analysis, a compartmental model is constructed as a representation of the body to describe the observed drug plasma concentration–time profile. It is assumed that the body is made up of one, two, or multi-compartments that have little physiologic or anatomic significance, but each compartment represents an amount of drug that kinetically behaves as it would be in a well-mixed,

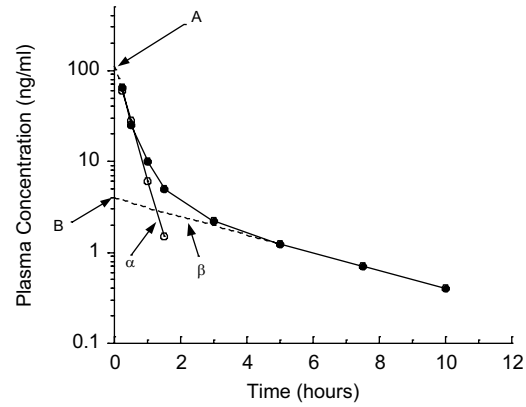

Fig. 1 Plasma concentration–time profile for a drug administered as an intravenous bolus dose and exhibiting a bi-exponential or two-compartment behavior. Residual values are denoted by the *open circle symbol*

homogeneous, and distinct volume. Compartmental analysis is useful to describe drug disposition, to estimate pharmacokinetic parameters, and to predict plasma concentrations following various schedules and doses of administration. The simplest scenario is single-dose intravenous bolus injection. After intravenous bolus injection, one-compartment model assumes that the drug distributes instantaneously to all body areas, while two-compartment model assumes that the drug distributes instantaneously into the central compartment and relatively slowly to the peripheral compartment. In general, for a drug exhibiting a mono-exponential decline of concentration–time profile, one-compartment model should be adequate, whereas, for a drug exhibiting a bi-exponential decline (Fig. 1), two-compartment or more complex model may be needed. The following sections present linear one- and two-compartment model with instantaneous input and first-order elimination as an example to illustrate the concepts and guidelines for compartmental modeling.

In linear compartmental models, the elimination rate and transfer rate of a drug from one compartment to another are governed by first-order kinetics. The rate of change in drug concentration or amount in a specific compartment can be described by differential equations. For one-compartment model with instantaneous input and first-order elimination, the rate of change in the drug concentration in the body is expressed as

$$dC / dt = -K \times C. \tag{21}$$

For two-compartment model with instantaneous input and first-order elimination from the central compartment (Fig. 2), the rates of change in the drug concentration in the central and peripheral compartment are expressed as Eqs. (22) and (23), respectively.

$$dC_1 / dt = k_{21}C_2 - k_{12}C_1 - k_{10}C_1, \tag{22}$$

$$dC_2 / dt = k_{12}C_1 - k_{21}C_2. \tag{23}$$

By using Laplace transforms, the differential equations can be integrated to give the equations for drug concentration in the system. For one- and two-compartment model with instantaneous input and first-order elimination, the drug concentration (C) at a particular time is expressed as Eqs. (24) and (25), respectively.

$$C = \frac{Dose}{V} \times e^{-Kt}, \tag{24}$$

$$C = \frac{Dose\left(\alpha - k_{21}\right)}{V_1\left(\alpha - \beta\right)} e^{-at} + \frac{Dose\left(k_{21} - \beta\right)}{V_1\left(\alpha - \beta\right)} e^{-\beta t}. \tag{25}$$

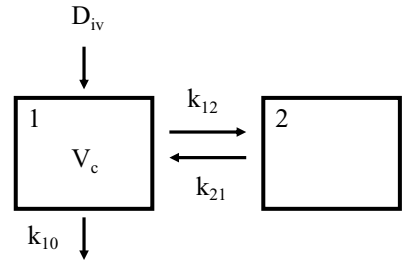

Fig. 2 Two-compartment model parameterized with micro-constants

Equation (25) can be simplified as

$$C = Ae^{-\alpha t} + Be^{-\beta t}, \tag{26}$$

where K is the elimination rate constant, V is the volume of distribution, C_1 and C_2 represent the drug concentration in the central and peripheral compartment, respectively, k_{10} is the elimination rate constant from the central compartment, k_{12} and k_{21} represent the transfer rate constants between the central and peripheral compartment, α and β are macro-disposition rate constants, and V_1 is the volume of distribution in the central compartment.

3.2 Parameter Estimation

Once an appropriate model is selected, the next step is to perform the curve fitting. Pharmacokinetic parameters are estimated by fitting the model to the observed time course of plasma drug concentrations for a given mode of drug input. The concentration–time curve can be fitted to the model using linear regression or nonlinear regression method. Typically, linear regression method is used for determining initial estimates of the parameters, while nonlinear regression method is used to determine the best estimates of the parameters by an iterative type of technique.

For simple models, it is possible to convert a curved line into a straight line. For example, Eq. (24) describes the plasma concentration of a drug exhibiting a mono-exponential decline. By taking the logarithm (natural log base e) of both sides of Eq. (24), Equation (27) is derived, which produces a straight line. The parameters K and V can be estimated from the slope and intercept of the line, respectively.

$$lnC = -Kt + \ln\frac{Dose}{V}. \tag{27}$$

For a drug exhibiting a bi-exponential decline [Eq. (26)], the method of residuals, also called curve stripping, is commonly applied to determine initial estimates of the parameters A, B, α, and β. Figure 1 depicts the method of residuals on the semilog plot of drug concentration versus time. If the ratio between the two rate constants α and β is big enough (e.g., $\alpha/\beta > 5$), at later time points (e.g., $t \to \infty$) the faster exponential term will become insignificant (e.g., $Ae^{-\alpha t} \to 0$), and thus the drug concentration can be described by a single exponential term [Eq. (28)].

$$C = Be^{-\beta t}. \tag{28}$$

As a consequence, at later time point the semilog plot of drug concentration versus time should be a straight line, and the parameters B and β can be determined from the intercept and the slope of this

line (Fig. 1). By knowing the equation of the extrapolated line, one can estimate the concentration on the extrapolated portion of the disposition line and subtract them from observed concentrations in the early phase to determine the residual values [Eq. (29)].

$$residual = C_{observed} - C_{extrapolated} = Ae^{-\alpha t}. \tag{29}$$

The semilog plot of residual versus time should produce a straight line with an intercept value for A and a slope value for α.

However, it is not always possible to convert a function of interest into a straight line, and even when it is possible, it can distort the curve-fitting process. Nonlinear regression is probably needed to determine the best estimates of the parameters of the model. This method typically uses initial estimates of the parameters as a start point and through a series of iterations achieves a minimum value for the objective function (e.g., weighted sum of square, WSS). Commonly used nonlinear regression algorithms, such as Gauss–Newton method, Nelder–Mead method, maximum likelihood, and nonlinear mixed effect model, are implemented in pharmacokinetic modeling software (e.g., WinNonlin, ADAPT II, NONMEM, or other commercially available programs (http://www.boomer.org/pkin/soft.html) for pharmacokinetic analysis).

Using nonlinear regression, the drug plasma concentration–time curve can be fitted to an equation that incorporates micro-constants [e.g., Eq. (25)] or macro-constants [e.g., Eq. (26)]. For example, for a linear two-compartment model (Fig. 2), the micro-constants include V_1, k_{10}, k_{12}, and k_{21}, while the macro-constants include A, B, α, and β. Macro-constants can be estimated from micro-constants and vice versa by the following equations [2]:

$$A = \frac{D_{iv}}{V_1} * \frac{(\alpha - k_{21})}{(\alpha - \beta)}, \tag{30}$$

$$B = \frac{D_{iv}}{V_1} * \frac{(\beta - k_{21})}{(\beta - \alpha)}, \tag{31}$$

$$\alpha = \frac{k_{21} * k_{10}}{\beta}, \tag{32}$$

$$\beta = \frac{1}{2} * \left[(k_{12} + k_{21} + k_{10}) - \sqrt{(k_{12} + k_{21} + k_{10})^2 - (4 * k_{21} * k_{10})} \right], \tag{33}$$

$$k_{10} = \frac{\alpha * \beta}{k_{21}}, \tag{34}$$

$$k_{12} = (\alpha + \beta) - (k_{21} - k_{10}), \tag{35}$$

$$k_{21} = \frac{(A * \beta) + (B * \alpha)}{A + B}, \tag{36}$$

$$V_1 = \frac{D_{iv}}{A + B}. \tag{37}$$

The following secondary parameters can be calculated from the model-estimated parameters using the equations listed below [2].

Half-life during the alpha (initial) disposition phase ($t_{1/2,\alpha}$):

$$t_{1/2,\alpha} = \frac{0.693}{\alpha}. \tag{38}$$

Half-life during the beta (terminal) disposition phase ($t_{1/2,\beta}$):

$$t_{1/2,\beta} = \frac{0.693}{\beta}. \tag{39}$$

Systemic clearance (Cl_s):

$$Cl_s = V_1 * k_{10}. \tag{40}$$

Area under the concentration–time curve (AUC):

$$AUC = \frac{A}{\alpha} + \frac{B}{\beta}, \tag{41}$$

$$AUC = \frac{D_{iv}}{Cl_s}. \tag{42}$$

Volume of distribution of peripheral compartment (V_2):

$$V_2 = \frac{k_{12}}{k_{21}} V_1. \tag{43}$$

Volume of distribution (V_d):

$$V_d = V_1 + V_2 = \frac{k_{12} + k_{21}}{k_{21}} V_1. \tag{44}$$

Bioavailability (F) can be estimated when a drug is given by both an intravenous and an extravascular route (e.g., orally, SQ, IM) using Eq. (45).

$$F = \frac{D_{iv} * AUC_{oral}}{D_{oral} * AUC_{iv}}. \tag{45}$$

In addition, bioavailability can be estimated as a structural parameter of a pharmacokinetic model with simultaneous fit of the oral and intravenous plasma concentrations. This approach was applied to estimate the oral bioavailability of 5-fluorouracil [3], cisplatin [4], and irinotecan [5].

3.3 Model Assessment

When fitting a model to the concentration–time data, the model should fit the data to some degree of precision and accuracy, demonstrate no bias, and follow the rule of parsimony [6–9]. The goodness of fit of the pharmacokinetic model is judged based on visual inspection of the observed and fitted concentration–time curve, examination of the dispersion of the weighted residuals, and inspection of the standard deviation (SD), coefficients of variation (CV), and confidence intervals (CI) of each estimated pharmacokinetic parameters. Ideally, there should be small, random differences between the observed and the predicted data, but no large or systemic deviations in the observed and fitted concentration–time curve. Bias can be detected by the inspection of the weighted residual plots that provide insight into whether the model consistently over- or underpredicts the actual concentration. The weighted residual plots should have a random appearance, but not have any discernable pattern. A weighted residual plot showing a pattern (e.g., a regular "U" or inverted "U" pattern or a tunnel pattern) may suggest that alternative models or weighting scheme should be considered. The SD, CV, or CI of each estimated parameter provides an estimate how well the data is described by the parameters of the specified model. A large CV (>20 %) or wide range in the CI could be due to (1) improper

model (e.g., too many or too few parameters in the model) or (2) insufficient (e.g., not extensive enough) or improper (e.g., poor time selection) sampling schema. Problem (1) can be resolved by selecting other models. Problem (2) can be resolved by performing better experiments using optimal sampling strategy (discussed in later section).

When choosing between several models, the rule of parsimony is followed in that the simplest model that can adequately describe the data should be chosen. Discrimination between the models is guided by minimization of the weighted sum of squared (WSS) residuals [Eq. (46)], Akaike information criterion (AIC) [Eq. (47)], or Schwarz criteria (SC) [Eq. (48)]. Basically, the model with the lowest value of WSS, AIC, or SC is considered as a better model.

$$WSS = \sum_{i=1}^{i=n} (Y_{observed,i} - Y_{estimated,i})^2 . W_i, \tag{46}$$

$$AIC = n \cdot \ln(WSS) + 2 \cdot m, \tag{47}$$

$$SC = n \cdot \ln(WSS) + m \cdot \ln(n). \tag{48}$$

In general, the increase in parameter number (e.g., when using more complex model) improves the WSS. To determine if the increase in parameter number produces a statistically significant decrease in the WSS value, an *F*-test is performed. The *F*-value is calculated using Eq. (49).

$$F(df_a - df_b, df_b) = \frac{WSS_a - WSS_b}{WSS_b} \times \frac{df_b}{df_a - df_b}, \tag{49}$$

where n is the number of data points, m is the number of parameters, df is the degree of freedom ($df = n - m$), and a and b represent the smaller and bigger model, respectively.

After the most appropriate model and weighting scheme are selected, a final step is model testing to determine the predictive ability and potential utility of the final model. A model is validated when it has been demonstrated that extrapolation (e.g., single dose to multiple dose or a change in the dose or infusion duration) is accurate and if study conditions are adjusted (e.g., renal function changes) that the model accommodates the changes and maintains the robustness.

4 Sampling Strategies

When designing a study, we want to collect samples at the optimal times that will give the best information about each of the parameters of the model and best discrimination between models. In phase I studies, the number of blood specimens taken and their timing must allow for the accurate description of the plasma disposition of the drug in individual subjects and for the estimation of individual pharmacokinetic parameter values. Since at the time of phase I drug testing the disposition of a drug in humans is usually unknown, it is necessary to employ an intensive (frequent) and extensive (prolonged) blood sampling scheme. To maximize the chances that all the phases of drug disposition are identified and measured, the following steps are undertaken: (1) sample intensively during drug administration and immediately following the discontinuation of drug administration; (2) sample at time points as far out after drug administration as is feasible; and (3) utilize highly sensitive assay methods. Intensive and extensive sampling schemes provide enough plasma concentration data to select among alternative models which best describe all plasma disposition phases of the drug (e.g., bi-exponential versus triexponential behavior) and allow for the detection and characterization of unexpected dispositional phenomena, such as enterohepatic recirculation.

In later stages of drug development, limited-sampling strategies are employed to allow estimation of pharmacokinetic parameters using a small number of plasma samples (e.g., 1–3). This is possible

only when the pharmacokinetic behavior of an agent is known. One approach to designing a limited-sampling strategy is by combining D-optimality with a Bayesian algorithm [10]. D-optimality uses optimal design theory to select a limited number of sampling times. A Bayesian algorithm then combines information from the limited-sampling scheme with prior information about the population pharmacokinetic parameter values (e.g., the average value and variance) to then estimate pharmacokinetic parameters for individual patients. A linear regression approach is another method to design limited-sampling schemes. This latter method generally allows for estimation of only a single pharmacokinetic parameter such as clearance or AUC. Disadvantages of the linear regression approach include the requirement for consistent timing of infusion duration and blood sampling and all data points must be obtained; all conditions are required for calculation of the pharmacokinetic parameter. The Bayesian algorithm is usually more robust and flexible and allows the description of the full pharmacokinetic profile and estimation of more than one pharmacokinetic parameter [11, 12]. The development of a limited-sampling strategy using a Bayesian algorithm may be approached in the following manner. First, a population pharmacokinetic model is developed and average values for each pharmacokinetic parameter and the variance about the pharmacokinetic parameter are determined. Next, the concentration–time data sets to be used for developing a limited-sampling scheme are randomly divided into two equal subsets, a training data set and a validation data set. Using the sample module of the software program ADAPT II (which employs D-optimality), a limited-sampling scheme is defined for the training data set. The validation data set is then used to validate the limited-sampling scheme. Individual plasma concentrations, at the selected time points, are fitted using a Bayesian algorithm as implemented in ADAPT II, where the Bayesian priors and covariance matrix are derived from the population pharmacokinetic model. Reference pharmacokinetic parameters for individual patients in the validation set are determined using the full pharmacokinetic profile and maximum likelihood estimation. The predictive performance of the limited-sampling strategy is evaluated by calculating the bias and precision of the Bayesian parameter [11, 12].

5 Examples of More Complicated Models

5.1 Models Incorporating Other Compartment in Addition to Plasma

One limitation of compartmental modeling is the potential oversimplification of body processes due to sampling limited to plasma or lack of sensitive analytical techniques. This could be solved by sampling from sites in addition to plasma. The modeling of concentration–time profiles involving samples from sites other than or in addition to plasma may require the development of pharmacokinetic models that are more elaborate than standard compartmental pharmacokinetic models. The compartmental models incorporating central and peripheral plasma compartment and a single cerebrospinal fluid compartment have been applied to fit simultaneously the plasma and cerebrospinal fluid concentration–time profiles of anticancer drugs such as topotecan [13] and erlotinib [14]. Figure 3 shows a three-compartment open model that simultaneously described topotecan lactone and total concentrations in the plasma and cerebrospinal fluid [13].

5.2 Nonlinear Pharmacokinetics

Nonlinear pharmacokinetic behavior can be a substantial source of variability in drug exposure and response. Linearity is evaluated by examining dose and time dependence. Dose dependence is assessed by normalizing plasma concentration–time profiles for dose and examining relationships between

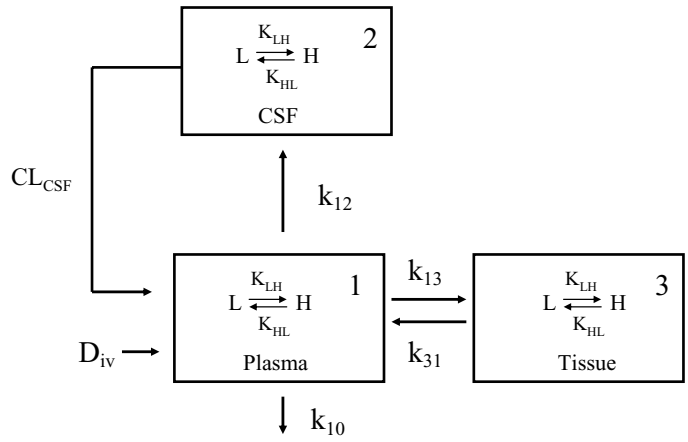

Fig. 3 Three-compartment model for topotecan lactone and total concentrations in the plasma and CSF. *Abbreviations*: Cl_{CSF} clearance of drug from the cerebrospinal fluid (CSF), K_{LH} and K_{HL} the forward and reverse rate constants for the lactone to hydroxy-acid conversion, respectively (adapted from reference [13] with permission)

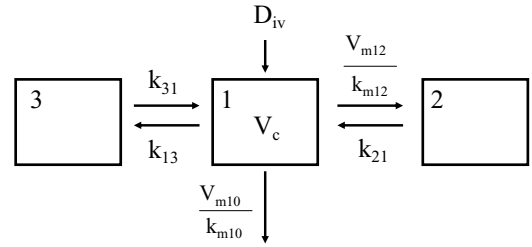

Fig. 4 Nonlinear three-compartment pharmacokinetic model that was fitted to docetaxel concentration–time profiles (adapted from reference [17] with permission)

dose and exposure parameters such as maximum concentration (C_{max}), steady-state concentration (C_{ss}), and the area under the concentration–time curve (AUC). The average value for each exposure parameter is examined and a determination is made if they increase proportionally with increasing dose. Relationships between dose and clearance, half-life, and volume of distribution are also assessed.

Time dependence is evaluated when a drug is given by prolonged infusion or on multiple dosing schedules. During prolonged infusions, a change in C_{ss} over time suggests a change in drug clearance. With repetitive dosing schedules, an increase in pretreatment trough levels over time (in the absence of expected achievement of steady state based on the drugs half-life and dosing schedule) or a change in a pharmacokinetic parameter value suggests a change in drug clearance. For example, changes in AUC or half-life following the first and subsequent doses may suggest that clearance is changing with time.

If apparent nonlinearity is not felt to reflect the dosing schedule, assay sensitivity, or interpatient variability, sources for true nonlinearity should be evaluated. Sources for dose- or time-dependent pharmacokinetics following oral or IV administration may include saturable gut wall transport or first-pass hepatic metabolism, saturable plasma protein and tissue binding, concentration-dependent renal excretion, capacity-limited metabolism, and enzyme induction or inhibition [15]. In addition, formulation effects may affect the apparent nonlinear behavior of a drug [16].

An advantage of compartmental analysis is that pharmacokinetic linearity is not assumed and can be incorporated into a model during any of the pharmacokinetic processes (e.g., drug absorption, distribution, metabolism, and elimination). Nonlinear models have been employed to describe nonlinear pharmacokinetic behavior of anticancer drugs such as docetaxel and a vascular-disrupting agent 5,6-dimethylxanthenone-4-acetic acid in cancer patients [17, 18]. Figure 4 illustrates a three-compartment model that includes Michaelis–Menten saturable distribution into the peripheral compartment and saturable elimination from the central compartment [17].

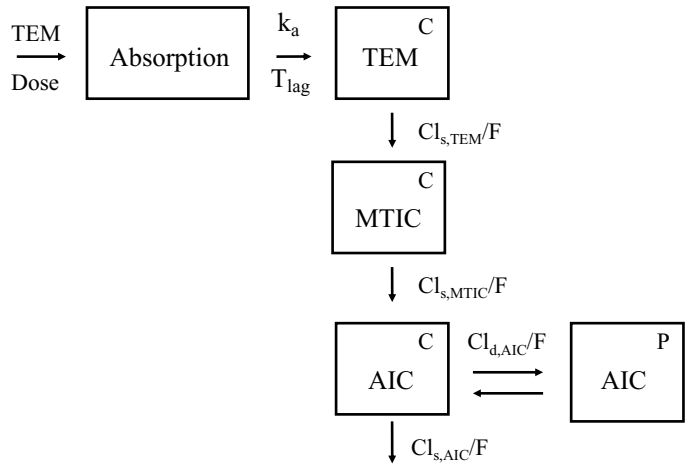

Fig. 5 Compartmental model for TMZ, MTIC, and AIC concentrations in the plasma. *Abbreviations*: *TMZ* temozolomide, *MTIC* 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide, *AIC* 4-amino-5-imidazolecarboxamide, $Cl_{s,TEM}/F$, clearance for the conversion of TMZ to MTIC, $Cl_{s,MTIC}/F$, clearance for the conversion of MTIC to AIC, $Cl_{s,AIC}/F$ AIC systemic clearance, $Cl_{d,AIC}/F$, AIC distribution clearance (adapted from reference [19] with permission)

5.3 Metabolite Kinetics

Many anticancer agents are extensively metabolized to inactive or active metabolites. Pharmacokinetic parameters for a drug metabolite are usually estimated using noncompartmental methods. The AUC ratio of metabolite to parent drug on a molar basis is then calculated to determine relative exposure of metabolite compared to parent compound. Pharmacokinetic parameters can also be estimated using compartmental models that describe the drug metabolite disposition. For example, the plasma disposition of temozolomide and its metabolites were characterized using a one-compartment linear model that had first-order absorption, first-order metabolite formation and elimination, and a peripheral distribution compartment for the metabolite, 4-amino-5-imidazolecarboxamide (Fig. 5) [19]. An alternative model for temozolomide has been described that incorporates clearance of parent drug via both chemical breakdown to 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide and renal clearance [20].

6 Conclusion

Pharmacokinetic modeling is a useful tool to be able to describe and predict concentration–time data. The sampling strategy is the most important first step to adequately describing the pharmacokinetics of a compound. If inadequate sampling occurs, various phenomena may be missed (e.g., a prolonged elimination phase). Although noncompartmental analysis does provide a quick and easy determination of the pharmacokinetic parameters, it is based on the assumption that the pharmacokinetics is linear. This assumption can be remedied by analyzing the concentration–time profile with compartmental analysis. Various pharmacokinetic programs are commercially available that can aid in compartmental analysis of data. Compartmental analysis can involve a simple linear two-compartment model or more complicated models that incorporate nonlinear processes. As early clinical trials incorporate more pharmacokinetic and pharmacodynamic endpoints, the role of pharmacokinetic modeling will continue to grow.

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Pharmacometrics

Satjit S. Brar and Joga Gobburu

Abstract Pharmacometrics is the science of quantifying disease, drug, and trial characteristics with the goal of influencing drug development and regulatory and therapeutic decisions. Techniques employing pharmacometric principles are increasingly being used, allowing for efficient utilization of prior experimental information and ultimately streamlining drug development. Using mathematical and statistical models, modeling and simulation allows a simplification of complex systems under investigation and may be able to predict the effects of various treatment options, and the corresponding consequence, on the future course of the disease process. The summation of information can be used to develop more efficient, and hopefully successful, clinical trials. This chapter summarizes the basic theory and application of pharmacometric techniques. Examples of where such pharmacometric principles have been successfully employed in oncology drug development are presented.

Keywords Pharmacokinetics • Pharmacodynamics • Regulatory • Drug development• Clinical pharmacology

1 The Science of Pharmacometrics

1.1 Introduction

Pharmacometrics is the scientific discipline which deals with the quantitative description of disease processes, drug effects, and the variability in drug exposure and response. Mathematical and statistical principles, along with trial information, are utilized to interpret pharmacological observations obtained

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The chapter was written while the corresponding author (Joga Gobburu) was at the FDA.

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from preclinical to clinical stages of drug development. Moreover, the pharmacometric approach integrates information across the various stages of drug development to ultimately influence therapeutic and regulatory decisions. In essence, the science of pharmacometrics is tailored to improving the efficiency and success in drug development.

The interdisciplinary science of pharmacometrics involves the collaboration of basic pharmacology principles, clinical pharmacology (pharmacokinetics/pharmacodynamics, PK/PD), pathophysiology, statistics, and computational techniques. The incorporation of mathematical and statistical models provides a bridge across the disciplines to explain pharmacological behavior and the inherent variability in drug response, for both desired and undesired effects. A compilation of techniques is used in pharmacometric analyses that primarily involve the modeling and simulation of data. These techniques include population pharmacokinetic analysis, exposure–response evaluation for drug efficacy and safety, clinical trial simulations, and disease progression modeling.

Several researchers have discussed the increasing importance of the use of modeling and simulation for enhancing drug development [1–5]. In oncology, PK/PD and physiological modeling and simulation are increasingly used to improve the understanding of the intricate relations of biological and physiological parameters that affect drug behavior at a molecular level. Moreover, the use of information obtained from the modeling and simulation exercises have been incorporated in clinical trial simulations that ultimately yielded plausible trial outcomes. A comprehensive text on pharmacometrics has been recently published, detailing the theory and different types of analyses performed with modeling and simulation [6].

The following chapter summarizes the theoretical concepts and methodologies employed in pharmacometric analyses during drug development and regulatory review. Specific examples are presented that successfully incorporate these pharmacometric principles in various aspects of drug development.

1.2 General Applications

The value of pharmacometric principles can be exemplified at all stages of drug development and during eventual regulatory review. The techniques used for data analysis creates the ability to translate information across the various stages. A major tenet of pharmacometric application to the drug development process has been eloquently described by Sheiner, coined the “learn-confirm” approach [7]. He asserts that the process of drug development should be science-driven by learning from experience and confirming what has been learned. This approach depends on the application of pharmacometric modeling and simulation to progress through the learn-confirm cycles.

The subsequent steps in the drug development process are devised incorporating the knowledge obtained from already acquired data and an explicitly defined model. Data are collected and pharmacometric models are built to describe data and confirm prior knowledge about the drug candidate. Modeling and simulation is then applied to acquire knowledge from new data to predict future outcomes for safety and efficacy. This process allows making informed decisions about future experiments and trial design.

Specifically, the potential applications of pharmacometric analyses range from candidate molecule selection, identification of biomarkers and surrogates, dosage/regimen selection and optimization, prognostic factor evaluation, benefit/risk evaluation, to clinical trial forecasting. A schematic demonstrating the various applications throughout the drug development stages is illustrated in Fig. 1. Pharmacometric methods provide a coherent, scientifically based framework to maximize the use of information and efficiency of decision making during the drug development and approval process.

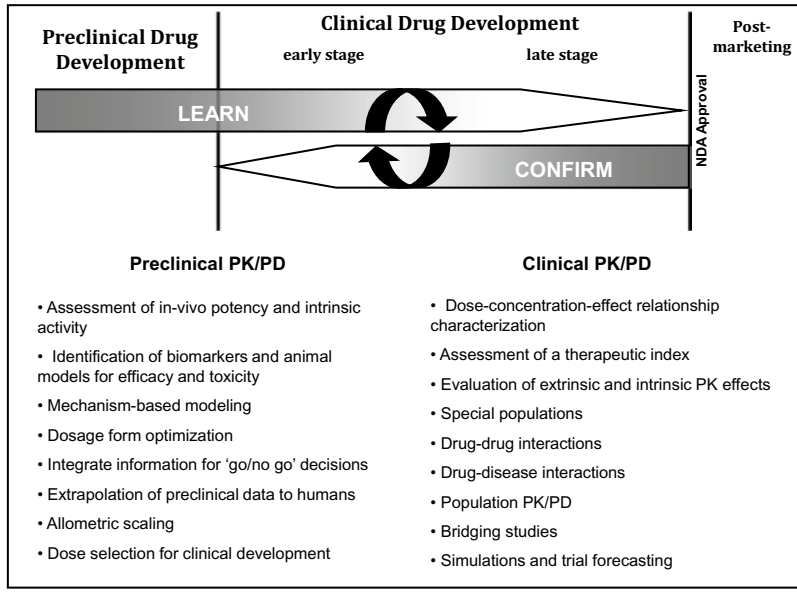

Fig. 1 Applications of the "learn–confirm" approach in drug development. *Adapted from* Meibohm et al. (2002)

1.2.1 Optimizing Antineoplastic Dosage Regimens

In oncology, the main purpose of designing an optimized dosing regimen is to destroy tumor cells and, at the same time, minimize the adverse effects of chemotherapy. Ideally, the fine balance of risk versus benefit for chemotherapy is explored via the administration of different dosing regimens. However, the exploration of several dosing strategies in clinical trials may be costly, unfeasible, and, in some cases, unethical. Simulation of chemotherapy exposures can be used to investigate different dosing schemes to ultimately select the optimal dosing regimen.

For several drugs, a single dosing scheme may not be able to achieve target exposures in majority of patients. This may necessitate dose individualization and therapeutic drug monitoring (TDM). Upon defining the concentration–effect relationship, the use of TDM can improve the clinical use of antineoplastic drugs, most of which have very narrow therapeutic indices and especially variable pharmacokinetics. Pharmacometric modeling can help realize this need and can also provide recommendations for the TDM strategy [8].

One of the most important uses of modeling and simulation is the development of a well-defined exposure–response relationship to support the approval of a dosing regimen not directly investigated in clinical trials. For majority of oncology therapies, the proposed labeling includes dosing regimens studied in registration trials. An exposure–response model can be used to explore intermediate doses that were not studied in clinical trials. This type of analysis, in conjunction with risk–benefit evaluation, may yield a regimen which may offer similar effectiveness with minimized toxicity. In optimizing the dosage regimen, it is important to note that further extrapolation to dosing regimens outside the studied dose range may not be appropriate. Nonetheless, a defined exposure–response model may help guide the design of additional clinical trials involving the antineoplastic therapy.

1.2.2 Future Trial Design

The two most common causes of failure in the late stage drug development are the lack of efficacy and unwarranted toxicity of the oncology agent investigated. Unsuitable trial design and a lack of

integration of prior knowledge are often reasons for the unsuccessful result of these trials. This knowledge gap restricts the information needed to inform the coherent design of clinical trials in human patients. Modeling and simulation provides a path to incorporate prior knowledge and offers a promising way forward to rationally design hypothesis-testing clinical trials.

Quantitative analyses using trial models and clinical trial simulations are useful for strategically designing oncology registration trials. This tailored, knowledge-driven, approach may provide decisive insight into aspects such as dosing (e.g., the number and separation of dose levels), trial design (e.g., adaptive vs. fixed, crossover vs. parallel design), determination of sample size and power (e.g., type I and II error), and evaluation of drug interactions and disease effects [9, 10]. Keeping these factors in mind, clinical trial simulation can aid in the realization of a rational clinical drug development program.

For example, prior information from the early clinical stages allowed for the development of an exposure–response model for degarelix, a gonadotropin-releasing hormone antagonist for the management of prostate cancer [11]. Modeling and clinical trial simulation led to suggestion of a new optimal dosing regimen for use in the registration trial. This integration of knowledge led to the eventual approval of the drug for use in prostate cancer patients [12]. In hindsight, the effectual pharmacometric analyses aided in the rational dosing and design of the trial, ultimately improving the potential for success. The degarelix example is detailed in Sect. 5.1.

1.2.3 Quantitative Disease–Drug–Trial Models

In addition to understanding the drug properties and exposure–response relation, knowledge of the time course of the disease status can aid in the clinical trial design and oncology drug development. Disease–drug-trial models are mathematical expressions of the time course of biomarkers and clinical outcomes, placebo effects, pharmacological effects of drugs, and trial execution characteristics [13]. These expressions can be used in concert to envisage the time course of disease in treated and untreated conditions. In turn, simulations using disease–drug-trial models may be able to predict the effects of various treatment options, and the corresponding consequence, on the future course of the disease process. The entirety of information can be used to develop more efficient, and hopefully successful, clinical trials.

Disease models that quantify the relevant biological system in the absence of drug are further discussed in Sect. 3. Drug models are intended to quantitatively characterize the pharmacology and exposure–response relationship for both efficacy and safety of drugs. In order to integrate information across the development stages, it is imperative that early studies focus on bridging exposure–response across patients, healthy subjects, animals, and in vitro results by performing adequate dose-ranging studies. In turn, the bridging of exposure–response across patients and healthy subjects can aid in designing better future trials for a potential oncology therapy.

Trial models account factors that determine patient characteristics and behaviors, such as inclusion/exclusion criteria, protocol adherence, premature discontinuation, and interdependence (covariance) of baseline variables. All these factors can appreciably influence clinical trial outcomes and should be considered prior to future trial design. Incorporation of these factors during the modeling and simulation of a clinical trial can contribute to providing a better foundation for designing future trials.

1.2.4 Prognostic Factors

In addition to dose-ranging studies, the clinical pharmacology characterization of a new drug involves several studies to identify significant prognostic factors (e.g., body weight, gender, food intake, and hepatic/renal impairment). In oncology, important prognostic factors include patient age, staging of

the disease (i.e., tumor size, grade, and location, presence of metastatic disease), and recurrence of the disease. These prognostic factors can help describe the intended study population, help formulate the study objectives, and ultimately influence the treatment strategy. The causal relationship between prognostic factors and the study endpoint may not be readily available from early drug development, but can be simulated from a previously developed drug model. This requires that prognostic factors be accounted for in the study analysis to evaluate results within and across studies.

The docetaxel exposure–response relationship in patients with cancer was successful in identifying a sub-population more prone to toxicity [14]. Results concluded that patients with elevated hepatic enzymes have a 27 % reduction in docetaxel clearance and are at a higher risk of grade 4 neutropenia. This significant finding was the impetus for dosing recommendations in the label for patients with liver insufficiency. The drug development program of docetaxel exemplifies the value added by the incorporation of prospective planning using modeling and simulation into clinical trials.

1.2.5 Special Populations: Pediatrics

The use of pharmacometric analyses has enabled the implementation of PK studies in special populations, where the number of samples to be obtained per subject is limited because of logistic, ethical, and medical concerns. In particular, modeling and simulation has facilitated drug development in the pediatric population. The prevalent application of pharmacometric analyses in pediatric PK studies can mainly be attributed to its capability to analyze clinical trials with sparse PK data collection, which are common features in pediatric studies.

The use of pharmacometric approaches has been encouraged by the regulatory incentives offered for performing pediatric PK studies during clinical development. The FDA offers a 6-month extension on the patent exclusivity for a new drug, once the sponsor fulfills the requirement of the written request to characterize the exposure–response relationship of the drug in pediatrics. When designing the pediatric trial, the integration historical information (i.e., a well-defined exposure–response relationship in adults) can guide study design and analysis for the use of the same drug in pediatrics. Modeling and simulation is an influential tool that can be used to provide reasonable trial design, rational dosing recommendations and useful labeling information in pediatrics when sufficient understanding of adult and pediatric pharmacology is available [15].

1.3 Model-Based Drug Development and Progressive Model Building

The learn-and-confirm paradigm suggests that the model-based drug development (MBDD) process allows the entire base of pertinent prior knowledge to be integrated into decision-focused recommendations for the future [7]. For example, MBDD can use the wealth of knowledge from predecessor drugs with a similar mechanism of action [16] to develop newer therapies in the same therapeutic class of compounds. Moreover, efficacy and safety drug models can be developed based on preclinical data of the new drug to inform study design for early clinical development. Prior clinical experience with structurally similar molecules can also provide information to serve this purpose. The models can be continually updated throughout clinical development, and thus the attributes of the new drug would correspondingly become better defined.

In a MBDD paradigm, models will be both tools and primary aims of drug development programs. Presently, population models are typically developed at the later to end stages of clinical development. A more practical way to economize time and costs to develop models is to update a model as new knowledge is accumulated. The use of a "progressive model building" (PMB) paradigm allows for this continuous incorporation of new information. PMB allows to carry forward knowledge

throughout the development of a given drug product. At the same time, PMB provides the ability to separate a big problem into several small components that are easier to solve. However, implementation of this paradigm requires an open collaboration of scientists from all disciplines and an institutional commitment to use the "current" model while designing the next trial.

2 Types of Data and Trial Designs

Throughout the drug development process, individual clinical studies are designed to answer specific questions and elucidate pharmacological attributes of the drug. Oncology trial protocols are based on prespecified standards and plans for types of data to be collected as well as analyses to be conducted. Thus, the trial design determines both the data collection and the data analysis methods.

In the early stages, the design of clinical trials is focused on evaluating the PK characteristics and toxicity profiles of the drug in question, making an attempt to define dose-limiting toxicities and the maximum tolerated dose (MTD). Competing treatment schedules and drug-combination strategies may also be explored during this time. Subsequent to obtaining initial safety and PK data, the drug candidate is evaluated for potential pharmacological activity within the specified population the drug is intended for (otherwise known as proof-of-concept trials). Upon deciding to proceed into the later stages of drug development, the focus of the trial design is to demonstrate efficacy compared to standard therapy in the intended population. During this stage of development, the safety aspects of the potential therapy can be further evaluated. At each stage, it is imperative that prior knowledge is efficiently utilized to design future studies. The quality of data obtained from each investigation compels the type of knowledge gained and the ability to utilize the information. Thus, optimal sampling schemes for exposure and endpoint measurement (safety and efficacy) should be devised as part of the clinical protocols.

2.1 Data

The frequency, schedule, and duration of data sampling govern the type of quantitative information that can be obtained from a trial. Generally, there are two types of data that can be acquired during clinical trials, rich data and sparse data. For PK–PD measurements, data are typically collected from trials conducted in a small number of patients over a short time duration. Usually, "rich data" (i.e., several samples from each subject) is collected under controlled conditions. With this sampling strategy, subject-level data can be analyzed independent of the others, in most cases, and then summarized. This kind of data is the best for elucidating the time course of drug exposure and response for the subsequent building of structural models (see Sect. 4.1 for details). Examples of studies that employ "rich-data" sampling are dose-proportionality studies and bridging studies that are performed to evaluate the impact of prognostic factors (e.g., food, renal/hepatic impairment, etc.) on the PK of a drug. Generally, 10–20 samples per subject are collected in these rich-data experiments.

Conversely, "sparse data" are collected in trials that are conducted to appraise the efficacy and safety of a drug, in a large number of patients and for relatively longer durations. The nature of these larger trials necessitates the infrequent sampling of PK–PD measurements for each individual. This sampling strategy poses a challenge to analyze data from each subject separately. Sparse data are most suited to building statistical models (see Sect. 4.1 for details). Examples of studies that collect sparse data are the late stage pivotal or registration trials. In such trials, relatively few samples (1–3) per subject are collected since obtaining several samples from each individual patient may not be feasible.

2.2 Trial Designs

Trial design in clinical oncology investigations have been summarized and deliberated in several publications [10, 17–21]. Specifics of trial design features for oncology drug development are described elsewhere in this handbook. This section provides a general summary of trial designs commonly employed in oncology drug development.

The three most frequently used trial designs are parallel, crossover, and titration. For a parallel study design, subjects are randomized to one of the several treatment options (i.e., placebo/control or different dose levels). While a parallel design will support the estimation of population PK–PD characteristics, individual subject-level characteristics are not easily obtained. In crossover study design, each subject receives a sequence of all treatment options. As this type of trial employs repeated measures within a given subject, this is the most powerful study design for estimating the individual exposure–response relationships. Crossover designed trials are generally longer in duration and may experience carry-over effects from previous treatments, necessitating sophisticated data analysis. Lastly, the titration design employs an incremental increase in dose to patients either until no additional benefit is observed or until dose-limiting toxicity occurs. This design is generally utilized in the initial stages of clinical development and permits the characterization of individual PK–PD parameters.

Trial design can also be governed by the way randomization to treatment is performed. Subjects can be randomized to receive a specified dose or concentration of the test drug or to a particular effect elicited by the drug. Henceforth, such trials are referred to as randomized dose-controlled (RDCT), randomized concentration-controlled (RCCT), or randomized effect-controlled (RECT) trials, respectively. In the case that a placebo control is considered unethical, an active control group can be employed in the trial.

In a RDCT, subjects are randomly assigned different doses of the drug. After randomization, data are collected throughout the trial and subsequently analyzed using appropriate statistical methods. These types of trials are commonly conducted due to the simple execution and analysis of the data.

For RCCT design, a set of target drug concentrations are chosen based on the exposure–response relationship established from prior studies. Using prior information about the drug pharmacological characteristics, target concentrations are chosen and subjects are randomized to one of these prespecified target concentrations [22]. Such a design necessitates an initial dose-titration period. During this period, the doses that ensure the attainment of concentrations within the specified target ranges (ex.: 5 ± 0.5 μg/L) is identified.

A deviation of the RCCT design is when doses may be prespecified based on a specific demographic variable (e.g., body surface area, BSA). This type of design is commonly performed in adult and pediatric oncology trials in which BSA-adjusted doses are routinely administered. Similarly, in an RECT, subjects are randomly assigned to a prespecified target effect level. In this case, the target effects are chosen based on prior knowledge of the drug's exposure–response relationship, and the dose is titrated accordingly.

RCCT and RECT designs have similar requirements for implementation. For these trials, it is necessary to utilize prior exposure–response information for selection of the appropriate target concentration or effect ranges. Moreover, trial conduct will be dependent on an efficient and sensitive analytical assay method with a short turn-around time, and sufficient number of formulation strengths to allow for dose adjustments as needed. Unfortunately, very few drug development programs utilize RCCT or RECT designs. This may be due to their relatively complicated execution and data analysis, compared with the RDCT design, as well as the cost of implementing TDM if the drug is approved [23, 24].

3 Disease Models

Model-based assessment of disease progression has become a significant aspect of drug development. Disease progress refers to the trajectory of a disease over time, which can be evaluated by observing the time course of a biomarker or other clinically relevant measure. This measure should reflect the status of the disease or the clinical status of a patient. A disease model is a mathematical representation of a biological system, in the absence of therapy, and attempts to quantify the time course of the disease. There are three chief sub-models that capture relevant aspects of disease modeling, primarily the relationship between biomarkers and clinical outcomes, the natural disease progression, and the placebo effect. In addition, there are three general approaches that can be applied to building any disease model. These are systems biology, semi-mechanistic, and empirical modeling. The main features of these approaches are summarized in Table 1.

3.1 Biomarkers and Clinical Outcomes

Biomarkers are commonly used as outcomes in clinical trials in lieu of the actual clinical endpoints, especially when clinical endpoints occur after prolonged periods of time. Therefore, the characterization of the relationship between biomarkers and clinical outcomes for a particular pathological condition is a vital aspect of disease modeling. Such models can then support trial design optimization and risk projection based on biomarker information. Systems biology models are very useful for this purpose [25]. Similar to physiologically-based models, systems biology models are based on the understanding of underlying biological system. The generated models attempt to mathematically represent the system at the molecular level, with an ability to account for pathological perturbations to the system. The model parameters are estimated from multiple detailed in-vitro and ex-vivo experiments.

Departing from complexity, empirical and semi-mechanistic models are generally data driven and do not consider details of the underlying and associated biological systems. Semi-mechanistic models simplify the system sufficiently enough to be able to describe the available data adequately. Empirical disease models are mathematical expressions used to interpolate between observed data and seldom relate to the underlying biology. Nevertheless, such simple models are useful and have been employed in making go/no-go decisions and in designing pivotal trials. The empirical parametric hazard model that describes the relationship between the change in tumor size and survival is one such example that

Table 1 Comparison of systems biology, semi-mechanistic, and empirical disease models

Feature	Systems biology models	Semi-mechanistic models	Empirical models
Source of information	Underlying biology with isolated detailed experiments	Typically one or more experiments	Typically one or more experiments
Complexity	Very complex	Relatively simple	Relatively simple
Validation	Very challenging	Relatively simple	Relatively simple
Resources	Extremely involved and diverse expertise needed	Less involved and fewer experts needed	Less involved and fewer experts needed
Scope	Flexible; often interrelationships with related systems also included	Narrow; do not consider related systems	Narrow; do not consider related systems. May not accommodate variations in experimental designs
Application	Target identification; dose selection trial design optimization; risk projection based on biomarker data	Dose selection; trial design optimization; go/no-go decisions	Dose selection; trial design optimization; go/no-go decisions

is used for this purpose. All types of models are useful, but it depends on the question being posed during development.

3.2 Natural Disease Progression

Natural disease progression modeling attempts to describe the change observed in the clinical outcome over a period of time. Drug treatments can modify the natural progression of the disease, and such models can provide insights into the time course and management of several diseases [26]. For example, the natural progression of Alzheimer's disease as measured by the Alzheimer's Disease Assessment Scale–Cognitive score (ADAS-COG) has been described using an empirical linear model [27]. In oncology, the time course of tumor growth has been characterized in patients with non–small cell lung cancer using a modified Gompertz model [28]. Using this model, in conjunction with their drug model, the investigators were able to predict tumor size changes during and after multiple cycles of chemotherapy. Mechanistic models are also being studied since they allow data collected under varied experimental conditions to be analyzed simultaneously. A mechanistic disease progression model for arthritis in rats has been proposed [29].

3.3 Placebo Effect

The effect observed in a placebo group refers to the psycho-socially induced biochemical changes in a patient's brain and body that in turn may affect both, the natural course of a disease, and response to therapy [30]. Although the placebo-effect is not directly associated to the disease, it can considerably impact outcomes observed in trials. For disease conditions that are measured symptomatically, such as pain and depression, this type of phenomenon is commonplace. Therefore, modeling the magnitude and time course of placebo effects can be valuable while projecting net drug effects and also aids in estimating sample size during trial design. Recently, a model that describes the time course of the Hamilton Depression Rating scale (HAMD-17) clinical score in the placebo arms of antidepressant trials, combined with a dropout mechanism, has been developed [31]. This model provides new insights on the validity of the results of several longitudinal registration trials currently used for new drug products.

For oncology trials, the placebo effect is not generally considered to be a significant factor in tumor response. In a review of 37 oncology trials, it was found that a placebo effect was observed with improvement in symptoms such as pain and appetite but rarely associated with positive tumor response [32]. Nonetheless, modeling of the placebo effect for trials associated with the treatment of symptomatic measures (e.g., pain) would aid in trial design of treatments intended to alleviate these associated problems.

4 Types of Pharmacometric Analyses

4.1 Conceptual Framework

Population PK–PD models involve both structural and statistical model components. Structural models account for the population parameters of the model or "fixed effects" and are deterministic in nature. A complete population PK–PD model incorporates four structural components including

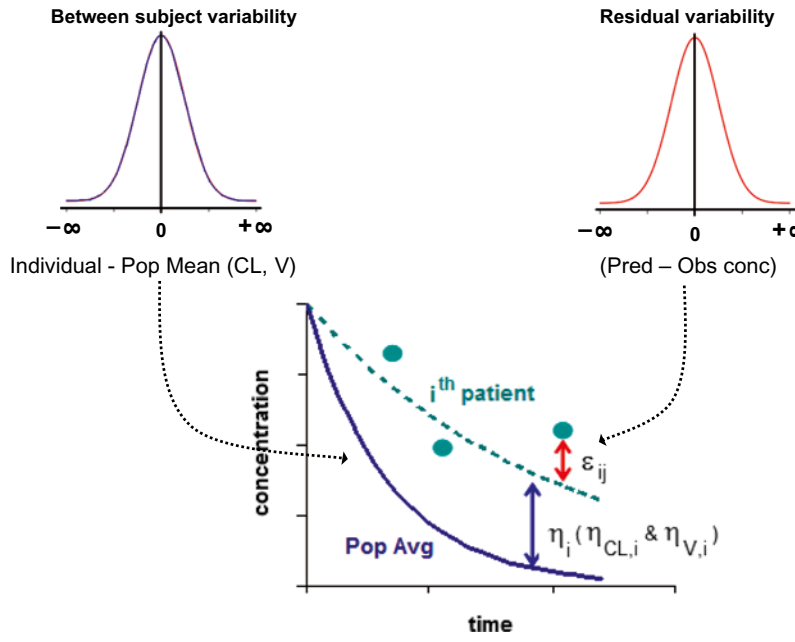

Fig. 2 Basic framework of nonlinear mixed-effect modeling

(1) a PK model, (2) a disease progression model, (3) a PD model, and (4) a covariate model. The average population parameters obtained from these models constitute the "fixed effect" portion of the population model and generally define the average value for a parameter in a population and/or the average relationship between measurable patient factors and pharmacokinetic and/or pharmacodynamic parameters. For example, parameters such as the typical value of systemic clearance for a 70 kg individual and the mean potency (i.e., EC_{50}) of a drug are classified as fixed effects. These components of the model do not account for the inherent variability seen with at the individual and observational levels.

To account for this variability, stochastic statistical models are generally implemented in population PK–PD models to describe the "random effects" seen with observational data. Three different statistical models within a population model are used to describe variability: between-subject variability (BSV) model, between-occasion variability (BOV) model, and within-subject variability (WSV) model. BSV, or interindividual variability, signifies the random unexplained differences between different subjects, while BOV signifies the deviance in an individual between different occasions. WSV, or residual variability, measures the remaining unexplained variability when all other sources of variability are accounted for. Also known as intraindividual variability, WSV may depict model misspecification and/or assay measurement error.

The parameters obtained from these statistical models are population models that quantify the random, unknown variation. The primary assumption with the random effect models is that the between-subject and between-occasion errors (η) are normally distributed with a mean of zero and a variance ω^2. Moreover, the within-subject or residual errors (ε) are normally distributed with a mean of zero and a variance σ^2.

In PK–PD, models that attempt to account for both fixed and random effects together are called nonlinear mixed-effect models. The concept of the mixed-effect model is illustrated in Fig. 2. In this example, consider a one-compartment model where the drug is given as an intravenous bolus and the

Table 2 Main features of the common population analysis techniques

Feature	Naïve averaged	Naïve pooled	Two-stage	One-stage
Uncertainty at observational level (and missing observations)	Ignores; mean will unduly be closer to outliers (extreme observations)	Ignores; mean will unduly be closer to outliers	Accounts; will not be influenced by extreme observations	Accounts; will not be influenced by extreme observations
Uncertainty at subject level	Ignores	Ignores	Ignores; subjects with more or fewer observations are all weighted equally	Accounts; subjects with more data are weighted more
Covariate exploration	Not easy; subjects can be divided into groups based on values of relevant covariates	Not easy; model with known relevant covariates can be imposed	Possible	Possible
Relative complexity	Low	Low	Low	High; needs training

volume of distribution (V) is identical in every individual (no BSV for V). Then, the concentration in the "ith" subject at the "jth" time point can be described using the following equations:

$$C_{ij} = \frac{Dose}{V}.e^{-\frac{CL_i}{V}.t} + \varepsilon_{ij} \tag{1}$$

$$CL_i = CL_{POP} + \eta_{CL,i} \tag{2}$$

In which, CL_i is the estimated clearance of the "ith" subject, CL_{POP} is the estimated population mean clearance, $\eta_{CL,i}$ is the difference between the population mean and individual clearances, and ε_{ij} is the residual error of the "jth" sample of the "ith" subject.

4.2 Population Analysis Techniques

A major objective of population analyses is to estimate population mean values of pertinent model parameters (i.e., mean CL and V) and variances (i.e., BSV for CL and V) as well as the unexplained, residual variability. Another goal of population analyses is to explain the BSV observed using patient-specific covariates such as body size, age, gender, and disease severity. Importantly, this type of analyses helps in estimating the individual parameters (such as CL_i and V_i) required to impute concentrations to perform PK–PD analysis and other simulations at a subsequent stage of analysis.

The most frequently employed methods for performing a population analysis are naïve pooled or naïve averaged analyses, two-stage analysis, and nonlinear mixed-effect (NM) analysis. The main attributes of these methods are summarized in Table 2.

In naïve pooled analysis, individual observations from all subjects are pooled (as if all the data came from a single *giant* subject) to obtain average PK parameters. In essence, a model without between-subject variability (BSV) and between-occasion variability (BOV) is fitted to the pooled data from all individuals. The naïve averaged analysis is a variation of this method which involves determination of the mean of the data at each time point. Both methods provide only the central tendency of the model parameters and the random effects are not estimated. These methods are used more often for preclinical data and are appealing because of their simplicity. On the other hand, since interindividual variability is not estimated and cannot be accounted for using covariates, the potential of naïve pooled analyses is very limited.

In two-stage analyses, the first stage estimates average parameters for each subject from the individual observations, while the second stage involves the estimation of the population mean and variance of the parameters, after adjusting for covariates, if necessary. In this second stage, relationships between patient covariates and parameters are explored. Estimates of both the central tendency and the interindividual variability can be obtained reasonably well. This method of analysis requires sufficient samples per subject to be collected (generally greater number of observations than the number of model parameters). This method assumes that the individual parameters estimated in the first stage are known without any uncertainty, which may not hold true. Moreover, this method of analyses is unable to model sparse observational data and concentration (or dose)-dependent nonlinear processes, which is a serious drawback.

In the nonlinear mixed-effect analysis, data from all individual subjects are simultaneously modeled to yield both population mean parameter and variance estimates. Since both stages of the two-stage method are executed in one step, the nonlinear mixed-effect technique is otherwise known as the "one-stage" method. Subsequent to this one-stage optimization, individual parameters are estimated. This type of modeling is the most robust technique for analyzing both experimental and observational data and does not share the disadvantages of the other aforementioned methods. A primary advantage of the nonlinear mixed-effect method is its capability to conduct meta-analyses, which are valuable in summarizing data across a drug development program. Disadvantages of this analysis method include the necessity of sophisticated software, requiring special training for its use, and that analysis using complicated models can be time consuming.

4.3 Model Qualification

All models are required to be qualified and deemed credible for further utilization. The term "validation" implies a procedure of paramount robustness and is generally not applicable to population PK–PD models. It is the simple fact that the true model and its parameters are not known which discourages the use of the word "validation" for such models. Therefore, the term "qualification" may be more suitable.

Prior to the commencement of any model building, the purpose for which the model is being developed should be clearly specified. A model and its corresponding set of parameters are deemed 'qualified' to perform a particular task if they satisfy certain pre-specified criteria. Various methods exist for exploring these criteria, many of which are graphical or statistical assessments of the observations in relation to measures of the model prediction. Application of a predictive check to a model and its parameters along with Monte-Carlo simulations is one of the effective methods used for model qualification [33–36].

Based on the purpose of the model, qualification techniques can evaluate the descriptive capacity and the ability for extrapolation of the given model. Adequate description of the experimental data will ensure that the proposed model and its parameters are qualified to make trustworthy inferences, within the range of the data studied. Routine diagnostic tests such as goodness-of-fit plots, summary statistics, and precision of the parameter estimates are generally used throughout the modeling process to improve and ultimately qualify a model.

Importantly, the physiological interpretation of model parameters is a significant aspect of model qualification. The model and its corresponding set of parameters should have a conceptual and physiological basis to perform the specified task on which the model was proposed. In addition, the credibility of the model and parameters should be ascertained and deemed satisfactory to a panel of subject matter experts. It is essential to note that there is no prescribed means of assessing whether a model can be used for extrapolation. The credibility of the model, i.e., whether the model was derived from plausible physiological principles that appear reasonable to a panel of experts, is important. Thus, a

model may be considered qualified to predict beyond the range of the data used for building the model, if the descriptive capacity of the model is acceptable and the model and its corresponding parameters are credible.

5 Case Studies

Pharmacometric analyses have been used at various stages of the drug development process in oncology. We present several case studies where such analyses have been employed and have had pragmatic value in decision making. Cases include drugs used for, or in conjunction with, chemotherapeutic agents. Table 3 summarizes all cases while a few selected cases have been elaborated further.

5.1 Degarelix: Optimizing a Dose for Prostate Cancer

Degarelix is indicated for the treatment of advanced prostate cancer. During clinical development, the primary endpoint used in clinical trials was testosterone < 0.5 ng/mL between day 28 and 1 year in 90 % of patients. The dosing goals were to suppress testosterone levels by day 28 of treatment initiation in at least 90 % of the patients and maintain this suppression through 1 year of therapy. The sponsor conducted five early and late phase dose-finding clinical studies but was unable to finalize an optimal dosing regimen. An end-of-phase 2A meeting was arranged between the FDA and the sponsor in March 2005 to discuss a better drug development plan for degarelix.

The aim of the pharmacometric investigation was to determine a rational dosing regimen that would maximize the effectiveness of degarelix in advanced prostate cancer patients. Population analysis was conducted to develop an exposure–response model for degarelix based on the five dose-finding studies conducted by the sponsor. The FDA suggested alternative dosing strategies and clarified the regulatory expectations of the NDA. For initial suppression of testosterone levels by day 28, a higher loading dose requirement was explored. A lower maintenance dose was derived to maintain the testosterone suppression through 1 year of drug therapy. Using a mechanistic PK–PD model and extensive clinical trial simulations, an optimal dosing regimen was suggested for the registration trial. All the pharmacometric analyses were conducted by the sponsor itself, under the guidance of the FDA. The model-based regimen was then evaluated in a registration trial that resulted in positive outcomes and led to the approval of degarelix for this indication.

Degarelix was approved for use in advanced prostate cancer based on a registration trial that employed a dosing regimen that was selected via modeling and simulation, which several prior studies failed to derive [11]. Trials in prostate cancer patients are challenging and costly and early interaction between the sponsor and the FDA enabled more cost-efficient drug development and a smoother review process.

5.2 Busulfan: Determination of Dosing for Pediatric Patients

Busulfex (an intravenous formulation of the drug busulfan) is used in combination with cyclophosphamide as an immunosuppressive conditioning regimen for bone marrow ablation prior to hematopoietic stem cell transplantation. The drug was initially approved for use in adults with chronic myelogenous leukemia. The dose-limiting toxicity associated with busulfan is potentially fatal hepatic venoocclusive disease (HVOD). Clinical studies suggested that a therapeutic window of

Table 3 Summary of several case studies where pharmacometric analysis has had an impact on decision making during different stages of drug development

Drug	Stage	Key questions	Impact	Comments
rPSGL-Ig [37]	Pre-clinical, Animal	☒ Molecule screening ☐ Experimental/trial design ☒ Dose selection ☐ Prognostic factor exploration ☐ Evidence of effectiveness ☐ Evaluation of benefit/risk	☒ Go/no-go ☒ Dose/regimen optimization ☐ Improved trial design ☐ Approval ☐ Labeling ☐ Dose selection-special population	Allometric models developed for PK parameters across animal species and used to predict pharmacokinetic (PK) and dose range for early phase (first-time-in-man) clinical trial.
Tacrolimus [38]	Pre-clinical, early and late clinical	☐ Molecule screening ☒ Experimental/trial design ☒ Dose selection ☐ Prognostic factor exploration ☒ Evidence of effectiveness ☐ Evaluation of benefit/risk	☐ Go/no-go ☒ Dose/regimen optimization ☒ Improved trial design ☒ Approval ☐ Labeling ☐ Dose selection-special population	Early phase trials starting dose based on two animal models (rat and dog). Target conc. range for Randomized Concentration Controlled (RCCT) trials and therapeutic drug monitoring (TDM) based on two animal models and in-vitro PD modeling. Final reduced starting dose predicted by simulations before onset of pivotal trial and confirmed by trial. Improved TDM and cost-efficiency by 3-4 fold reduction in number of blood samples drawn.
Docetaxel [14]	Early clinical	☐ Molecule screening ☐ Experimental /trial design ☒ Dose selection ☒ Prognostic factor exploration ☐ Evidence of effectiveness ☐ Evaluation of benefit/risk	☐ Go/no-go ☐ Dose/regimen optimization ☐ Improved trial design ☐ Approval ☒ Labeling ☒ Dose selection-special population	Identification of a sub-population (liver impairment patients) more prone to grade 4 and febrile neutropenia. Dose reduction recommendations in the label for patients with liver insufficiency to improve the safety profile of the drug.
Myco-phenolate mofetil [39, 40]	Early clinical	☐ Molecule screening ☒ Experimental/trial design ☒ Dose selection ☐ Prognostic factor exploration ☐ Evidence of effectiveness ☐ Evaluation of benefit/risk	☐ Go/no-go ☒ Dose/regimen optimization ☐ Improved trial design ☐ Approval ☐ Labeling ☐ Dose selection-special population	Exposure-response model based on a pilot study used to determine the dosing regimen for a late phase RCCT.

Degarelix [11]	Late clinical	☐ Molecule screening ☐ Experimental/trial design ☒ Dose selection ☐ Prognostic factor exploration ☒ Evidence of effectiveness ☐ Evaluation of benefit/risk	☐ Go/no-go ☒ Dose/regimen optimization ☐ Improved trial design ☒ Approval ☐ Labeling ☐ Dose selection-special population	Modeling and simulation were performed based on five phase 1/phase 2 studies to explore alternate dosing strategies. Joint work with FDA via End of Phase 2A meeting led to selection of final dosing regimen for registration trial that eventually led to approval of the drug.
Busulfan [41, 42]	Late clinical	☐ Molecule screening ☐ Experimental/trial design ☒ Dose selection ☐ Prognostic factor exploration ☐ Evidence of effectiveness ☐ Evaluation of benefit/risk	☐ Go/no-go ☐ Dose/regimen optimization ☐ Improved trial design ☐ Approval ☒ Labeling ☒ Dose selection-special population	2-step pediatric dosing regimen proposed and incorporated into the label: 1.1 mg/kg for patients weighing ≤ 12 kg and 0.8 mg/kg (adult dose) for patients weighing > 12 kg. TDM strategy proposed and included in label to enhance therapeutic targeting.
Everolimus/cyclosporine [41]	Late clinical	☐ Molecule screening ☐ Experimental/trial design ☒ Dose selection ☐ Prognostic factor exploration ☐ Evidence of effectiveness ☒ Evaluation of benefit/risk	☐ Go/no-go ☒ Dose/regimen optimization ☐ Improved trial design ☒ Approval ☐ Labeling ☐ Dose selection-special population	Modeling and simulation employed to project likely outcomes of altered dosing schemes. New dosing regimen proposed that reduced renal toxicity while maintaining efficacy thereby improving the benefit/risk profile of therapy than seen in registration trial. New regimen to be evaluated in future trial based on cardio-renal advisory committee recommendation.
Zoledronic acid [1]	Post-marketing	☐ Molecule screening ☐ Experimental/trial design ☒ Dose selection ☒ Prognostic factor exploration ☐ Evidence of effectiveness ☐ Evaluation of benefit/risk	☐ Go/no-go ☐ Dose/regimen optimization ☐ Improved trial design ☐ Approval ☒ Labeling ☒ Dose selection-special population	The original NDA and post-marketing reports suggested an increased risk of renal deterioration with drug use. Risk of renal deterioration was modeled and all analyses suggested a correlation with drug exposure. Hence dose adjustments were recommended in mild and moderate renal impairment patients. The Dosage and Administration and Warnings sections of the drug label were revised based on the modeling and simulation results.

(continued)

Table 3 (continued)

Drug	Stage	Key questions	Impact	Comments
Micafungin [41]	Early & Late clinical	☐ Molecule screening ☐ Experimental/trial design ☒ Dose selection ☐ Prognostic factor exploration ☐ Evidence of effectiveness ☒ Evaluation of benefit/risk	☐ Go/no-go ☒ Dose/regimen optimization ☐ Improved trial design ☒ Approval ☒ Labeling ☐ Dose selection-special population	The relationships between dose and effectiveness as well as toxicity (liver enzyme elevations) were modeled. Based on the analysis, a 150 mg dose was recommended (between 100 and 150 mg options studied) for approval for the treatment of esophageal candidiasis. A statement in the label was added indicating the greater potential for liver toxicity at this approved dose.
Oral suspension product for prophylaxis of invasive fungal infections in high-risk patients [41]	Late clinical	☐ Molecule screening ☐ Experimental/trial design ☒ Dose selection ☒ Prognostic factor exploration ☐ Evidence of effectiveness ☐ Evaluation of benefit/risk	☐ Go/no-go ☒ Dose/regimen optimization ☐ Improved trial design ☐ Approval ☒ Labeling ☐ Dose selection-special population	An optimal dosing strategy to avoid clinical failure in majority of patients was sought since very large variability in exposures was seen across patients. Modeling revealed a significant exposure-clinical failure relationship that was not confounded by any known demographic covariates indicating that TDM may maximize effectiveness for all patients. Conditions to optimize drug absorption and importance of ensuring adequate plasma concentrations were included in the label. A post-marketing study to evaluate the benefit of implementing TDM was planned.
Drug to treat a mild, moderate, or severe life-threatening disease [41]	Late clinical	☐ Molecule screening ☒ Experimental /trial design ☐ Dose selection ☐ Prognostic factor exploration ☒ Evidence of effectiveness ☐ Evaluation of benefit/risk	☐ Go/no-go ☐ Dose/regimen optimization ☒ Improved trial design ☐ Approval ☐ Labeling ☐ Dose selection-special population	3 registration trials with inconsistent results and a flawed exposure-response model led to development of a new model by the FDA. Stratification by disease severity sowed that most patients with mild disease were non-responders while consistent effectiveness was seen in patients with moderate and severe disease, leading to the equivocal evidence from previous trials. A future study in only moderate and severe disease patients was recommended.
Pro-drug to treat a life-threatening disease [43]	EOP2A	☐ Molecule screening ☐ Experimental/trial design ☒ Dose selection ☒ Prognostic factor exploration ☐ Evidence of effectiveness ☐ Evaluation of benefit/risk	☐ Go/no-go ☒ Dose/regimen optimization ☐ Improved trial design ☐ Approval ☐ Labeling ☐ Dose selection-special population	A non-inferiority trial failed to establish effectiveness primarily due to suboptimal dose selection. Modeling and simulation revealed that body weight was an important prognostic factor for effectiveness and toxicity and per kg dosing of both test and reference drugs would allow a more appropriate investigation of non-inferiority. Indirectly, an optimal dosing strategy for the reference drug for wider application across other development programs was derived.

900–1,500 μmol/L/min in adults was appropriate to balance occurrence of HVOD and leukemic relapse and failure to engraft. The FDA issued a written request (WR) to the sponsor to determine the PK of busulfan in pediatrics (aged 4–17 years) and the optimal dosing regimen in this population that would achieve target exposures.

Using modeling and simulation, the investigation sought to determine an appropriate dosing strategy for busulfex in pediatric patients. A population PK study was conducted to characterize the PK of intravenous busulfan in pediatrics and to provide dosing recommendations . Clinical studies indicated that the therapeutic window was considered to be similar for pediatric patients. However, this was confounded by the increased variability in the PK of oral busulfan seen in pediatric patients compared with adults. Hence, a target therapeutic window with a lower, more conservative threshold for toxicity, than in adults, was used for pediatric patients (900–1,350 μmol/L/min). Body weight, age, gender and body surface area were explored for their impact on pediatric dosing. Simulations suggested that the mg/kg and mg/m^2 based dosing regimens were similar in their efficiency. Exposures obtained with different dosing regimens with 1 to 7 dosing steps including various combinations of weights and doses were evaluated. All the dosing regimens explored had, at best, 60 % patients achieving target exposures after the first dose. Notably, the model revealed that between-subject variability is large (25 %) while the within-subject variability is low (6 %), indicating that the BSV is the key determinant of therapeutic success. This finding coupled with the narrow therapeutic window for busulfan, supports implementation of therapeutic drug monitoring for optimizing drug therapy.

Based on the model predictions, and practical considerations, a two-step dosing regimen was proposed from this study: 1.1 mg/kg for patients weighing $\leq$ 12 kg and 0.8 mg/kg (adult dose) for patients weighing $>$ 12 kg. In addition, considering that about 40 % patients may not achieve target exposures after the first dose, even with the optimized regimen, TDM was proposed to enhance therapeutic targeting. Instructions for dosing and TDM were incorporated into the drug label. This recommended dosing strategy has not been directly tested in clinical trials.

5.3 Disease Progression Model for Non-small Cell Lung Cancer

Lung cancer had the highest cancer-related death rate during the past decade, with rates surpassing that of colon, breast, and prostate cancer combined. Despite the novel efforts and large costs towards finding treatments, anticancer drugs have one of the lowest rates of successful drug development at only 5 % [44]. Even compounds reaching Phase III clinical trials have a failure rate of about 60 %. To facilitate the drug development of novel therapies for NSCLC, a tumor size (i.e., biomarker) and survival (i.e., clinical outcome) model was developed utilizing data from across a number of NSCLC trials [45]. This model can facilitate clinical screening of novel compounds and provides a tool that drug developers can use to perform clinical trial simulations to improve the design of future trials.

The goal of the pharmacometric analyses was to ascertain if there is a relationship between tumor size progression and survival in patients with NSCLC. Four drug registration trials for NSCLC containing nine different treatments were used to develop pharmaco-statistical models that link survival to baseline risk factors and changes in tumor size during treatment. The purpose of developing these models is to leverage prior quantitative knowledge to facilitate future drug development of other NSCLC regimens. Eleven risk factors were screened based on a Cox proportional hazard model. Tumor size dynamics were modeled with a mixed exponential decay (i.e., shrinkage) and linear growth (i.e., progression) model to estimate tumor sizes of individual patients over time. Survival times were described with a parametric survival model incorporating key risk factors and tumor size change as predictors. Results showed that baseline tumor size and the Eastern Cooperative Oncology Group (ECOG) score were consistent prognostic factors for survival. The mixed tumor-shrinkage/progression model was able to describe individual patient tumor size well, especially in the initial

stages of treatment initiation. The overall parametric survival model included the ECOG score, baseline tumor size, and week-eight tumor size change as significant predictors for patient survival time. The survival model developed from one treatment group predicted the survival outcomes for the other eight treatment groups, despite the different mechanisms of action and the fact that they were studied in different trials. When included in the parametric model, tumor size change at the eighth week allows early assessment of activity of an experimental NSCLC regimen.

A detailed description of the model and the simulation results is included in the proceedings of the Clinical Pharmacology Advisory Committee meeting [46]. The survival model and the tumor dynamic model will be beneficial for screening early clinical development candidates, simulating NSCLC clinical trials, and optimizing trial designs. Specifically, the model can be applied to simulate pivotal trials in order to make go/no-go decision early in development, project effect sizes and dose selection.

6 Future Perspective

Throughout both the registration trial and the regulatory review stages, late-phase attrition rates in drug development are alarmingly high [1, 47]. A primary reason for this failure rate is the lack of efficient planning during the early phases of drug development. It has been shown and therefore is a belief that timely application of pharmacometric methods during the drug development and approval process can improve future development plans and reduce these attrition rates [1, 2, 5, 13, 44, 48–50]. However, modeling and simulation should neither be used to substitute clinical trials altogether, nor as a tool to salvage failed trials for regulatory approval. During the initial stage of drug development, communication between the FDA and drug sponsors may help in more efficient planning of drug development. It is expected that the end-of phase 2A (EOP2A) meetings will facilitate this goal via more rational dose selection and reduction in number of cycles involved in the NDA review (FDA 2003).

In oncology, quantitative disease–drug–trial models are a valuable tool for improving future drug development. These models will be increasingly employed to design future trials using clinical trial simulations. Models can be used to perform simulations of expected survival based on tumor shrinkage, or other biomarker, for an investigational drug in early clinical studies. Refinement of these models and simulations with emerging data from new clinical studies will assist with key oncology development program decisions, including optimized dose selection and improved design of survival trials. As adequate experience is gained with a particular disease–drug–trial model suite, a standardized template can be created for the data and analysis submission for that indication. Given the limited resources, consortia on focused topics may be an effective approach toward developing such model suites. The Predictive Safety Testing Consortium (PSTC) is one such effort in this direction.

Increased partnership between the industry, academia, and the FDA is essential for the growth and wider application of pharmacometrics. In addition, increased interaction across the board between experts, such as clinicians, pharmacometricians, and statisticians, is imperative for better appreciation of this field.

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Pharmacodynamic Modeling

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Abstract The pharmacodynamics of anti-cancer agents, as opposed to direct acting medications, pose a unique set of challenges. These challenges include; delayed response, inadequate number of response measurements, and limited set of biomarkers for effect or toxicity. However these challenges can be overcome and more advancements have allowed for limiting the aforementioned challenges. Pharmacodynamic assessment in early stage oncology clinical requires a study design optimized not only for the determination of clinical response and toxicity, but also for optimal measure of pharmacokinetic and pharmacodynamic measures. Pharmacodynamic models, study design, and pharmacodynamic biomarkers for anti-cancer clinical trials are discussed.

Keywords Cancer • Chemotherapy • Biomarkers • Pharmacodynamics

1 Introduction

Pharmacodynamics is the mathematical relationship between a drugs concentrations and its pharmacological and clinical response. The goal of pharmacodynamic modeling is to allow prediction of an individual patient's clinical outcome(s) based on the dosage regimen administered and that patient's specific clinical response given the known pharmacokinetic behavior of the medication prescribed. However, the term pharmacodynamics is often misused to show the response to the drug based on a time course or a dose administered in lieu of pharmacokinetics. Optimal clinical trial design, accurate pharmacokinetic parameter estimation, and appropriate pharmacodynamic analysis are required to meet this goal.

Drug concentrations used for pharmacodynamic analysis are typically plasma or serum concentrations. However, concentrations may be measured at other available sites such as cerebrospinal fluid, ascites fluid, or other easily accessible tissues or fluids. Pharmacokinetic modeling allows prediction of plasma drug concentrations following dose administration. However, application of a pharmacodynamic model alone or in combination with a pharmacokinetic model allows determination of drug

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response at a specified concentration, or based on a specified measure of drug exposure at any time after the administered dose. With this knowledge, the optimal dosage regimen of the drug to be given to a specific patient or patient population can be determined and therapy can be monitored effectively.

The pharmacodynamic study of anticancer agents is beset with several pitfalls and hurdles making the analysis more difficult. These include narrow therapeutic index, delayed response to therapy, difficult to measure direct effect markers and biomarkers, difficult to extrapolate exposure parameters to the site of action, small sample size, and heterogeneous patient populations within early phase clinical trials. The therapeutic index is defined as the ratio of the concentration causing severe toxicity to the concentration at which the drug exhibits the desired pharmacological effect. The therapeutic index for cytotoxic drugs is very narrow or even nonexistent. Anticancer agents typically cause severe and potentially life-threatening toxicities prior to reaching the concentration required for complete remission or "cure." The desired effect of "cure" based on tumor cell kill is not easily measured and is often delayed. For drugs exhibiting an immediate response, an effect can theoretically be observed simultaneously with drug concentrations. However, simultaneous measurement of cell kill based on imaging is a time-dependent process, often taking weeks to detect a change in tumor mass. The lack of a simultaneous and easily measured direct effect, such as tumor burden in many cancers, also limits pharmacodynamic modeling for classical cytotoxic agents. Although for some solid tumors CT scan is an effective measure of response, the measure of tumor shrinkage is delayed and may cause unnecessary continuation of therapy for a drug that might be under dosed or inactive in a particular patient.

An alternative measure of pharmacodynamic response would be a well-validated biomarker. Although the search for reliable and easily measured biomarkers is underway, one main hurdle for identifying reliable biomarkers remains that each drug class and tumor type may possess a different "ideal" biomarker. Moreover, in cancer research, the correct exposure marker may be difficult to identify. Traditional direct response models can reliably predict clinical or pharmacological effect by modeling the measured effect versus the measured or predicted plasma concentration. However, several well-established models for anticancer agents have shown that atypical exposure measures such as time above a threshold concentration, maximum concentration (C_{max}), or area under the concentration versus time curve (AUC) are more applicable [1, 2].

Finally, a major drawback of reliable pharmacodynamic modeling for anticancer agents is due to lack of an ideal clinical trial design. Phase I studies for anticancer agents using healthy volunteers are neither feasible nor would this population exhibit the appropriate pharmacodynamic response. Early phase clinical trials for anticancer drugs employ a small and heterogeneous population and often a heterogeneous population for phase II investigation. Together these drawbacks have hampered the field of cancer chemotherapy pharmacodynamics and limited the usefulness of the results obtained to truly optimize therapy by predicting appropriate response based on drug pharmacokinetics. However, recent advances in clinical trial design and simulation, availability of more practical and accurate measures of tumor response, and identification of viable biomarkers are paving the way to allow optimal pharmacodynamic modeling for cancer therapy.

Novel therapies targeting tumor angiogenesis, invasion, metastases, or signal transduction present the additional challenge that they are not meant to directly kill the tumor cell but alter the biological process which can consequently enable tumor survival. Thus, current studies of anticancer agents are identifying specific pharmacological biomarkers to assess pharmacodynamics. The goal of anticancer drug pharmacodynamic modeling is to optimize the dose such that maximal benefit can be obtained with minimal toxicity. Currently drugs used to treat cancer are most often dosed on body surface area (mg/m^2), weight basis (mg/kg), or as a flat-fixed dose (mg). Clearly, this is not the safest and most effective way to dose cancer therapies. Clinical investigation of anticancer agents has shown that pharmacokinetically and pharmacodynamically guided dosing are feasible strategies [3–7]. These methods would represent an improvement if they were clinically possible and cost effective. Thus, guiding the regimen of an anticancer agent based on its individual pharmacodynamic parameters would represent the most efficient means to dose these therapies [8].

2 Classical Pharmacodynamic Models

2.1 Individual Pharmacodynamic Models

Most clinical pharmacodynamic studies of anticancer therapies approach data analysis in a non-modeling fashion. One typical approach utilizes segregation of patients by response and statistical comparison of the difference in mean or median systemic exposure (C_{max}, or AUC) values (Fig. 1). Initial evaluation of an effect can often most efficiently be conducted by investigation of pharmacokinetics within groups with the largest differences in outcomes. For instance, it may be easier to see differences in the pharmacokinetics of a potential hepatotoxin when the assessed population contains groups of patients who experience no hepatic toxicity or extreme toxicity as compared to a population of patients with all degrees of toxicity. Obviously, later studies will need to determine the feasibility of identifying patients who will subsequently have only mild toxicity compared to morbid effects.

Given the limited nature of the phase I studies and the discontinuity of some of the pharmacodynamic measures, the statistical approach may be the only practical method of pharmacodynamic assessment. Comparisons between pharmacological response value and drug concentration can be modeled using a cumulative linear logistic (logit) model. For pharmacological response parameters described best by a binary function [i.e., response (+) or no response (+)], a simple logit model or a Wilcoxon Rank-Sum analysis may be the most appropriate method to assess differences in response based on exposure. A cumulative logit model analysis might be performed to assess the association between level of clinical response and pharmacokinetic exposure parameter. The ordinal data parameters of treatment response categorized as complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) as previously defined would be used for this analysis. Similarly a cumulative logit model might be used to assess the effect of plasma concentrations on the ordinal parameters of toxicity grade (NCI grade I through IV). If the toxicity data are better described by a binary function, a simple logit model can be used to assess the toxicity as a function of changed pharmacological parameter.

Data measures using a continuous variable may employ comparison of two or more groups using a paired *t*-test or analysis of variance (ANOVA) assuming the data meet the criteria for parametric testing. An alternative method places patients into discrete groups based on their degree of systemic exposure (e.g., mean or median value of exposure) and then compares any difference in the pharmacodynamic response amplitude or duration between the groups (Fig. 2).

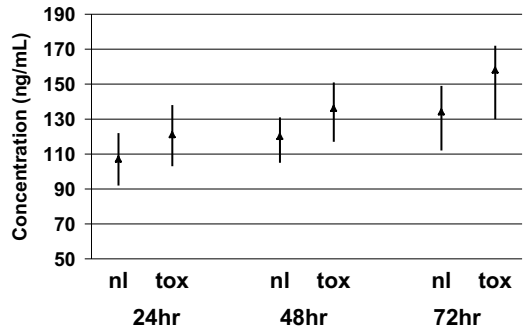

Fig. 1 Example of a pharmacodynamic segregation analysis. Patients were given a potentially nephrotoxic chemotherapeutic agent by continuous infusion over 3 days. Renal toxicity was assessed as a change from baseline. Daily, steady-state concentrations were segregated into groups based on the presence (tox) or absence (nl) of postexposure nephrotoxicity. *Reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press* (*edition 1*), *Totowa*, *N*., *2004*.)

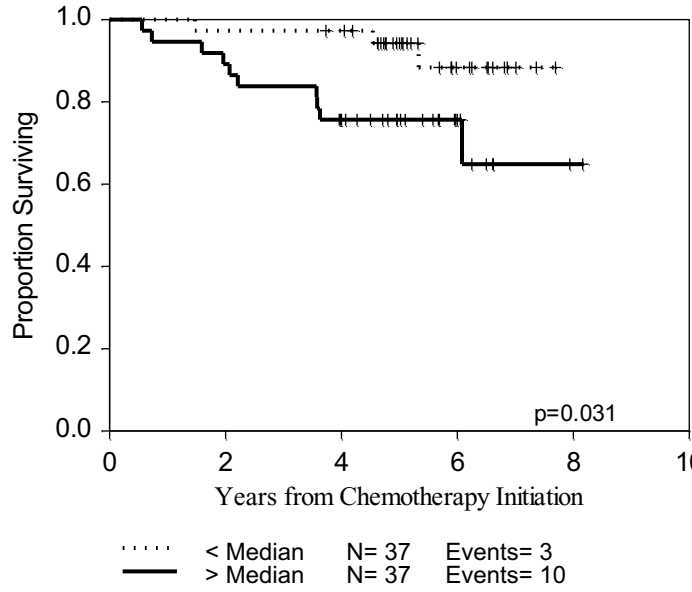

Fig. 2 Example of a pharmacokinetic segregation analysis. Kaplan–Meier survival analysis of patients who were segregated based on the median population systemic exposure to a prodrug (lower values = more exposure to active moiety). *Reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004.*)

Determination of an appropriate approach to model an association with systemic exposure and pharmacodynamics is often initially conceived by the outcome measures and the mechanistic basis for the effect. In situations in which one has multiple response outcomes for one patient (e.g., time-dependent change in biomarker versus changes in drug concentration), the evaluation of each patient's data set can be done via standard two-stage approach. It is typical with oncology studies to have just one pharmacodynamic outcome for each patient (e.g., survival). In the latter setting, the approach would be to evaluate patients in a single stage approach such as grouping all the systemic exposure and survival data points into one file and evaluating at the same time.

Model selection should be based on the mechanistic relationship between the drug and the pharmacodynamic measure expected. Many models used in oncology are based on receptor occupancy (Fig. 3). In these situations one expects no observable effect until a minimal exposure is achieved, followed by a nonlinear increase in effect, a pseudo-linear change in response, and a maximization of the effect, above which no significant increase in response is noted.

Despite the difficulties with pharmacodynamic measures for cancer therapy, the Hill equation (1) [9] and its variants have been used to describe the pharmacodynamic relationship for many oncologic drugs.

$$E = \frac{E_{\max} * C^{s}}{EC_{50}^{s} + C^{s}}, \tag{1}$$

where E is the effect response, $E_{\max}$ is the maximum effect response, C is a measure of drug exposure/concentration, EC_{50} is the concentration/exposure producing one-half the maximum effect, and s is the Hill constant.

These relationships can take the form of direct or indirect-response models and can relate various drug exposure parameters, such as discrete concentration at the time of the response (C), $C_{\max}$, time above a threshold concentration, or AUC, to the pharmacodynamic response variable. Pharmacodynamic response entails changes in circulating plasma proteins involved in tumor growth and metastases, toxicity measurements, radiologic response, or clinical response. Another feasible method employs comparisons between pharmacological response value and drug concentrations obtained from the various dose or exposure groups using a regression analysis to assess validity.

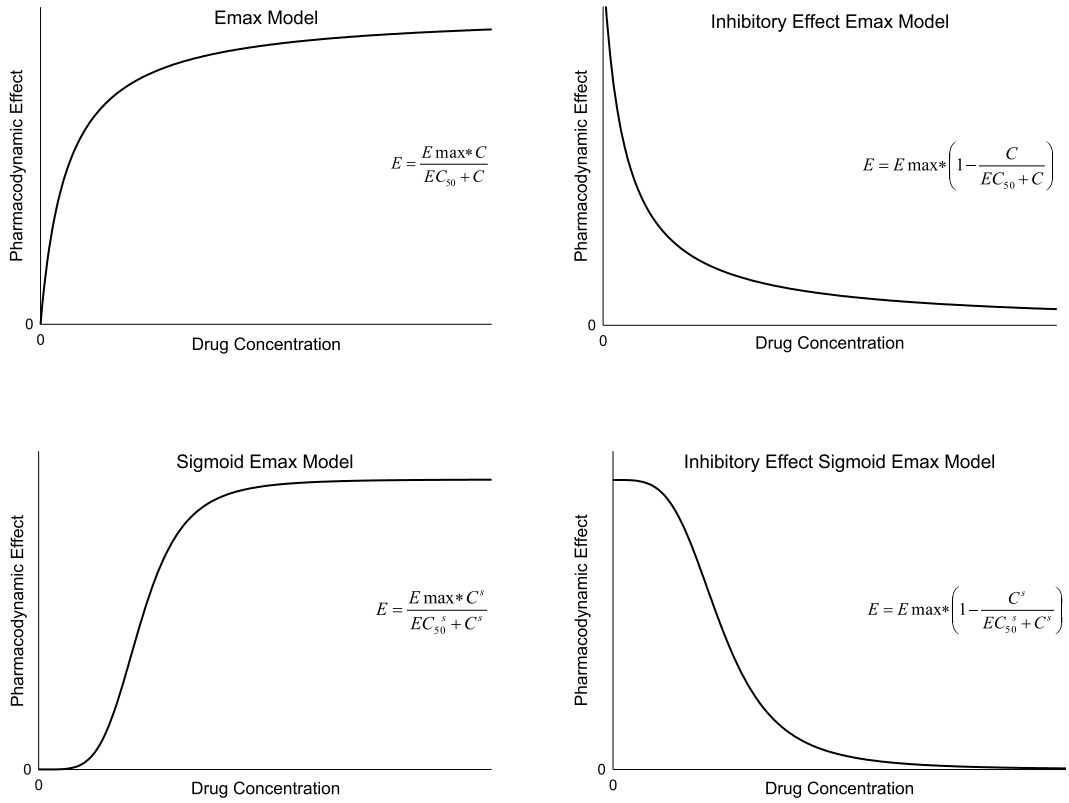

Fig. 3 Examples of pharmacodynamic modeling approaches based on receptor interaction theory. *Reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004.)*

Although it may be relatively easy to identify a relationship between pharmacokinetics and pharmacodynamics, the strength of the association may not be sufficient to justify a therapeutic target without further analysis and data interpretation.

2.2 Pharmacodynamic Model Assessment

Arguably, one of the most appropriate techniques to assess a model's utility is the evaluation of bias and precision [10]. The bias can be easily calculated as the mean prediction error (me) (2), and the precision can be measured as the root-mean-squared prediction error (rmse) (3). Determination of these parameters will enable accurate assessment of how relevant the model will be for future clinical use.

$$me = \frac{1}{N}\sum_{i=1}^{N} pe_i, \tag{2}$$

$$rmse = \sqrt{\frac{1}{N}\sum_{i-1}^{N} pe_i^2}, \tag{3}$$

where me is the mean prediction error, rmse is the root-mean-squared prediction error, and pe is the error of the predicted pharmacodynamic parameter.

2.3 Sampling and Measurement

Pharmacokinetic studies are typically designed with rigor to ensure adequate evaluation of patient-specific parameters such as systemic exposure. Such detail is typically not applied to many pharmacodynamic endpoints; thus their accuracy is not as well controlled. The reasons for this vary from lack of attention to adequate evaluation of such variability to dealing with logistical issues. A good example is modeling of pharmacokinetic data with the pharmacodynamic endpoint of chemotherapy-induced myelosuppression. Routine clinical practice would entail weekly WBC monitoring for many drugs. However, in order to establish a good association between the nadir WBC and chemotherapy systemic exposure, one may need to monitor the WBC at least several times per week or ideally once daily.

Routine lab tests conducted by accredited clinical labs have sufficient quality control such that their data could be utilized in pharmacodynamic studies. However, tests conducted for research purposes on pharmacodynamic endpoints may not have sufficiently stringent controls or acceptable variability (e.g., <15 % (20 % at limit of quantitation) is acceptable for the analytical range of drug concentrations) in order to conduct pharmacodynamic modeling. This could be due to inexperience of the lab conducting the test or the nature of the test itself.

The accurate assessment of effect is essential to the success of a pharmacodynamic model. This is best accomplished if the measure of effect is made at the effect site. These measures can safely be performed for many diseases. For example, a pharmacodynamic study of a proton pump inhibitor may include direct measurement of gastric pH by the placement of a nasogastric tube for sample collection of gastric fluids at appropriate time points. However, the assessment of antitumor effect of an antitumor agent in patients with solid tumors may require repeated tumor biopsy; the risk of tumor biopsy often outweighs the benefit. Although bone marrow biopsy is more easily performed than a solid tumor biopsy, serial samples are typically not obtained. To perform a robust pharmacodynamic study, repeated measurements would be necessary, which is not an option in patients with either solid malignancies or leukemias. Thus the clinical pharmacologist must rely on surrogate measurements for pharmacological response in patient plasma or circulating white blood cells. Changes in transcription or protein expression can be measured in peripheral lymphocytes, but the question is whether changes in normal circulating cells reflect the pharmacology at the tumor site. Preclinical correlative studies are required to validate these types of studies. In tumor xenograft models, the pharmacologist can measure changes in proteins and/or messenger RNA at the tumor site and in the circulating lymphocytes and plasma concentration determination simultaneously. If properly controlled, these preclinical studies can identify the most appropriate surrogate biomarker(s) for clinical pharmacodynamic studies.

2.4 Clinical Trial Design for Early Phase Pharmacodynamic Evaluation

Human pharmacodynamic modeling is most commonly conducted during clinical phase I trials. The reasons for this are twofold: (1) extensive pharmacokinetic data is available, and (2) the largest range of doses is explored in phase I. The doses administered in phase I studies typically vary from ones not exerting any measurable biological effect to those producing intolerable toxicity, thus providing a broad range of dose or systemic exposure versus response profile. If a relationship exists, it should be evident with such a strategy; however this is not an optimal approach to validate associations between systemic exposure and outcome. This is due to the fact that since there is almost always a direct correlation between dose and systemic exposure, associations of the latter term with pharmacodynamic measures may simply be a reflection of the dose–effect relationship. The optimal setting for evaluation of a correlation between systemic exposure and a pharmacodynamic parameter would be in situations where the dose is fixed for all patients (or normalized to body size) such as phase II and

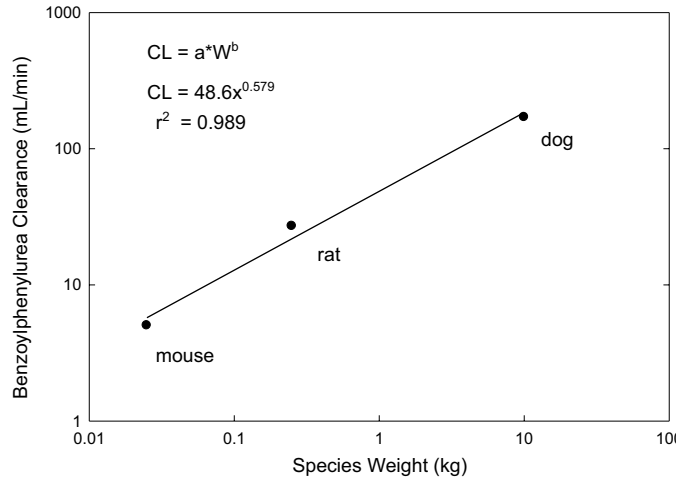

Fig. 4 Example of allometric scaling of benzoylphenylurea clearance. Where *a* allometric coefficient; *W* weight, *CL* clearance, and *b* allometric exponent (Unpublished data, Bauer KS 2002)

III trials. Despite this issue, pharmacokinetic–pharmacodynamic associations are important in many contemporary phase I studies since biomarkers may be used as the sole determinant of dose selection for phase II studies. Later phase studies will typically evaluate relationships between targets demonstrating usefulness on earlier studies with tumor response and/or survival.

The typical phase I trial for first-in-human anticancer agents employs a dose escalation using a modified Fibonacci scheme using a 3 + 3 enrolment strategy. Thus, at lower dose levels, it is likely that only three patients per dose level will be enrolled, leaving a small sample set from which to assess the potential pharmacodynamic response. Often times the sampling scheme is established based on the pharmacokinetic parameters determined in rodents from preclinical toxicology and pharmacology studies. Assumptions are often made that the drug will behave in a similar manner. However it is infrequent that an allometric approach is used to scale up the dose selection and sampling scheme based on scaled clearance and volume of distribution. Allometric scaling could be used to predict the human clearance, volume of distribution, and thus drug exposure (Fig. 4) [11, 12]. By using a typical scale up using only one species, one could predict human clearance and volume of distribution as

$$Y = aW^b \tag{4}$$

where Y is the human PK parameter of interest, a is the allometric scaling coefficient (log a is the y-intercept), W is the body weight (average human body weight), and b is the allometric exponent (b is the slope). a and b are derived by log–log regression of the known species PK parameter versus species weight (Fig. 4).

Studies have shown that the starting dose in humans can be determined based on a single species, assuming a b of 0.67 using the FDA guidance "Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers" [13] using the following approach:

$$HED = NOAEL * \left(W_a / W_h\right)^{1-b}, \tag{5}$$

where HED is the human equivalent dose, NOAEL is the no observed adverse effect dose in the most sensitive species, W_a is the average weight of the most sensitive species, W_h is the human weight, and (1–b) is the allometric slope factor (1–0.67), or 0.33.

Predicted pharmacokinetic parameters (e.g., clearance and volume) and a more rigorous sampling scheme could be employed to accurately define the pharmacokinetic parameters in human. Likewise, information obtained from preclinical pharmacological and biomarker studies could be used to predict an optimal sampling scheme for the identified markers of pharmacodynamic effect. Even by

using these strategies, one cannot always obtain a reasonable prediction for human PK as demonstrated in the first-in-human studies with UCN-01 [14]. Thus, it is recommended that first-in-human studies use a real-time pharmacokinetic evaluation plan such that the drug concentrations and PK parameters can be determined following each dose escalation and allow for protocol update to most accurately study the pharmacokinetic and pharmacodynamic of anticancer agents in this setting. An alternate approach to human pharmacokinetic parameter determination uses a micro-dosing technique expected to have no toxicologic effect on the subject while allowing for determination of pharmacokinetic parameters in humans prior to initiating the phase I trial. This type of clinical assessment is now termed a phase 0 clinical trial (see Phase 0 Trials in Oncology Chapter).

3 Population PK/PD Models

3.1 Population Models

Later phase evaluation of pharmacokinetic–pharmacodynamic effects may employ sparse pharmacokinetic sampling approaches. These could entail utilization of Bayesian algorithms or traditional population-based models such as those employed by NONMEM. There are several contemporary large studies that have utilized this strategy to identify potentially important physiologic markers which are associated with inter-patient variability in pharmacokinetics. The typical approach is to follow up this type of analysis with a study which then validates the relationships using traditional full assessments of pharmacokinetics and pharmacodynamics. For more information on population models, see the Pharmacometrics: The Science and Application in Oncology Drug Development Chapter.

4 Classical Pharmacodynamic Measures

If the ultimate goal of pharmacodynamic study is to optimize the drug regimen, then the primary markers of effect representing this endpoint for anticancer agents would be absolute tumor burden. However, this is very difficult, if not impossible. Using categorical measures of toxicity and response, establishing easily obtainable continuous measures for toxicity and identifying surrogate markers and biomarkers for effect are the only measurements that can be performed. The type of data obtained from the effect measures may be continuous, scalar, categorical, or discrete/binomial variables. The type of variable plays a major role in the choice of pharmacodynamic model that can be used. Thus, for any anticancer agent being studied, the investigator must determine the best choice of endpoint to be measured based on sampling feasibility, site of sample acquisition, availability of resources for measurement determination, cost, and, ultimately, how well it relates with actual clinical response.

The typically used categorical measures for toxicity are CR, PR, SD, and PD. An additional parameter of minor response (MR) may also be used on occasion. Clinical toxicity is a scalar variable with a value of 1–5 based on a predetermined grading scheme such as the NCI common toxicity criteria. Measures of toxicity are often a simple measure, while surrogate biomarkers must take into account the mechanism of action of the agent. As previously mentioned, one of the challenges in the study of pharmacodynamics of cancer therapy is the delayed measurable effect of decreased tumor burden (e.g., tumor shrinkage) or measurable toxicity (e.g., decreased blood cell counts). For classical cytotoxic chemotherapies, several relatively noninvasive easily measured indices have been established (Table 1). However, molecularly targeted agents, which act on specific signal transduction pathways or cellular and molecular processes, require more complex measures to determine the early effects of

Table 1 Examples of cytotoxic chemotherapeutic agents and reported pharmacodynamic measures

Drug	Pharmacodynamic measure	References
Carboplatin	Thrombocytopenia, leukopenia	[15, 16]
Cisplatin	Nephrotoxicity	[17]
Docetaxel	Neutropenia	[18]
Doxorubicin	Neutropenia	[19]
Etoposide	Leucopenia, neutropenia	[20]
Fluorouracil	Leucopenia, mucositis	[21]
Ifosfamide	Neurotoxicity (orientational disorder)	[22]
Irinotecan	Diarrhea, neutropenia	[23]
Methotrexate	Mucositis	[24]
Paclitaxel	Neutropenia, peripheral neuropathy	[25]
Topotecan	Neutropenia	[26]

Table 2 Examples of investigational agents and pharmacodynamic measures assessed

Drug	Pharmacodynamic measure	References
Antiangiogenesis agents		
Carboxyamidotriazole	Serum VEGF, serum bFGF	[6]
Col-3	Plasma MMP-2, plasma MMP-9, plasma VEGF	[27]
Semaxanib	Urine VEGF, urine bFGF	[28]
Signal transduction		
BAY 37-9751	Phosphorylated ERK1 and ERK2 (in lymphocytes)	[29]
Cetuximab	KRAS mutations	[30]
Crizotinib	EML4-ALK fusions (in tumors using FISH)	[31]
Gefitinib	Activated EGFR, activated MAPK (skin biopsy)	[32]
Tipifarnib	Farnesyl protein transferase activity (bone marrow)	[33]
Vemurafenib	BRAF V600E mutation	[34]

the drug in the clinical setting. Since many of these new drugs are not directly cytotoxic and target aberrant cellular processes and pathways, they may not exhibit the hematologic toxicity seen with the classical cancer chemotherapies. However, they are not without side effects; nonetheless, identifying an easily obtainable marker for effect may present more of a challenge (Table 2). For agents targeting tumor angiogenesis, markers of angiogenic signaling might be measured in the plasma, urine, or in biopsy tissues if available. For agents targeting specific signaling pathways, a downstream protein or event might be measured in lymphocytes, skin, or tumor tissue. One approach for monitoring agents that are designed to stimulate apoptosis is a radiolabeled annexin V product. Annexin V has been shown to bind to phosphatidylserine, an intracellular membrane-associated protein. During apoptosis phosphatidylserine is expressed on the external cell membrane. By labeling annexin V with a radioimaging agent such as 99Tc, the rate of apoptosis can be measured [35]. This approach may allow quick, noninvasive, and direct measure of tumor response in patients with a number of solid tumors. This technology could lead to the ideal pharmacodynamic marker, a means to directly determine tumor burden in real time. In addition to the mechanism of action of the agent providing the means for determining a biomarker, the disease itself may also produce a specific biomarker for tumor burden. In the cases of prostate cancer and ovarian cancer, prostate-specific antigen (PSA) and CA-125 are commonly used as clinical biomarkers for disease progression and can also be used as pharmacodynamic measures for drug response.

Once the optimal endpoint for the agent has been selected, practical issues involving the clinical setting and sampling issues are then addressed to appropriately fit the model to the data. The endpoints for pharmacodynamic assessments are typically determined by what is suspected to be the

drug's primary target for effect. Broad classifications include receptor binding, alteration of enzymes/proteins, membrane interactions, or transport blockade. Target identification will allow for speculation as to the immediacy and duration of response. For example, if a drug is known to stimulate an alpha adrenergic receptor, an immediate effect may be anticipated, but one which may display altered response over continuous exposure time. Tachyphylaxis and hypersensitivity reactions are good examples of time-dependent pharmacodynamic effects. Perhaps the most difficult to evaluate are chronic pharmacodynamic effects of cancer therapy. Examples of such chronic effects include delayed growth effects, impaired learning, cardiac toxicity, and secondary malignancies.

It is important to realize that pathophysiologic factors may also influence the pharmacodynamic effect in an individual patient. For instance, a person who has been previously exposed to multiple cycles of myelosuppressive chemotherapy is likely to experience a greater effect from subsequent myelosuppressive agents due to depleted bone marrow reserves.

5 Pharmacodynamic Biomarkers

5.1 Oncology Biomarkers

Complexity of treatment decision-making in malignancies is in part due to intra-patient variability in response to chemotherapy and in part due to the complex and heterogeneous biology of malignancy. Without the ability to identify patients who may or may not benefit from a certain therapy, there is a high possibility of exposing the nonresponders to significant toxicity. For example, more than 50 % of patients undergoing chemotherapy for advanced and metastatic colorectal cancer do not show significant evidence of benefit. Identifying a measurable indicator (e.g., appropriate biomarker), which acts as a surrogate marker to chemotherapy response variability, allows us to decide between implementing an immediate treatment and deferring therapy and to select the appropriate chemotherapy agent(s) as well as modifying the regimen if needed [36–38]. Individualized anticancer therapy combined with biomarker-based monitoring for the efficacy of the regimen holds enormous potential for optimizing anticancer therapy and reducing cancer-related mortality and morbidity [39].

Biomarkers include an array of different modalities such as, but not limited to, physical symptoms, mutated DNAs and RNAs, processes such as cell death or proliferation, and serum or tissue concentrations of molecules or secreted proteins [36]. Although a biomarker can be a physical or physiological parameter, the term is now typically refers to molecular biomarkers [37].

Biomarkers are defined as molecules that are objectively measured and evaluated to be used as surrogate indicator of normal or disease processes as well as pharmacological responses to therapy [40]. Biomarkers can be prognostic, predictive, or surrogate. A prognostic biomarker identifies patients with differing risks of a specific outcome, while a predictive biomarker can be used to predict the response to a given therapy [41, 42]. A surrogate biomarker can be used "to substitute as an intermediate for a clinically meaningful endpoint" [42]. Predictive and surrogate biomarkers are more important in direct treatment decision-making strategies [42].

Proteins as biomarkers, measured in serum and/or tissue, can be used as indicators of the existence, progression, or recurrence of cancer. In addition to early detection of cancer, measurement of panels of protein biomarkers holds promise for personalized cancer therapy, treatment monitoring and optimization, and identifying response to therapy or progression of the disease [39, 42]. A single protein biomarker can be cancer and non-cancer specific, such as elevated prostate-specific antigen (PSA) in prostate cancer, benign prostatic hypertrophy (BPH), or prostatitis, or overexpressed in several disease states, such as interleukin 6 (IL-6), in oral, prostate, lung, multiple myeloma, and renal cell cancers [39, 43]. Therefore, a single biomarker might not offer adequate predictive value, and panels of proteins may be necessary when reliable cancer detection and monitoring is required [39]. However,

detection of panels of biomarkers is complicated not only because both normal and elevated serum levels of biomarkers need to be accurately measured but also because their concentrations may vary widely in the serum [39].

A comprehensive biomarker pipeline includes six essential process components including candidate discovery, qualification, verification, research assay optimization, biomarker validation, and commercialization [37]. In summary, a widely used coherent and comprehensive process for novel cancer protein biomarker's discovery and validation has three phases: discovery, verification, and validation [44].

5.2 Biomarker Identification

Pharmacodynamic studies investigate the molecular, biochemical, and physiological effects of the drug on the organism. Such investigations enable identifying how the drug binds and modulates its target, initiates the mechanism of action, and produces a therapeutic or secondary effect. Therefore, pharmacodynamic studies can help to determine and quantify these biological effects which, consequently, help to determine the optimal biological dose in vivo. Since a therapeutic agent can also affect the downstream signaling pathways, identifying reliable biomarkers would permit prediction of the individual response of each patient. A reliable biomarker needs to be a marker of both sensitivity and resistance to a therapeutic compound. Biomarkers are identified during preclinical in vitro or in vivo studies. Thousands of potential biomarkers can be generated by utilizing high-throughput techniques and powerful discovery and screening technologies, such as DNA microarrays and proteomic profiling of various biological sources such as tissue, proximal biological fluids, cell culture supernatants, and serum [39, 44, 45]. Several proteomic technologies have allowed significant progress in cancer biology including matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), surface-enhanced laser desorption/ionization (SELDI), and material-enhanced laser desorption/ionization (MELDI). These are relatively simple and high-throughput techniques that analyze with high sensitivity and specificity intact proteins expressed in complex biological mixtures, such as serum, urine, and tissues [46]. The generated list of candidate proteins can then be shortened to about 50–100 molecules by applying a set of predefined criteria, involving semiquantitative assessments, multiple bioinformatic analysis, and literature search. These candidates are then moved along to verification phases, during which their discriminatory potential for differentiating cases from controls in order to diagnose cancer, predict prognosis, stratify therapy, or detect recurrence is assessed through quantitative analysis in about 50 to several hundred patients. Unfortunately, the majority of these potential candidates are rejected either because they cannot discriminate between cases and controls or because they are outperformed by other clinical biomarkers currently in use [44].

5.3 Biomarker Validation

A clinically relevant biomarker needs to be directly useful in modifying treatment algorithm. Although a host of different biomarkers have been verified, only a few hold actual clinical validity. Some of these biomarkers are discussed below. In non-small cell lung cancer (NSCLC), perfusion CT can be considered a reliable biomarker because it provides direct quantification of vascular function and consequently provides assessment of perfusion and angiogenesis in lung cancer [47]. EGFR gene copy number can also be assessed as a potential biomarker associated with survival in patients with advanced NSCLC receiving single-agent treatment with EGFR tyrosine kinase inhibitors (TKIs) [40]. Some of the prognostic and predictive markers in colorectal cancer (CRC) include carcinoembryonic

antigen (CEA), lactic dehydrogenase (LDH), guanylyl cyclase C (GCC), high-frequency microsatellite instability (MSI-H), loss of heterozygosity (LOH), and thymidylate synthase (TS) [48].

Circulating tumor cells (CTCs) are considered as a biomarker to assess the severity of cancer. CTCs, "defined as nucleated cells lacking CD45 and expressing cytokeratin," are isolated tumor cells disseminated from the tumor, e.g., breast cancer, which can be identified and measured in the peripheral blood. While CTC level does not correlate with radiographic measurable disease burden, it correlates with the extent of metastatic bone involvement and can reflect the outcome [49].

A high association has been established between expression level of class III b-tubulin and disease progression in patients with metastatic breast cancer who are receiving epirubicin/paclitaxel [50].

The only protein biomarker currently recommended by the American Cancer Society as an early cancer screening tool is PSA. The danger zone for PSA serum concentration is 4–10 ng/mL, a level indicating the possibility of early stage prostate cancer, while normal levels are typically 0.5–2 ng/mL. Late stage prostate cancer is characterized by values of 10–1,000 ng/mL [39]. However, several novel biomarkers have been investigated in castration-resistant prostate cancer including LDH, hemoglobin, Ras/Raf mutations, tubulin mutations, androgen receptor splice variants, CTCs, c-met/HGF activity, androgen synthesis precursor levels, DNA repair defects, Myc amplifications, bone turnover markers such as high urine N-telopeptide and trap-5b, p 16 levels, and ki-67 [42, 51, 52].

GCC, a brush border membrane receptor found exclusively on the epithelial cells of the small intestine and colon, is overexpressed in intestinal cancer, as well as CRC metastatic to the liver and lymph nodes. Quantitative GCC mRNA analysis in lymph nodes by RT-PCR can identify micrometastases with a sensitivity of 93 % and a specificity of 97 % in patients with resected colorectal cancer [41].

Presence of high-frequency microsatellite instability (MSI-H), a phenotype characterized by impaired DNA mismatch repair, is associated with improved survival for patients with MSI-H tumors [41].

CA 19-9 is the most sensitive and specific biomarker for pancreatic cancer. It does not hold any value for diagnosis; however, higher preoperative CA 19-9 levels correlate with lower resectability, more advanced stage, and lower survival [41].

5.4 Validated Cancer Chemotherapy Biomarkers

Upon identification and verification, candidate biomarkers must be evaluated and validated in order to determine their real utility [45]. Usually 2–5 proteins are moved forward into the final phases of clinical validation, in which they are tested by means of established quantitative assays with high analytical sensitivity and specificity in a large cohort of clinically relevant samples (several hundred to thousands of samples per group), collected either retrospectively or prospectively [44]. Ideally, a cancer biomarker's "repeatability, robustness, and accuracy (sensitivity, specificity)" must be validated through analytical measures while considering the "performance characteristics of the test itself, storage conditions, stability, inter- and intra-patient variability (signal to noise), and internal and external validity" in several phase three clinical trials [42]. A fully validated cancer biomarker must have high clinical specificity and sensitivity (e.g., >90 %) to avoid false positives and false negatives which are crucial parameters in avoiding misdiagnosis [39]. A validated biomarker allows confirmation of the pharmacological and biological mechanism of action in patients, contributes to optimal biological dose selection, identifies the best schedule of administration, or minimizes the secondary effects [45]. Furthermore, by permitting prediction of efficacy and safety of a specific compound, biomarkers provide a platform to optimize pharmacological development, to increase the confidence in each step of the validation, and to contribute to decision-making strategies in the final approval of a drug [45]. Immunohistochemistry techniques are important in biomarker validation in cancer tissues [39]. Serial tumor samples or indirect biological samples, such as peripheral blood or mucous or skin biopsies, can be analyzed by PD biomarker analysis involving Western blot or ELISA-based assays to determine the biological effect and to validate their equivalence [53].

Plasma provides a source for many biomarkers such as CTCs, proteins, and metabolites; however, it might have low sensitivity and measurements can be compromised by variability in sample collection as well as handling and storage. Normal tissue surrogates, such as hair follicles, can also be considered; however, they may not exhibit the targeted pathway expressed in tumor cells [54]. Noninvasive imaging endpoints offer great advantage in this regard for assessing PD markers of drug activity. And different imaging techniques can be used to measure blood volume, blood flow, and several semiquantitative and quantitative kinetic hemodynamic parameters. Such techniques include CT, MRI, PET, single-photon emission computed tomography (SPECT), ultrasound, and near-infrared optical imaging [55].

Functional imaging techniques can also be used in measuring biological effect(s). These techniques are based on the noninvasive monitoring of target lesions by procedures such as CT, PET, or MRI [45]. For example, to monitor pathophysiologic changes in tumor vascular structure and functionality in response to antiangiogenic agents, molecular and functional imaging techniques, such as dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI), PET, and dynamic contrast-enhanced computed tomography (DCE-CT), have been developed [53]. Additionally, noninvasive imaging techniques can be used to monitor metabolism, proliferation, and cellularity of the tissue and, therefore, can be used as a method for metabolic imaging of pharmacodynamic biomarkers [54]. Moreover, perfusion computed tomography may provide prognostic and predictive pharmacodynamic information by measuring tumor perfusion and neoangiogenesis after administration of antiangiogenic agents. Currently the primary biomarker validation research has focused on early detection and prognosis of cancer; many of these biomarkers, as reasonable effect parameters, can be used for pharmacodynamic modeling to guide dosing and predict response to anticancer therapies.

6 Model Implementation

6.1 Model Implementation and Assessment

Attention to methods employed is vital in order to ensure that the parameters selected to evaluate relationships between drug exposure and pharmacodynamic response are adequately described. Typical drug exposure parameters utilized in oncology studies include total systemic exposure(AUC), maximal drug concentration, minimal concentration (for multidose), and concentration above a target value. Preclinical data (both animal and cell cultures) can often be useful to assist in determination of the optimal parameter. Most sampling schemes will be adequately constructed to allow estimation of AUC. However, accurate determination of time above a target concentration or other exposure parameters mandate a degree of attention to the concentration versus curve profile and the sampling strategy with an emphasis on duration of sampling to assess the terminal portion of the curve.

A variety of pharmacokinetic programs are used to conduct pharmacodynamic modeling. Many programs (e.g., WinNonlin, Pharsight, Inc.; ADAPT II, BMSR, UCSF) have embedded the standard receptor-based equations in their model libraries. Pharmacodynamic modeling is often conducted based on changes from a baseline value of a measurable biomarker. Measurable physiologic biomarkers can be affected by diurnal changes, day-to-day variability, as well as a variety of other factors including diet and coadministration of other medications. It is good practice to obtain at least two baseline values for pharmacodynamic evaluations that involve continuous data of an endogenous biomarker. Initial model selection also involves determination whether the model will be based on a direct effect or an inverse (inhibitory) relationship. The process of identifying the most appropriate model involves a statistical approach similar to that used for pharmacokinetic model identification (see Pharmacokinetic Modelling Chapter for more details).

While it is relatively easy to identify relationships between pharmacokinetics and pharmacodynamics, the strength of the association is often not sufficient to justify utilization of the data for establishment of a therapeutic target.

6.2 Data Interpretation

Establishment of a relationship between pharmacokinetics and pharmacodynamics is typically used to justify investigation of therapeutic drug monitoring in the clinical setting. Simply establishing the link does not mean that individualization of doses can be applied easily and accurately. However, the data may be utilized to determine the optimal schedule of administration and provide clues toward the likely biological mechanism of action. For example, if the time of plasma exposure experienced above a potentially cytotoxic concentration relates to response better than the maximal observed concentration, it would suggest a cycle-dependent mechanism and a prolonged exposure regimen may be preferred for future studies based on these data. Thus, the pharmacodynamic assessment of anticancer agents will rely heavily on effect marker determination, data analysis, and appropriate clinical trial design.

7 Conclusion

Pharmacodynamic modeling of anticancer agents presents a unique set of challenges from measurement to model building. However, diligence has proven that appropriate models can be produced to accurately predict the pharmacological effects of these drugs at given drug exposures and times. The real remaining challenge is the development of simple and cost-effective means to use these models to individualize cancer therapies to achieve maximum benefit and minimal toxicity.

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Protein Binding

Alex Sparreboom and Walter J. Loos

Abstract Incorporation of pharmacokinetic information of anticancer drugs into routine patient care can contribute to drug dosage individualization and account for differences among patients in rates of drug metabolism and/or excretion. The standard analytical methods for measuring concentrations of drugs in plasma determine drug bound to plasma proteins as well as free drug dissolved in plasma water. For this reason, the relationship between total drug concentration in plasma and treatment outcome (i.e., toxicity and efficacy) will only be good if the degree of plasma protein binding of the agent is constant, or if so little drug is protein bound that changes in binding make insignificant changes in unbound concentration. A review of available literature data indicates that, in general, protein binding of anticancer drugs is not of principal clinical relevance. However, there are several instances in which monitoring of unbound concentrations might be useful: (1) agents demonstrating protein concentration-dependent binding, (2) agents that bind irreversible or near covalently, (3) when formulation excipients modulate unbound drug levels, and (4) metabolically interconversible agents. While available evidence suggests that for these agents unbound drug levels correlate better with clinical effects than total plasma concentrations, there are insufficient data to justify the recommendation of the routine use of unbound drug concentration monitoring for most of these agents at present.

Keywords Protein binding • Anticancer drugs • Drug monitoring • Pharmacokinetics • Albumin • Unbound drug

1 Introduction

During the last few decades, the value to clinical practice of determining plasma concentrations of chemotherapeutic agents has been convincingly demonstrated for several important drugs [1]. Such tests are generally not appropriate for drugs of limited effectiveness and potency and in patients who respond well to the usual dosage regimen of a drug. They are also superfluous for drugs whose

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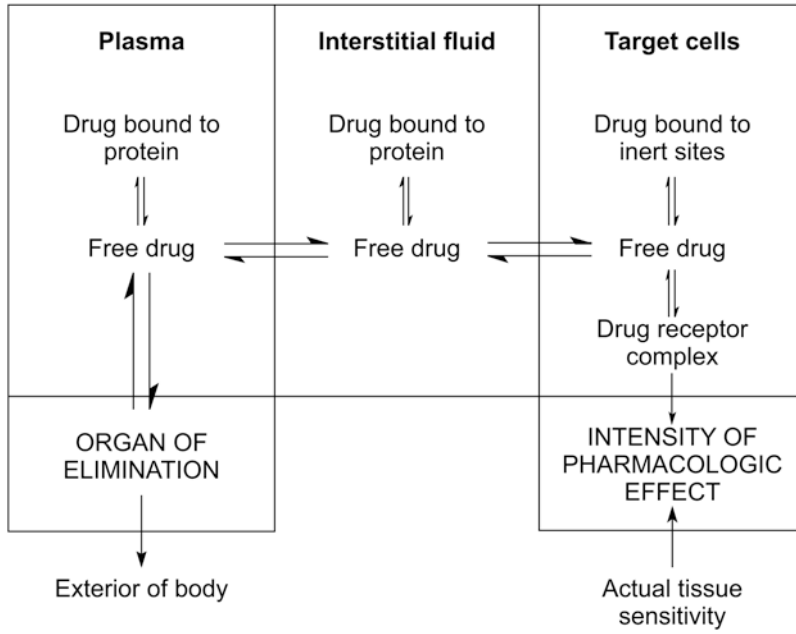

Fig. 1 Representation of the diffusion equilibria that occur to relate the concentration of drug in the plasma to the drug concentration at the site of action and subsequent intensity of drug effect (after M.M. Reidenberg; reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004)

intensity of action can be judged accurately during their clinical use and whose dosage can be adjusted on that basis. Nevertheless, a broad area of clinical usefulness remains. Measurement of plasma concentrations generally clarifies the picture when usual doses of a drug fail to produce therapeutic benefits or result in unexpected toxicity. It has been proven particularly helpful in patients with hepatic or renal function disorders in whom the relation between dosage and plasma concentration may be grossly abnormal, or when drugs are being administered concomitantly and may be altering each other's metabolic fate [2, 3]. Clearly, determinations of drug concentrations in plasma will become more widely applicable as we expand our knowledge of the pharmacological correlates of plasma levels to clinical outcome for more drugs. One problem in achieving individual dose adjustment is identifying and interpreting what constitutes the therapeutic concentration of a drug in the plasma. The intensity of effect is usually related to the concentration of the drug in the plasma water phase, since this establishes the diffusion gradient for the drug to get to its site of action. The relationships of drug–plasma protein binding to the process that establishes the concentration of drug at the active site are shown in Fig. 1. Surprisingly, only in a few instances, plasma protein binding can significantly affect pharmacokinetic processes, such as distribution and elimination by renal and/or hepatic mechanisms, and thus have important pharmacodynamic implications [4]. Here, we discuss (1) the methodological aspects of protein–ligand interactions, (2) the relation between protein binding and drug disposition, and (3) the clinical relevance of free drug monitoring in cancer patients.

2 Drug–Protein Interactions: General Considerations

Within the blood, drugs can bind to many components including blood cells, particularly erythrocytes and platelets, and plasma proteins. As a consequence of the binding, the concentration of drug in the whole blood, in plasma (C_p), and unbound drug in plasma water (C_u) can differ greatly. Binding of

drugs to proteins is usually instantaneous and reversible so rapid that an equilibrium is established within milliseconds. Consequently, the associated (bound) and dissociated (unbound) forms of a drug can be assumed to be at equilibrium at all times and under virtually all circumstances. If there is a perfusion limitation, dissociation of the unbound drug and diffusion of this species across membranes occur so rapidly that delivery of drug, rather than protein binding itself, limits the transport.

The degree of drug binding to plasma proteins is frequently expressed as the ratio of the bound concentration to the total concentration. This ratio has limiting values of 0–1. Drugs with values >0.9 are said to be highly bound, and those with values <0.2 are said to show little or no plasma protein binding. However, the value of the fraction of drug in the plasma that is bound to proteins (f_u) is usually considered of greater utility in therapeutics than is that for bound drug:

$$f_u = C_u / C_p. \tag{1}$$

Binding is a function of the affinity of the protein for the drug. Because of the limited number of binding sites of a protein, binding also depends of the molar concentrations of both drug and protein. Assuming a single binding site of the protein, the association is simply summarized by the following reaction:

$$[Drug] + [Protein] \leftrightarrow [Drug - Protein\ complex]. \tag{2}$$

From mass law considerations, the equilibrium is expressed in terms of the concentrations of unbound drug, unoccupied protein, and bound drug (C_{bd}) by the association constant K_a, which is a direct measure of the affinity of the protein for a given drug. It is possible from binding data to obtain information of K_a by fitting observed data to the following equations for saturable (3) and nonsaturable binding (4):

$$C_{bd} = {}^{m}\sum_{I=1} \left(n_i P \times K_i \times C_u\right) / \left(1 + K_i \times C_u\right), \tag{3}$$

$$C_{bd} = \left(nK\right) \times C_u \tag{4}$$

where C_{bd} and C_u are expressed as molar concentrations, m is the number of binding site classes, n the number of saturable binding sites per mole of protein in the ith class, P the molar concentration of protein, K the association constant, and nK the contribution constant of nonspecific, nonsaturable binding on one site.

The usual approach of drug–protein-binding studies is to fit experimental data to (4) and to plot them as linear regression to C_{bd} versus C_u or in a transformed representation in the form of C_{bd}/C_u versus C_{bd} (i.e., the Scatchard plot) [5]. Both approaches have specific limitations, including the oversimplification of ligand attachment to the binding site(s) by fitting of curvilinear plots with straight lines or conversely, the detection of visionary, biochemically, or pharmacologically not interpretable acceptor heterogeneity [6, 7]. In addition, experimental artifacts may cause curvilinearity of the Scatchard plot, and low-affinity binding components might be overlooked by an incorrect data analysis. Although the Scatchard plot is likely to be further used for quantitative evaluation purposes in the future, a number of alternative graphical representations have been proposed, including the Bjerrum plot [8, 9].

3 Methodological Aspects

3.1 General Considerations

The various techniques available for quantitation of protein–ligand interactions are usually based on one of the following procedures: (1) separation of free- and protein-bound fraction of ligand (i.e., determination of the free drug concentration), (2) detection of a change in a physicochemical

property of the complexed ligand, or (3) detection of a change in a physicochemical behavior of the binding protein [10]. In contrast to non-separation methods, the separation methods allow the study and description of not only the characteristics of primary high-affinity sites but also the concomitant presence of secondary low-affinity binding sites [11]. Although the identification of binding structures and the calculation of binding structures and the calculation of binding parameters in vitro can provide useful quantitative or qualitative information, only combined in vitro and in vivo data can give a comprehensive picture of the impact of binding on a drug's overall pharmacokinetic profile.

3.2 Conventional Methods

Protein binding of anticancer drugs is most commonly determined by equilibrium dialysis, ultrafiltration, or ultracentrifugation. All of these methods are based on the separation of free drug from bound drug under equilibrium conditions and have their own merits and disadvantages [11]. Equilibrium dialysis is based on establishment of an equilibrium state between a protein-containing compartment and buffer compartment, which are separated by a semipermeable membrane. Although this technique is often regarded as the reference method for protein–ligand interactions, no available experimental data support this supposed superiority. In particular, the method has a number of problems, including the long time needed to attain equilibrium (e.g., more than 24 h) [12, 13], volume shifts [14, 15], and nonspecific adsorption to the test device [11]. Several simple and inexpensive dialysis systems employing small volumes have been developed employing microtubes that can be constructed in any laboratory at a minimal cost and that can be discarded after use [16].

Ultrafiltration has been introduced widely for routine monitoring of free drug, since it offers significant advantages over equilibrium dialysis, including short analysis time, ease of use, and lack of dilution effects and volume shifts, although a major controversy involves that stability of the binding equilibrium during the separation process [17]. Ultracentrifugation is an alternative to both equilibrium dialysis and ultrafiltration, since it eliminates the problems associated with membrane effects and enables the separation of the free and protein-bound fraction without addition of buffer systems and dilution problems. Discrepant results have been reported between equilibrium dialysis and ultracentrifugation related to sedimentation, back diffusion, viscosity, and binding to lipoproteins in the supernatant fluid [18, 19].

3.3 Other Methods

The progress in chromatographic technology, particularly affinity chromatography [20] and micellar chromatography [21], has led to the development of various automated systems for routine monitoring of free fractions of drugs in biological fluids. Although these procedures have received only limited attention in cancer pharmacology, binding data obtained by such methodologies offer much higher precision and reproducibility than those measured using conventional techniques [11]. Because of its speed, efficiency, and selectivity, capillary electrophoresis is currently the most dynamically growing analytical technique in this area, and applications include affinity capillary electrophoresis [22], capillary affinity gel electrophoresis [23], and packed-capillary electrochromatography with immobilized protein-stationary phase [24].

Despite reports from some authors of a good correlation between binding parameters obtained by separation methods as compared to spectroscopic methods [11], this approach is successful mainly for high-affinity binding sites and is poorly sensitive to low-affinity interactions. Nevertheless, these methods facilitate insight into three-dimensional protein structure and conformational variations of a

protein molecule resulting from ligand attachment. The most widely used methods for the purpose of studying protein–ligand interactions in this group are those based on fluorescence spectroscopy [25, 26] and nuclear magnetic resonance (NMR) spectroscopy [27] as well as a number of chiroptical methods such as optical rotary dispersion or circular dichroism [28, 29]. Rather exceptionally, some other methods have been used for protein-binding studies, with respect to unique features of the ligand or to reveal specific qualitative or quantitative aspects of the interaction. Examples include the use of polarography [30], calorimetry [31], stopped-flow analysis [32], fluorescence-polarization immunoassay [33], biomolecular interaction analysis mass spectrometry [34], or (dextran-coated) charcoal adsorption-based procedures [35, 36]. Several physiologically based approaches have also been put forward for the determination of the nonprotein-bound fraction of drugs in dynamically functioning living biological systems. However, these kinds of measurements, which include analysis of saliva [37], cerebrospinal fluid [38], red-cell partitioning [39, 40], and capillary ultrafiltration [41], are only of limited general utility for therapeutic monitoring of free drug levels.

Because of its experimental versatility, techniques based on microdialysis [42, 43] offer at present the most promising methodological alternative for monitoring of dynamic changes of free drug in vivo. Microdialysis is a minimally invasive sampling method based on the diffusion of analytes from the interstitial compartment through a semipermeable membrane and allows for the evaluation of blood, tumor, and tissue disposition of drugs. The concept of microdialysis has been optimized in neurological research where microdialysis was used to monitor neurotransmitter concentrations in liquor. Microdialysis has been shown to be applicable in oncology as well [44, 45]. In chapter "Regional Drug Delivery for Inoperable Pulmonary Malignancies", the microdialysis technique is discussed in more detail.

4 Binding as a Disposition Factor

Variability in systemic drug binding has frequently been demonstrated in man [4, 46, 47]. However, the significance of this variability to drug disposition and pharmacodynamics depends largely upon the drug's pharmacokinetic characteristics. The impression gained from the literature is a tendency to overemphasize the general importance of the binding phenomenon. However, only in cases of highly protein-bound agents, that is, more than 90 %, binding might be important in a practical sense. Many investigators, in attempting to extrapolate from in vitro to in vivo, lose sight of the fact that the plasma compartment comprises a relatively small fraction of the total volume available for drug distribution and that protein–drug complexes of rather extraordinary stability must be formed to reduce substantially the amount of active, diffusible, unbound drug.

Many authors have reported a correlation between the elimination rate of a drug and the percent bound to plasma proteins and that individual differences in plasma binding were associated with pronounced variations in the elimination rate constant [4]. In comparing different drugs, however, there may be a pitfall. One cannot assume that just because a drug is highly bound to plasma proteins that it will have a long half-life. For example, the anticancer agent chlorambucil is 99 % bound to albumin and yet the median half-life is only 1.3 ± 0.9 h [48]. Such a short half-life, for such a highly bound drug, has not been explained, but makes one wary about making predictions about other drugs. It is also noteworthy that if a drug is bound to only one class of binding sites on a protein molecule, the carrying capacity of the plasma for the drug is limited to one times the molar equivalent of the plasma's protein content. For albumin, this is in the order of $6–7 \times 10^{-4}$ M, which for a compound with a molecular weight of 300 (such as cisplatin) is equivalent to a plasma concentration of 200,000 ng/mL. Although, theoretically, at higher concentrations the unbound fraction would increase very rapidly above this threshold, the expected plasma levels after therapeutically relevant doses are several orders of magnitude lower than this. Indeed, for almost all drugs the total plasma concentration required for

a clinical effect is much less than 0.6 mM, so that albumin-binding sites are far from saturation. It is important to realize, however, that some drugs, including tolbutamide and some sulfonamides, induce their effects at plasma concentrations at which the binding to protein is approaching saturation. On the other hand, saturable binding might occur if drugs are mainly bound to proteins other than albumin, such as α_1-acid glycoprotein.

5 Binding Proteins

Apart from neutral, lipid-soluble drugs that can be associated with the globulins of lipoprotein complexes by solution in the lipid component, plasma protein binding consists usually in the interaction of ionized polar or nonpolar groups of a drug with corresponding groups of a protein (Table 1 and Fig. 2). Most anticancer drugs are organic chemicals that are either weak acids or weak bases. The demonstration that plasma from uremic patients had markedly decreased binding of organic acids but not of organic bases has led to grouping drugs into one or the other of these classes for the purpose of drug-binding studies and analysis [49]. Most of the binding of acidic drugs is to human serum albumin (HSA), and multiple binding sites for drugs have been identified [50, 51]. Agents that compete for binding at one of these sites do not necessarily change the binding properties of any of the other sites. In contrast, basic drugs bind to HSA to only a small extent but to other plasma proteins to a much greater extent. α_1-Acid glycoprotein (AAG, orosomucoid), an acute-phase reactant, is a major binder for many basic drugs. This was first recognized by Fremstad et al. [52] when they observed an increase in the plasma protein binding of quinidine in patients following surgery. Other plasma proteins, including γ-globulin and lipoproteins, also bind some basic drugs, although, overall, their relative importance is low.

Besides changes in drug binding connected with structural alteration of a protein molecule, the most important changes in the free fraction of a drug are related particularly to disease-induced variations in plasma protein levels. For example, significantly and clinically important changes in binding have been demonstrated for drugs with hepatic flow-dependent extraction [53, 54]. In addition, it has been demonstrated that the plasma protein binding of several anticancer drugs is altered in patients with cancer. The primary changes in drug-binding proteins seen in these patients are an increase in AAG [55, 56] and a decrease in HSA concentration [57]. The physiologic role of AAG is not clear,

Table 1 Major plasma protein fractions[a]

Protein	Amount (mg/dL)	Molecular weight
α_1-Acid glycoprotein[c]	40–100	42,000
Serum albumin[b]	3,500–4,500	67,000
α_1-Globulins	300–600	40,000–60,000
α_2-Globulins	400–900	100,000–400,000
β-Globulins	600–1,100	110,000–120,000
γ-Globulins	700–1,500	150,000–200,000
Lipoproteins	Variable	200,000–2,400,000
Fibrinogen	3,000	340,000
Prothrombin	100	69,000
Transcortin	3.0–7.0	53,000

[a]The total plasma protein content is 7,000–7,500 mg/dL. Many different proteins are found in blood plasma; only the major classes are listed
[b]Might be decreased in cancer patients
[c]Might be increased in cancer patients

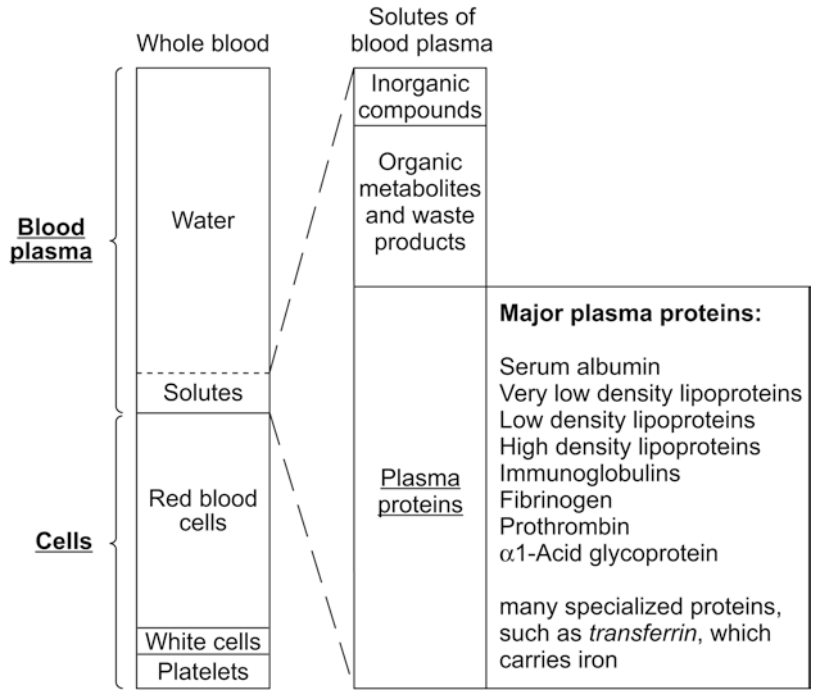

Fig. 2 Composition of human blood. The plasma fractions contain about 10 % of dissolved solids, of which about 70 % consists of plasma proteins (after AL Lehninger; reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004)

although it is elevated in several degenerative and malignant conditions. In addition, the plasma levels of AAG vary widely in healthy individuals, and females seem to have a slightly lower AAG level than males. Interestingly, the affinity of lidocaine for the presumed AAG binding site was higher in plasma from cancer patients compared with healthy controls [58]. Although the amino acid composition was similar to normal human AAG, the carbohydrate side chains were different, possibly accounting for the difference in affinity. Although it seems likely that ageing does not have a clinically remarkable impact on protein binding of drugs [59], the genetically determined modifications of proteins exhibit dramatically altered binding behavior. This has been observed in vitro for genetic variants of AAG interacting with various drugs, including tricyclic antidepressives [60] and quinidine [61]. Diurnal variation in AAG concentration may also contribute to inter- and intraindividual variability in binding characteristics and should be considered for their accurate interpretation [62].

In contrast to AAG, HSA levels vary less than twofold in healthy individuals, although in cancer patients, this range may be substantially larger. Hence, a decrease in HSA may lead to an increase in the unbound fraction for drugs normally highly bound to this protein. In patients with cancer, the HSA levels may be decreased because of decreased synthesis, increased plasma volume, and increased catabolic rate [57]. Other pathophysiologic processes can also lead to a decrease in plasma protein binding and an increased fraction unbound. Specifically, hyperbilirubinemia can displace drugs from binding sites on HSA [63] and lead to an increase in unbound drug concentrations, although this appears to be clinically relevant only at bilirubin concentrations above 10 mg/dL [64]. Similar to the observed variants of AAG, a large number of alloalbumins have been discovered [65, 66], which exhibit either no change in binding properties [67] or reduced binding affinity due to slight variations in protein conformation [68].

6 Binding of Anticancer Drugs

The degree of binding of anticancer drugs can vary over the entire range from essentially no binding for drugs like bleomycin and thiotepa to almost all of the drugs present in plasma being bound to proteins as with teniposide and vinblastine (Table 2). The interindividual variation in plasma protein binding of anticancer drugs is usually quite small in metabolically normal individuals. Therefore, protein binding is not an important consideration in therapeutic drug monitoring. It is also important to realize that the clinical significance of protein-binding displacement interactions has been severely overstated and based largely on in vitro interaction data [115, 116]. In addition, when drugs are not highly protein bound or when the more easily measured total drug concentration provides a consistent and accurate reflection of the free concentrations with little interpatient variability, monitoring the unbound drug concentration is not necessary. Drugs that are highly protein bound are most likely to show wide variations among patients in the unbound drug concentration and are the most likely candidates for monitoring unbound drug concentrations. In very few instances, the total concentration is not reflective of the unbound drug level. For some anticancer agents, this situation arises if (1) the agent demonstrates protein concentration-dependent binding, (2) irreversible or near-covalent binding occurs after therapeutic doses of an anticancer drug, (3) formulation vehicles (e.g., liposomes and nonionic surfactants) change the binding characteristics of the formulated agent, or (4) the agent demonstrates metabolic interconversion.

6.1 Protein Concentration-Dependent Binding

The epipodophyllotoxins etoposide and teniposide are both extensively bound to plasma proteins (Table 2). Whereas etoposide is approximately 95 % bound in patients with normal serum albumin and bilirubin, an even higher extent of binding has been observed for teniposide, with >98 % bound to plasma proteins [85, 106]. Interestingly, wide interindividual variability in the percentage-unbound etoposide has been reported in patients with cancer (range, 5–45 %) [117]. In addition, a significant interaction between both HSA and, to a lesser degree, total bilirubin with the free fraction was identified [3, 118]. Although concentration-dependent binding of etoposide was not observed in vitro, the binding ratio was significantly correlated with HSA levels [119, 120]. The addition of exogenous bilirubin to donor plasma supported competitive binding to HSA as the mechanism for the effect of bilirubin on etoposide protein binding. A pharmacokinetic model for prediction of etoposide plasma protein binding in humans, based on HSA and total bilirubin levels, has been prospectively validated in cancer patients, with only slight bias toward overestimation of the free fraction in patients with normal bilirubin or low HSA levels [97]. The clinical implications of the variable etoposide protein binding were illustrated recently in a study of 28 adult cancer patients [121]. The systemic exposure to unbound etoposide more precisely correlated with measures of hematologic toxicity than total drug levels. In addition, patients with HSA levels <35 mg/dL had substantially larger area under the curves of unbound etoposide than patients with normal HSA, bilirubin, and serum creatinine values [122]. Since this increase in systemic exposure was associated with more severe neutropenia, these findings suggest that unbound etoposide concentrations might be indicated for therapeutic drug monitoring, particularly in patients with aberrant binding (e.g., in case of hypoalbuminemia). Similarly to what has been observed for etoposide, the percentage-unbound teniposide is highly variable among patients and has a strong inverse linear relationship with HSA levels [123]. Furthermore, systemic exposure to unbound teniposide correlated significantly with hematologic toxicity, whereas exposure measures based on total drug were not as well correlated [123]. Thus, it is likely that prospective monitoring of

Table 2 Plasma protein binding of small molecule anticancer agents

Agent	% Unbound	Binding matrix	V (L/kg)[a]	$T_{1/2}$ (h)[b]	References
Amsacrine	3 %	HSA, AAG, γ-GL	~2.5	2.6	[69]
Bleomycin	>99 %	Plasma	0.27 ± 0.09	3.1±1.7	[70]
Bortezomib	17 %	Plasma	21 ± 11	98 ± 145	[71]
Brequinar	2 %	HSA	0.11–0.27	13–18	[72]
Busulfan	72 %	HSA	0.99 ± 0.23[c]	2.6 ± 0.5	[73]
Carboplatin	10 %	HSA	0.24 ± 0.03	2.0 ± 0.2	[74]
Chlorambucil	1 %	Plasma	0.29 ± 0.21	1.3 ± 0.9	[75]
Cisplatin	<5 %	HSA, TF, γ-GL	0.28 ± 0.07	0.5 ± 0.1	[76]
Cyclophosphamide	87 %	Plasma	0.78 ± 0.57	7.5 ± 4.0	[77]
Cytarabine	87 %	Plasma	3.0 ± 1.9	2.6 ± 0.6	[78, 79]
Dasatinib	4 %	Plasma	~33[c]	3–5	[80, 81]
Docetaxel	<2 %	HSA, AAG, HDL	1.8 ± 1.2	14 ± 7.5	[82]
Doxorubicin	15–25 %	HSA	17 ± 11	26±17	[83, 84]
Etoposide	4 %	HSA	0.36 ± 0.15	8.1 ± 4.3	[85]
Erlotinib	3–7 %	HSA, AAG	3[c]	36	[80, 81]
Everolimus	25 %	Plasma	1.5[c]	18–32	[86, 87]
5-Fluorouracil	>95 %	HSA, α,β,γ-GL	0.25 ± 0.12	0.2 ± 0.07	[88]
Gefitinib	5–9 %	HSA, AAG	19[c]	48	[80, 81]
Ifosfamide	45 %	Plasma	0.50 ± 0.20	3.8–8.6	[89]
Imatinib	5–8 %	HSA, AAG	4[c]	18	[80, 81]
Irinotecan	65 %	HSA	3.4–6.4	12 ± 3.0	[90]
Lapatinib	<1 %	HSA, AAG	30[c]	24	[81]
Melphalan	71–80 %	HSA, AAG	0.45 ± 0.15	1.4 ± 0.2	[91, 92]
6-Mercaptopurine	81 %	HSA, AAG	0.56 ± 0.38	0.9 ± 0.4	[93]
Methotrexate	54 %	HSA	0.55 ± 0.19	7.2 ± 2.1	[94, 95]
Nilotinib	2 %	Plasma	8[c]	17	[81]
Oxaliplatin	13–21 %	HSA, γ-GL	5.0 ± 1.9	240±54	[96]
Paclitaxel	2–8 %	HSA, AAG, HDL	2.0 ± 1.2	16 ± 8.9	[97, 98]
Pazopanib	<1 %	Plasma	NA	31	[99, 100]
Pemetrexed	19–27 %	Plasma	0.25	4–5	[101]
SN-38	2 %	HSA, AAG	NA	24 ± 6.0	[90, 102, 103]
Sorafenib	<1 %	Plasma	NA	25–48	[80, 81]
Sunitinib	5–9 %	Plasma	30[c]	40–60	[80, 81]
Tamoxifen	<2 %	HSA, β-GL	50–60[c]	96–264	[104, 105]
Teniposide	<1 %	HSA	0.22 ± 0.05	9.0 ± 3.0	[106]
Thiotepa	90 %	HSA, HDL	0.71 ± 0.18	2.1 ± 0.4	[107]
Topotecan	79 %	HSA	0.40–2.45	3.5 ± 1.5	[102, 108]
Trimetrexate	2 %	HSA, AAG	0.33 ± 0.18	13.0 ± 5.0	[109]
UCN-01	<0.02 %	AAG	0.11 ± 0.08	1,370 ± 280	[110, 111]
Vinblastine	<1 %	AAG	1.4–27	29 ± 12	[112, 113]
Vinorelbine	12 %	AAG	51–76	45 ± 12	[114]

Abbreviations: HSA human serum albumin, *AAG* alpha-1-acid glycoprotein, *HDL* high-density lipoprotein, *GL* globulin, *TF* transferrin, *NA* not available

[a]Mean distribution volume

[b]Terminal disposition half-life

[c]Distribution volume divided by oral bioavailability

epipodophyllotoxins as a selective approach to therapy optimization might be useful. However, additional studies are required to further define relationships between exposure to unbound etoposide and pharmacodynamic outcome of treatment (i.e., side effects and antitumor efficacy).

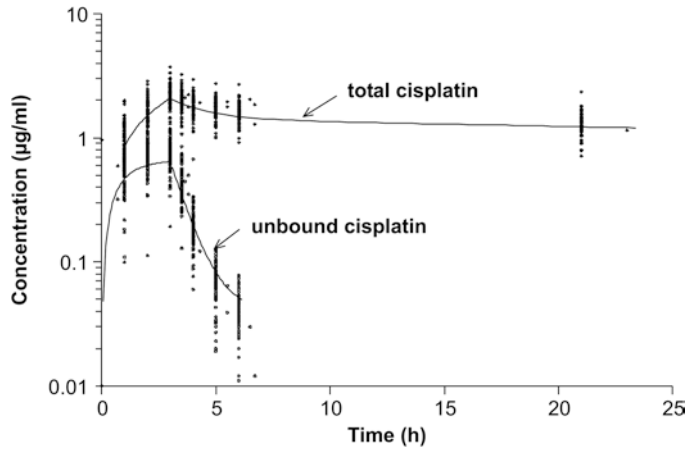

Fig. 3 Representative plasma concentration–time profiles of unbound and total cisplatin in patients treated with a 3-h intravenous infusion of cisplatin at a dose of 70 mg/m^2 (unpublished data, Erasmus MC—Daniel den Hoed Cancer Center, Rotterdam, The Netherlands; reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004)

6.2 Irreversible Binding

Platinum-containing anticancer drugs, including cisplatin, carboplatin, and oxaliplatin, are currently the only agents for which unbound concentrations are routinely measured and for which the relation between unbound drug and therapeutic effects has been extensively studied. In body fluids, these agents are readily attacked by nucleophiles with exchange of one or both chloride ligands to form high and low molecular weight complexes. For example, one day after intravenous administration of cisplatin, 65–98 % of platinum in plasma is protein bound [124, 125], while no unbound platinum has been detected at any time in plasma of patients after slow 20-h infusions [125]. The extent of protein binding also results in significantly lower urinary excretion and an increased tissue deposition of platinum [125]. It has been demonstrated that the unbound fraction is affected by many factors. Plasma components such as HSA, hemoglobin, transferrin, and γ-globulin were previously suggested to be the main ligands for cisplatin [126], and the binding of cisplatin to HSA was considered to be essentially irreversible, although this has recently been questioned. Nevertheless, the concentration–time curves of unbound cisplatin in plasma and total cisplatin (bound to plasma proteins plus unbound) do not run in parallel (Fig. 3). This suggests that the clearance of cisplatin is restrictive and that for a representative calculation of the area under the curve and clearance, the unbound cisplatin concentrations should be used.

In contrast to cisplatin, the concentration–time profiles of unbound and total carboplatin are similar over the first 6 h after drug administration, with the distribution half-life being similar for the different species (approximately 1 h). Thereafter, the concentrations of total platinum remain higher, indicating that protein binding is relatively slow. Indeed, the protein binding of carboplatin averaged 10 % at the end of the administration and increased progressively to reach more than 90 % at 24 h after the end of infusion. The extent of binding of related platinum analogue, oxaliplatin, to plasma proteins in cancer patients has also confirmed these results and showed that at 5 days posttreatment, plasma protein binding was estimated to be >95 % [96]. Overall, these results suggest that plasma protein binding and the pharmacokinetic behavior of platinum analogues are determined by (1) the stability of the leaving

ligand and thereby the chemical reactivity and intrinsic cytotoxicity of the complex and (2) the nature of the carrier ligand that influences the binding and distribution characteristics of the molecule. Regardless of the exact nature of these processes, the protein-binding studies conducted with platinum-containing anticancer agents may provide a firm scientific basis, for the safe and effective use of these agents in the clinic.

A striking example of very extensive binding of an agent to human plasma proteins has been UCN-01 (7-hydroxystaurosporine), a protein kinase C inhibitor, which is presently under clinical investigation as an anticancer drug. The clinical pharmacokinetic behavior of UCN-01 after administration as a 3- or 72-h infusion to cancer patients in initial phase I trials displayed distinctive features that could not have been predicted from preclinical data [110]. Specifically, the distribution volume (0.08–0.16 L/kg) and the systemic clearance (0.05–0.25 mL/h/kg) were extremely low, in contrast to the large distribution volume and rapid clearance in experimental animals. In vitro protein-binding experiments have demonstrated that these discrepant findings were directly attributable to a near-covalent binding of UCN-01 to human AAG, with an association constant in the order of 8×10^8 M^{-1} [111]. Clearly, the implication of such pharmacological features of UCN-01, that is, the extremely low unbound concentrations and long exposure in cancer patients following its administration, will need to be further evaluated in both preclinical and clinical studies in order to find exposure measures that can be linked to treatment outcome.

6.3 Drug Formulation Interference

The use of liposomes (i.e., microparticulate carriers that consist of one or more lipid bilayer membranes enclosing an internal aqueous phase) as a drug delivery system has been an area of increasing interest in anticancer drug development and has significant implications for pharmacokinetic monitoring. Over the last decade, the use of anticancer agents encapsulated in liposomes has proven useful in attenuating toxicity while maintaining or increasing efficacy of certain compounds, thus enhancing the therapeutic index [127, 128]. A complete evaluation of such trials will require a comprehensive plasma pharmacokinetic analysis. There are several factors contributing to the complexity of the pharmacological handling of drugs delivered by liposomes after intravenous administration: (1) circulating drug is present in three distinguishable forms (i.e., liposomal associated, protein bound, and unbound) and (2) plasma clearance occurs as a result of various processes with different elimination rates (i.e., tissue uptake of liposomes carrying the drug, leakage of drug from liposomes, and clearance of unbound drug). It has been argued that pharmacokinetic studies with such agents limited to the analysis of total drug concentrations in plasma are not informative enough and may even be misleading, since pharmacological effects are mainly related to the level of free drug in the plasma. A small number of reports have addressed this issue for liposomal-formulated anticancer agents (e.g., doxorubicin and vincristine) and have demonstrated that the vast majority of drug present in the circulation after injection of liposomal preparations remains entrapped with the lipid carrier [129, 130]. At present, various reliable analytical procedures based on high-performance liquid chromatography preceded by ultrafiltration or solid-phase extraction have been reported to separate unbound from liposome-associated drug [131, 132]. Clearly, implementation of such techniques in the future would significantly increase the capability to rigorously evaluate the complete pharmacokinetic behavior of liposomal anticancer drugs in a clinical setting.

Similar to liposomal entrapment, anticancer drugs can also be sequestered by other formulation excipients, such as micelles composed of nonionic surfactants used in pharmaceutical preparations of intravenous dosage forms. The most extensively studied example of this kind is encapsulation of the antimicrotubule agent paclitaxel (Taxol) with its formulation vehicle, Cremophor EL, a polyoxyethylated castor oil. Initially, it was found that paclitaxel binds extensively (about 95 %) to human plasma

in vitro at clinically relevant concentrations (0.1–6 μM) in a concentration-independent manner [97]. These studies also indicated that HSA and AAG contributed about equally to overall binding, with a minor contribution from lipoproteins. Subsequently, it was demonstrated that this in vitro protein-binding phenomenon was substantially altered in the presence of Cremophor EL [98]. Furthermore, a recent clinical pharmacokinetic study with paclitaxel has shown that after intravenous drug administration over 3 h (at the recommended dose of 175 mg/m^2), the principal fraction of the agent in blood is associated with the hydrophobic interior space of Cremophor EL micelles [133]. Since the clearance of this formulation vehicle itself is schedule dependent (with a significant increase in its clearance with prolongation of the infusion duration from 1–3 to 24 h), this type of drug sequestration is likely to affect paclitaxel pharmacokinetics with alternative infusion duration [134]. An assay method for separation of unbound and bound (i.e., Cremophor EL plus protein-associated) drug based on equilibrium dialysis with a tracer of tritiated-paclitaxel followed by liquid-scintillation counting has become available recently and implemented in retrospective analysis of clinical samples from patients treated with paclitaxel [135]. A population pharmacokinetic model for unbound paclitaxel following its administration after 1-, 3-, and 24-h infusions has demonstrated that systemic exposure to unbound drug correlated significantly with neutropenia and could explain the schedule-dependent hematologic pharmacodynamics of this agent (i.e., more severe bone marrow suppression with prolongation of infusion duration) [136].

6.4 Metabolic Interconversion

Another aspect of the relevance of anticancer drug–plasma protein binding is seen with agents that are enzymatically or chemically converted back and forth from metabolites or degradants to the administered drugs (i.e., interconversion) (Fig. 4). Usually, irrespective of which form of such agent is administered, both the parent and interconversion product are present in the plasma. How quickly the equilibrium is established and where the ratio lies depend not only on the kinetics of interconversion but also on the irreversible loss of each species from the body as well as on the binding to plasma proteins. One example of an anticancer agent undergoing interconversion is camptothecin, a pentacyclic structure with a lactone functionality that not only is essential for antitumor efficacy but also confers a degree of instability in aqueous solutions [137]. This agent, as well as its analogues, can undergo a pH-dependent reversible interconversion between the lactone form and a ring-opened carboxylate form [138]. The equilibrium between the lactone and carboxylate forms of camptothecins is solely dependent not on pH but also on the presence of specific binding proteins, notably HSA [139]. Investigations have shown that HSA had a significant preference for the carboxylate form of camptothecin compared with albumin from five other animal species [140]. However, structural modification to the camptothecin ring structure seen with irinotecan, its metabolite SN-38, and topotecan diminished interspecies differences in stabilization of the carboxylate forms [140, 141]. In the case of the related agent, 9-aminocamptothecin, the lactone moiety appears to be stabilized by murine serum albumin but not by HSA, with 35 ± 6.2 % being present in the pharmacologically active lactone forms in the presence of murine serum albumin and only 0.63 ± 0.10 % in the presence of HSA [142]. Since the lactone and carboxylate forms of these various analogues have very distinct pharmacokinetic profiles due to variable binding to HSA [137], it has been proposed that separate measurement of both drug forms has clinical importance [143]. To ensure adequate measurements of the pharmacologically active lactone forms of the camptothecin analogues in pharmacokinetic studies, blood samples have to be processed directly after sampling at the site of the patient by either (1) direct analysis of the samples, (2) direct extraction of the lactone form from the plasma, or (3) stabilizing the lactone to carboxylate ratio. This latter procedure is clearly preferable, since it is the least laborious approach [143].

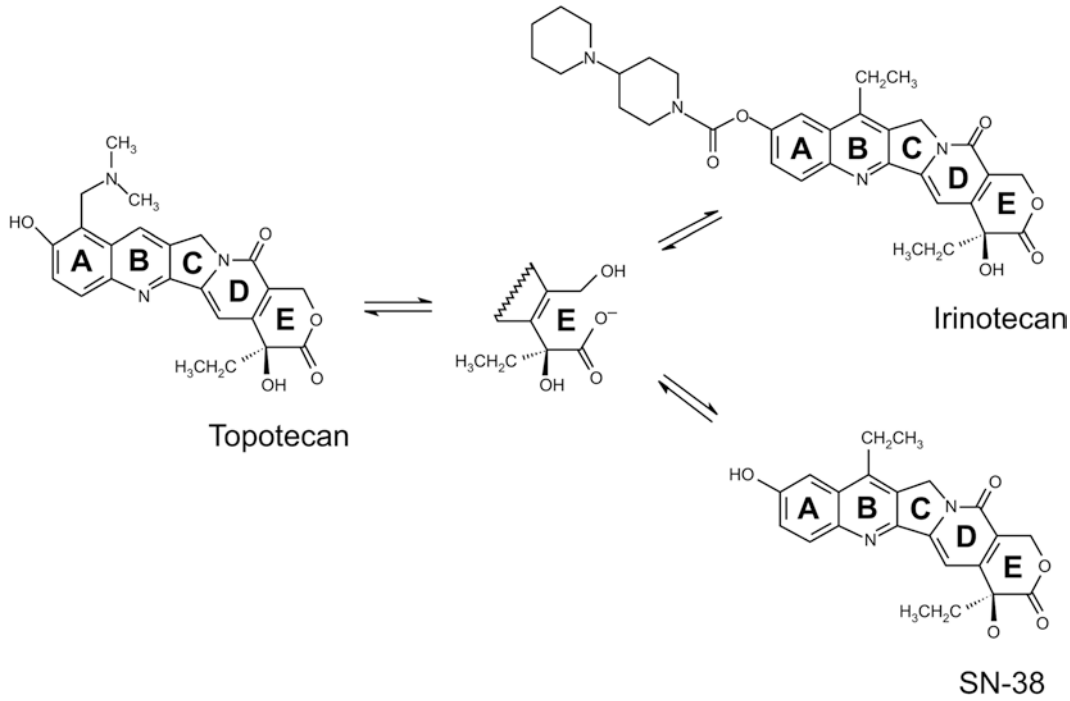

Fig. 4 The concept of metabolic interconversion, exemplified by the lactone and carboxylate forms of topotecan, irinotecan (CPT-11), and its pharmacologically active metabolite SN-38 (Reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004)

7 Overall Significance

Knowledge of the protein binding of anticancer drugs may have significant clinical relevance in a very limited number of cases. In general, plasma protein binding is unimportant for monitoring levels of poorly bound drugs (i.e., less than 90 %), and when the total drug concentrations reflect the unbound levels (i.e., when binding is concentration independent and reversible). In these circumstances, the practicing physician should regard protein binding of any drug with the minor degree of attention it deserves. For highly protein-bound drugs, knowledge of the parameters that influence the binding is important in interpreting the plasma concentrations of such agents. For some anticancer drugs, including epipodophyllotoxins, platinum analogues, paclitaxel, and liposomal-formulated agents, the therapeutic implications of binding to proteins (or other macromolecules) seem to be clearly defined. However, with the exception of some very interesting clinical data regarding etoposide and a few studies with paclitaxel, it seems that we have learned relatively little regarding unbound drug concentration–effect relationships. While available evidence suggests that unbound concentrations correlate better with clinical effects than total plasma concentrations, there are insufficient data to justify the recommendation of the routine use of unbound drug concentration monitoring for any of these agents at present. Nonetheless, for new anticancer agents as well as their (active) metabolites, it will be imperative that the extent and variability of protein binding be documented in an early phase of drug development to allow, if indicated, accurate determination of the relationship between unbound drug exposure and pharmacodynamic effect (i.e., toxicity and efficacy). Recent advances in techniques to

determine unbound drug concentration have greatly simplified the task of monitoring this parameter in clinical practice (reviewed in [144, 145]). Eventually, the utility of such monitoring must be carefully considered in the environment of cost containment in which clinicians must currently function.

8 Summary

The major purpose of therapeutic drug monitoring is to enable drug dosage individualization for differences among patients in rates of drug metabolism and/or excretion. Most standard analytical methods for measuring concentrations of drugs in plasma determine drug bound to plasma proteins as well as free drug dissolved in plasma water. For this reason, the relationship between total drug concentration in plasma and treatment outcome (i.e., toxicity and efficacy) will only be good if the degree of plasma protein binding of the agent is constant, or if so little drug is protein bound that changes in binding make insignificant changes in unbound concentration. Available literature data indicates that, in general, protein binding of anticancer drugs is not of principal clinical relevance. However, there are several instances in which monitoring of unbound concentrations might be useful: (1) agents demonstrating protein concentration-dependent binding, (2) agents that bind irreversible or near covalently, (3) when formulation excipients modulate unbound drug levels, and (4) metabolically interconversible agents. While available evidence suggests that for these agents unbound drug levels correlate better with clinical effects than total plasma concentrations, there are insufficient data to justify the recommendation of the routine use of unbound drug concentration monitoring for any of these agents at present.

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Metabolism (Non-CYP Enzymes)

David Jamieson, Sally A. Coulthard, and Alan V. Boddy

Abstract The fate of xenobiotics, and therefore the efficacy or toxicity of chemotherapeutics, may be dictated by the action of metabolizing enzymes. The metabolism of drugs is categorized into reactions that chemically modify a compound (phase 1) or conjugate a compound with a small reactive biomolecules to yield a polar product amenable to excretion (phase 2). While oxidation by cytochrome P450 enzymes is the primary route of metabolism for many drugs, many additional enzymes may modify the structure and thus function of a wide range of agents. The primary function of these non-CYP enzymes may be detoxification, which may coexist with an endogenous biochemical function. While drug metabolism can lead to a loss of efficacy, there are also numerous commonly used cancer chemotherapeutic agents where metabolism is essential for the generation of the active compound. This review outlines what is known about the metabolism of anticancer drugs by non-CYP enzymes and discusses the potential impact of gene expression and genotypic variation of metabolizing enzymes on efficacy and toxicity.

Keywords Phase I • Phase 2 • Metabolism • Detoxification • Prodrugs • Pharmacogenetics

1 Introduction

1.1 Scope

Drug metabolism is relevant to the pharmacology of anticancer drugs to the extent that it influences the delivery of active drug species to the tumor or to sites of potential toxicity (Fig. 1). The chemical modification of xenobiotics may be viewed as a means to increase the hydrophilic nature of the substrate molecule or to introduce chemical substituent moieties, which are then better substrates for subsequent conjugation. Although the division is not absolute, these reactions may be characterized as chemical modification (phase I) or conjugation reactions (phase II). A significant proportion of phase I reactions are oxidative, and the majority of oxidative metabolic reactions are mediated

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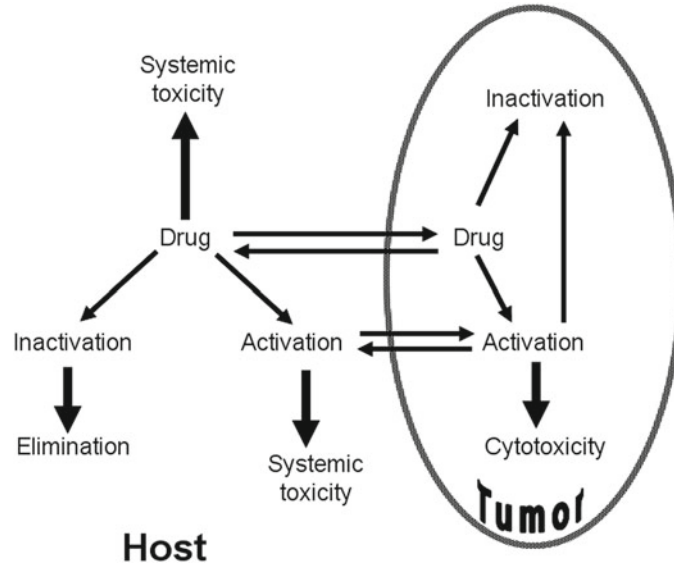


Fig. 1 Potential role for non-CYP metabolism in the pharmacology of chemotherapeutic agents. Pathways for drug activation and inactivation in the host and tumor tissues (Reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004)

by the cytochrome P450 (CYP) superfamily of enzymes. The CYP enzymes are the subject of the next chapter.

A number of phase I metabolic reactions, both oxidative and non-oxidative, are mediated by enzymes other than those in the P450 family. These include oxidases, reductases, dehydrogenases, methyltransferases, and esterases. The phase II conjugation reactions are catalyzed by transferase enzymes that attach glucuronyl, glutathione, sulfonyl, or acetyl groups to suitable substrate sites on the drug molecule. These enzymes are named for their function, rather than for their membership of a genetically homologous family of proteins. For a given reaction, there do exist different genetically related isoforms, such as the UDP-glucuronosyltransferases (UGT).

1.2 Potential Influence

Drug metabolism is primarily a process of drug inactivation, the resulting metabolites being both less active than the parent compound and more rapidly eliminated from the body. The implication for metabolic reactions of this type is that individuals who have low or absent enzyme activity for a particular reaction will be at increased risk of unacceptable toxicity. Conversely, individuals in whom the relevant enzyme is highly active or induced will inactivate the drug faster, and so will have a lower probability of responding to treatment.

Exceptions to this general rule of inactivating metabolism include prodrugs, such as irinotecan, which is cleaved by esterases to yield an active metabolite. There are also examples of drugs whose metabolites have clinically significant potency or where metabolites are more toxic than the parent drug. These exceptions will be discussed in detail where appropriate.

The chapter is organized according to the division between phase I and phase II reactions, with subsections on enzymes classified by the type of reaction catalyzed or on specific enzymes with limited or exclusive substrate specificity. Issues of drug inhibition and induction and the genetics of each of the enzymes will be discussed where relevant and where sufficient characterization of the enzyme has been performed.

2 Phase I Reactions

2.1 Non-CYP Oxidation

2.1.1 Aldehyde Dehydrogenase

The aldehyde dehydrogenase (ALDH) family of enzymes comprises more than 19 members, with a number of associated pseudogenes [1]. The primary function of ALDH is to oxidize potentially toxic aldehydes to carboxylic acids which are either excreted directly or are substrates for phase II conjugation reactions. ALDH enzymes can be either cytosolic or mitochondrial and can use NAD^+ or $NADP^+$ as cofactors. Expression of ALDH varies for the different isoforms, but ALDH1A1 and ALDH3A1 are mainly expressed in brain, heart, liver, kidney, and lung. ALDH expression and activity have also been found in tumors.

The most significant role for ALDH enzymes in the pharmacology of chemotherapeutic agents is probably interruption of the activation pathway of cyclophosphamide and ifosfamide. The activation of these oxazaphosphorines is initiated by CYP450-mediated 4-hydroxylation, tautomerization to an aldehyde intermediate, and spontaneous release of DNA-alkylating species (Fig. 2). The aldehyde intermediate is oxidized by ALDH enzymes to an inactive carboxylic acid.

The role of ALDH in inactivating the intermediate aldophosphamide was identified nearly 20 years ago [2, 3], leading to the identification of ALDH expression in tumors [4] and erythrocytes [5] and the suggestion that ALDH might confer protection to bone marrow following gene transfection [6]. ALDH1 and ALDH3 isoforms are primarily responsible for resistance to oxazaphosphorines, which

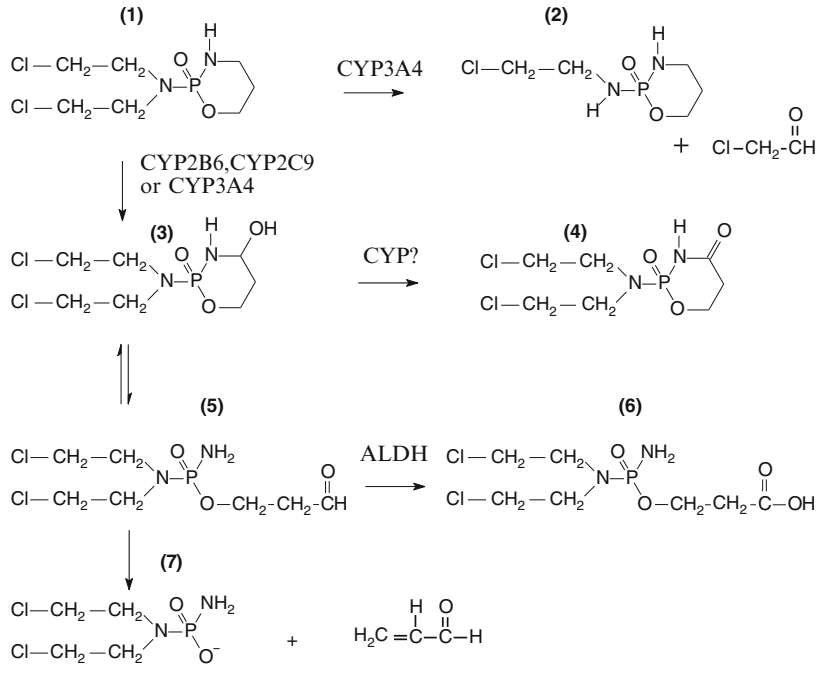


Fig. 2 Metabolism of cyclophosphamide (1), showing inactivation of aldophosphamide (5) by ALDH enzymes to the inactive carboxy form (6). Oxidation to inactive dichloroethyl (2) and keto (4) metabolites and to active 4-hydroxy (3) and phosphoramide mustard (7) forms mediated by CYP450 enzymes (Reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004)

may be reversed by the ALDH inhibitor disulfiram. Antisense oligonucleotides to ALDH1 suppress enzyme activity and increase sensitivity of chronic myelogenous leukemia K562 and lung adenocarcinoma A549 cell lines to 4-hydroxycyclophosphamide in vitro [7]. In a clinical investigation, breast cancer metastases in patients previously treated with cyclophosphamide had higher ALDH1A1 expression, but not higher ALDH3A1 expression, than metastases from patients who had not been treated with cyclophosphamide. High ALDH1A1, but not ALDH3A1, expression was also predictive of poor response to cyclophosphamide treatment for metastatic breast cancer but was not associated with altered prognosis in the absence of cyclophosphamide therapy [8]. While expression of ALDH1A1 may be predictive of response to cyclophosphamide therapy, a 17 bp deletion promoter polymorphism in ALDH1A1 and a non-synonymous SNP in ALDH3A1 are not [9].

The redefinition of ALDH1 as the primary marker of the breast cancer stem cell phenotype has indirectly facilitated investigations into resistance to chemotherapy. ALDH1 expression may be associated with both poor prognosis and an aggressive tumor phenotype, that in the pre-Herceptin era would have had limited chemotherapeutic options (Her2+ and HR−ve) [10]. High expression of ALDH1 has also been shown to be predictive of poor response to neoadjuvant treatment of breast cancer with paclitaxel, cyclophosphamide, epirubicin, and 5-fluorouracil (5-FU). As all patients received the same chemotherapeutic regimen, it was impossible to resolve if the efficacy of any individual drug was effected by ALDH1 expression [11].

Another DNA-alkylating drug, procarbazine, is activated to azoxy-intermediate metabolites. These azoxy compounds are substrates for and are inactivated by both ALDH and xanthine oxidase (XO) [12].

The role of ALDH1A isoforms enzymes in the synthesis of retinoic acids [13] is intriguing, given the differentiating and even cytotoxic effects of retinoids against some tumors. In turn, all-t*rans*-retinoic acid downregulates the expression of ALDH1A1 and ALDH3A1 in lung cancer cell lines and sensitizes the cell to 4-OH-cyclophosphamide [14].

2.1.2 Flavin-Containing Monooxygenases

These enzymes are involved in a number of oxidation reactions and have some overlap in terms of substrate specificity with CYP isoforms. Until recently the only significant action of flavin-containing monooxygenases (FMOs) with relevance to the pharmacology of cancer treatment that has been identified is the N-oxidation of tamoxifen (Fig. 3) [15, 16], which is associated with activation to a reactive carcinogen. This reaction is mediated by FMO1, which is expressed in the intestine and kidney [17], and FMO3 [18], which is highly expressed in the liver [19]. Genetic variants of both FMO1 and FMO3 with altered oxidation activity have been reported [20, 21]. The significance of this oxidation is uncertain, however, as the tamoxifen-N-oxide metabolite of FMO catalysis is readily reduced back to the parent compound by heme-containing proteins including CYPs and hemoglobin [22].

More recently FMO3 has been shown to catalyze the N-oxidation of dasatinib to a minor metabolite that is found in vivo following administration of the drug [23]. It may be that the contribution of FMO to the oxidation of anticancer drugs has been underestimated due to the thermal lability of FMO in the absence of NADPH [24].

2.1.3 Xanthine Oxidoreductase

Xanthine oxidoreductase (XOR) is a collective term for two forms of the same gene product. *Xanthine dehydrogenase* exists as a homodimer and can readily be converted to *xanthine oxidase* by oxidation of essential thiol residues, followed by protease cleavage of a 20 kDa subunit from each monomer [25]. The endogenous substrate for XOR is xanthine, resulting ultimately in oxidation to uric acid,

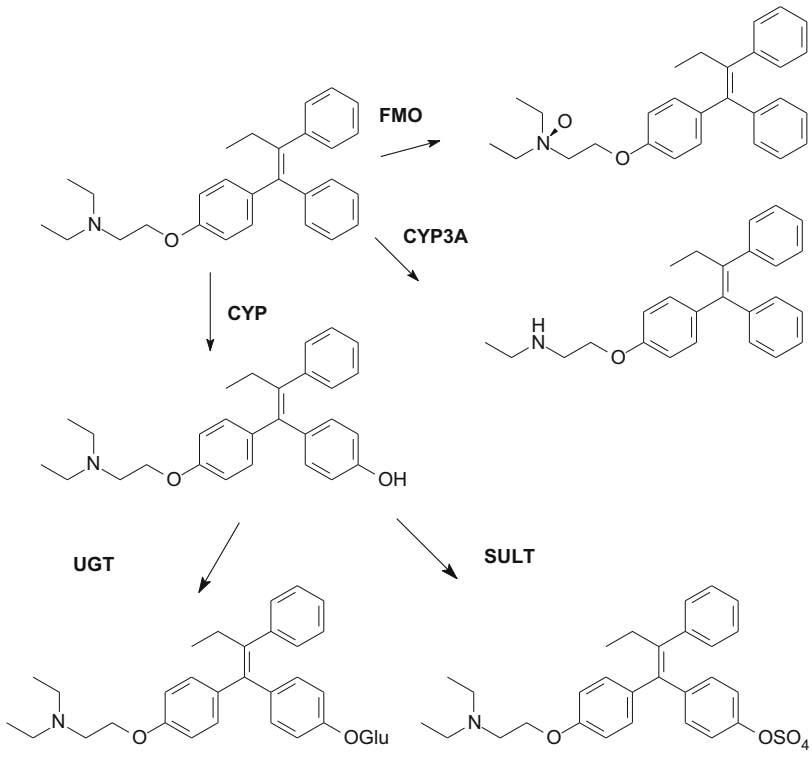
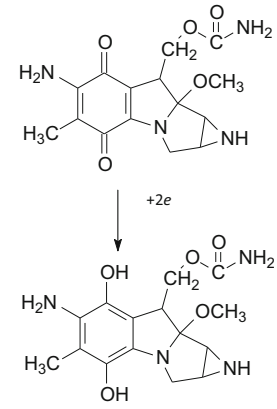


Fig. 3 Metabolism of tamoxifen, including N-oxidation by FMO. Competing reactions include CYP-mediated N-demethylation and 4-hydroxylation. The latter is followed by phase II conjugation by either glucuronosyl (UGT) or sulphonyl (SULT) transferases (Reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004)

Fig. 4 Activation of mitomycin C by two-electron reduction (Reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004)



with corresponding reduction of NAD^+. Xenobiotic substrates include purines, pyrimidines, heterocycles, and aldehydes [26].

XOR can activate the bioreductive class of drugs, the prototype of which is mitomycin C (Fig. 4) [27]. This area is discussed in more detail in the section on NQO1. Other cancer chemotherapy agents which are substrates for XOR include doxorubicin, which may be activated to reactive oxygen species under aerobic conditions [28] or inactivated to an aglycone, under hypoxic conditions (Fig. 5) [29].

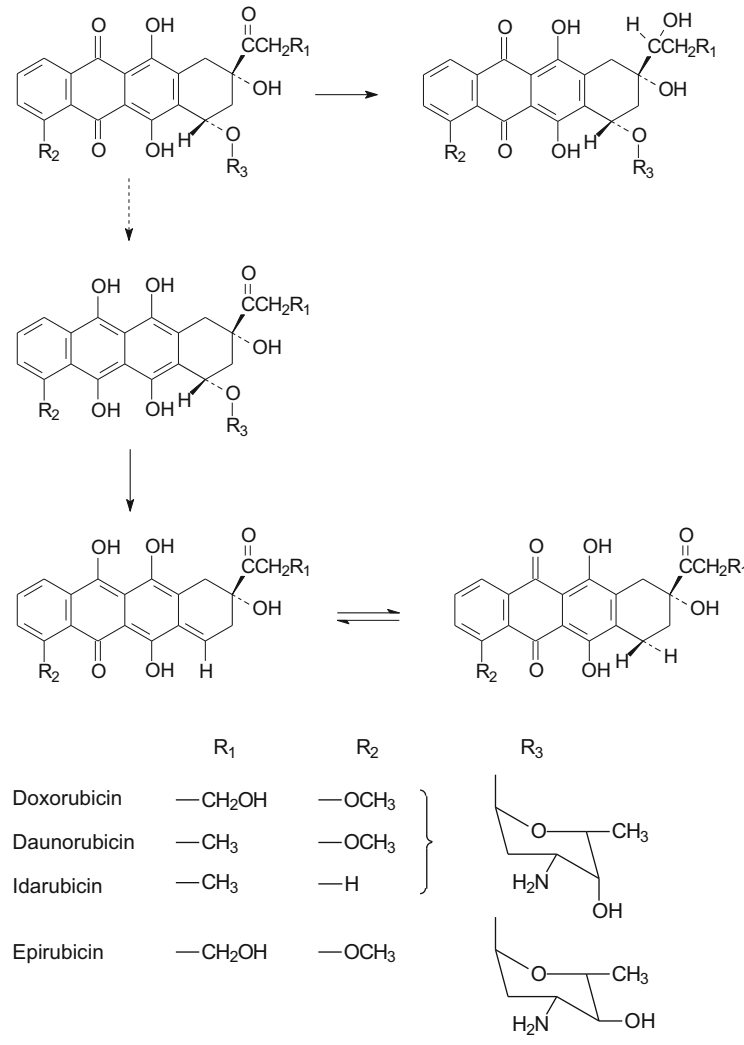


Fig. 5 Metabolism of anthracyclines. Illustrated are both ketone reduction by carbonyl reductase enzymes and quinone reduction to hydroquinones by reductase enzymes (Reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004)

For purine analogues, which are cytotoxic by incorporation into DNA or by inhibition of *de novo* purine synthesis, xanthine oxidase (XO) may mediate an important inactivating pathway of metabolism. For 6-mercaptopurine (6-MP) (see Sect. 2.3), XO catalyzes the formation of thioxanthine and thiouric acid (Fig. 6). Coadministration of the XO inhibitor allopurinol, which may be clinically indicated in lymphomas, results in impaired metabolism of 6-MP [30]. Methotrexate also inhibits XO activity [31] and concurrent treatment with methotrexate increases the plasma concentration of 6-MP after oral dosing [32]. Activity of XO is low in extrahepatic tissues including circulating blood cells and in the bone marrow and is therefore unlikely to affect the activity of the thiopurine drugs in the lymphocytes [33–35].

2.1.4 Myeloperoxidase

Physiologically myeloperoxidase (MPO) catalyses the halogenation of hydrogen peroxide to yield hypochlorous acid. This occurs in the phagosome of neutrophils during an antibacterial immune

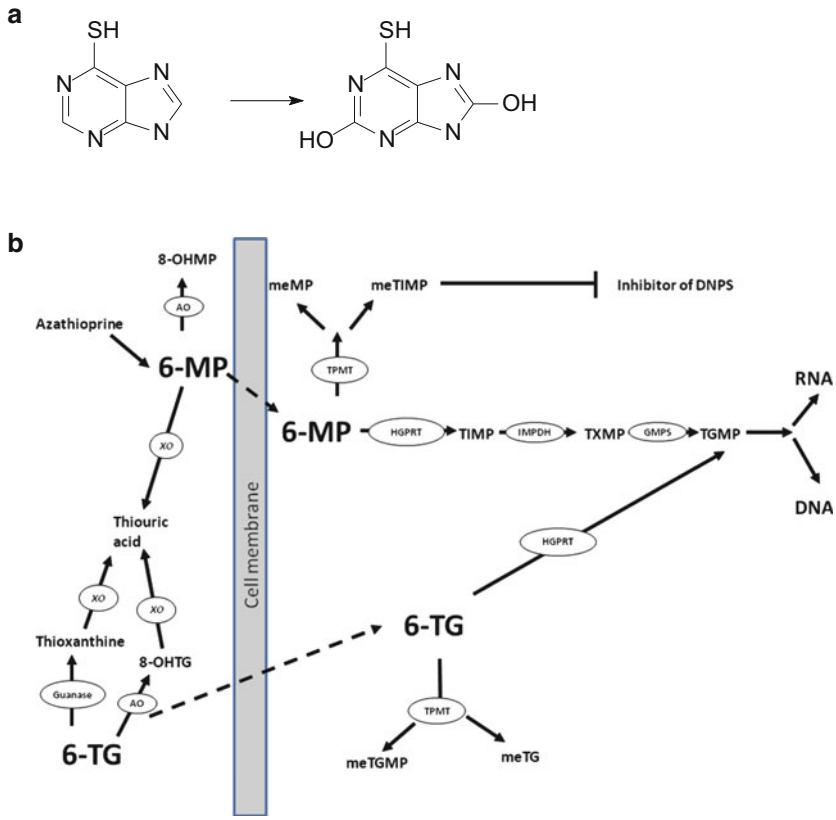


Fig. 6 (**a**) Metabolism of 6-mercaptopurine by xanthine oxidase to thiouric acid. (**b**) Metabolism of 6-MP and 6-TG in human ALL cells. AO, aldehyde oxidase; XO, xanthine oxidase; 8-OHTG, 8-hydroxythioguanine; 8-OHMP, 8-hydroxymercaptopurine; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; IMPDH, inosine monophosphate dehydrogenase; GMPS, guanosine monophosphate synthase; TPMT, thiopurine methyltransferase; TIMP, thioinosine 5′-monophosphate; TXMP, thioxanthine monophosphate; TGMP, thioguanosine monophosphate; meTG, methylthioguanine; meTGMP, methylthioguanine monophosphate; meMP, methylmercaptopurine; meTIMP, methylthioinosine monophosphate; DNPS, de novo purine synthesis (Reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004)

response but pathologically can also damage host tissue. MPO is oxidized by hydrogen peroxide as an intermediate stage prior to the production of hypochlorous acid, which is capable of oxidizing small molecules [36]. MPO can oxidize etoposide to yield highly reactive phenoxyl radicals, which in turn can oxidize glutathione in HL60 cells [37]. Additionally, etoposide metabolites form DNA topoisomerase II complexes [38]. It has been hypothesized that MPO expression may promote therapy-related acute myeloid leukemia following treatment with etoposide, but this has not been investigated clinically [39].

2.1.5 Aldehyde Oxidase (AO)

AO may play a role in the formation of 7-hydroxymethotrexate [40] or of O-6-benzyl-8-oxoguanine (Fig. 7) [41]. The latter is the major metabolite of O-6-benzylguanine, an inhibitor of methylguanine methyltransferase which is responsible for the repair of DNA alkylation [42].

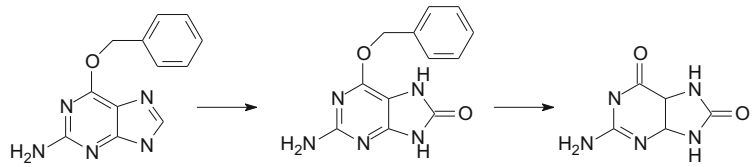


Fig. 7 Formation of 8-oxo-6-benzylguanine from O6-benzylguanine by aldehyde oxidase and subsequent debenzylation (Reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004)

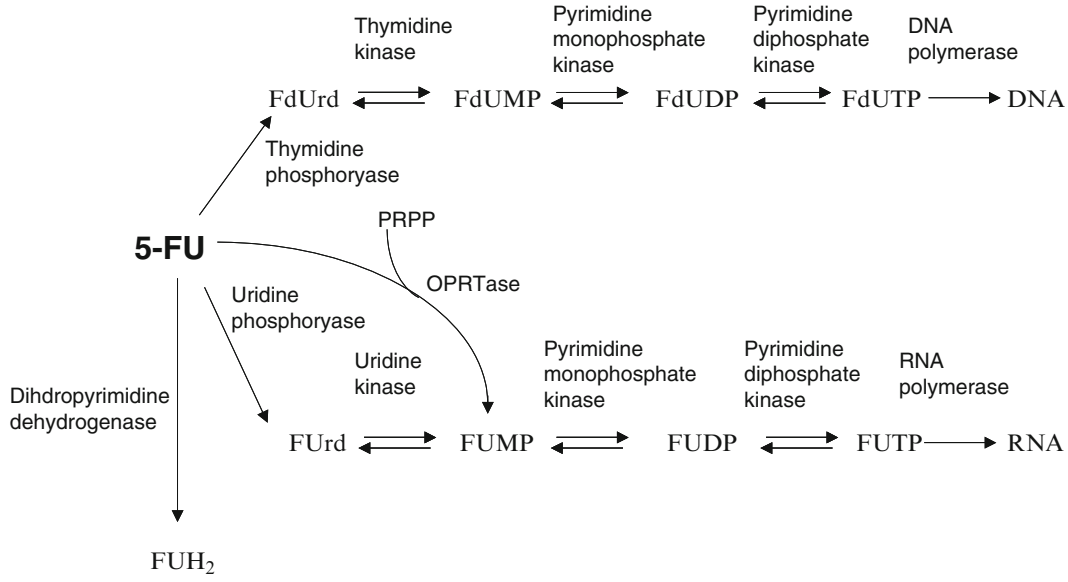


Fig. 8 Intracellular activation of 5-FU, 5-fluorouracil; FdUrd, 5-fluorouridine; FdUMP, fluorodeoxyuridylate; FdUDP, fluorodeoxyuridine diphosphate; FdUTP, fluorodeoxyuridine triphosphate; FdUrd, 5-fluoro-2′-deoxyuridine; FUMP, fluorouridine monophosphate; FUDP, fluorouridine diphosphate; FUTP, fluorouridine triphosphate; FUH2, dihydrofluorouridine; PPRP, phosphoribosyl phosphate (Reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004)

2.2 Dihydropyrimidine Dehydrogenase

Dihydropyrimidine dehydrogenase (also known as dihydrouracil dehydrogenase, dihydrothymine dehydrogenase, uracil reductase, DPD, or DYPD; E.C. 1.3.1.2) is the initial and rate-limiting enzyme in the three-step pathway of uracil and thymidine catabolism, leading to the formation of β-alanine [43]. Individuals who are totally deficient in this enzyme may present with a nonspecific clinical picture of cerebral dysfunction and persistent urinary excretion of excessive uracil, thymine, and 5-hydroxymethyluracil. DPD is also the principle enzyme involved in the degradation of the chemotherapeutic agent 5-fluorouracil (5-FU or 5-FUra) [44]. DPD converts over 80–85 % of 5-FU to dihydro-5-fluorouracil (5-FUH_2), an inactive metabolite (Fig. 8).

5-FU undergoes anabolism to cytotoxic nucleotides, 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP), fluorouridine triphosphate (FUTP), and fluorodeoxyuridine triphosphate (FdUTP). FdUMP forms a stable covalent complex with 5,10-methylenetetrahydrofolate and thymidylate synthase (TS), thereby blocking the formation of dTMP [45]. Consequently, there is depletion of dTTP, which is

needed for both DNA synthesis and repair. FUTP and FdUTP are misincorporated into RNA or DNA, respectively.

The liver has the highest DPD activity (mean, 705 nM/g tissue/h using 5-FU as the substrate), with minimal activity found in the kidneys, spleen, lung, colon, colon tumors, pancreas, breast tissue, breast tumors, bone marrow cells, and peripheral leukemic cells [46]. DPD activity has also been reported in a significant proportion of malignant cells [47]. DPD activity in both peripheral blood mononuclear cells and liver from normal individuals shows no significant difference with respect to age, gender, or race [48]. In the blood the highest level of DPD is found in monocytes, followed by that of lymphocytes, granulocytes, and platelets, whereas no activity could be found in erythrocytes [49].

Within both healthy populations and cancer patients, a large degree of variation (8–21 fold) in peripheral blood mononuclear cell (PBMC) DPD activity has been observed [48, 50–52]. The frequencies of partial and total DPD deficiency in the general population are estimated to be 3–5 % and 0.1 %, respectively [48, 50]. Total DPD deficiency is heritable as an autosomal recessive trait and has been attributed to homozygosity or compound homozygosity for characterized polymorphisms in the DPYD gene. The phenotypic impact of DPD deficiency in pediatrics is variable and not all children with complete DPD deficiency develop symptoms [53]. Individuals who are heterozygous for DPYD mutations are also asymptomatic [53].

The DYPD gene is over 950 kb containing 23 exons with about 3 kb of coding region [54–56] and has been located to chromosome 1p22 [57]. To date at least 21 variant alleles have been described in the gene coding for DPD [58–64]. DPYD*2A, a G to A mutation in the 5′ splicing recognition sequence of intron 14, results in a 165 base-pair deletion of exon 14 and translation to a truncated protein. This is the most widely reported mutation associated with DPD deficiency [60].

5-FU was introduced as a chemotherapeutic agent over 40 years ago and remains one of the most widely prescribed cancer chemotherapy drugs for the treatment of cancers of the digestive tract, breast, and head and neck [65]. Following administration, about 85 % undergoes catabolism via DPD into biologically inactive metabolites that are excreted in the urine and the bile [66, 67]. Plasma clearance of 5-FU is directly proportional to activity of DPD in peripheral blood lymphocytes [68]. There is little evidence that DPD activity impacts on the efficacy of 5-FU. However, 5-FU can result in grade 3/4 hematotoxicity in 30 % of patients following bolus administration with 0.5 % drug-related deaths occurring irrespective of route of administration [69]. Low DPD activity or a DPYD genotype associated with low enzyme activity are in turn associated with an increased likelihood of toxicity. Despite this association the clinical utility of a low DPD phenotype is limited due to low sensitivity and a poor positive predictive value of current assays [70]. A recent prospective trial investigating DPYD genotype in cancer patients treated with 5-FU reported a sensitivity of 5 % and PPV of 46 %. The study suggested that DPYD genotype testing may be more predictive of severe 5-FU hematotoxicity in men than women, with a PPV of 83 %, but sensitivity was still only 10 % indicating that a DPD-independent mechanism is also responsible for sensitivity to the drug [71].

As the liver is the major site for catabolism of 5-FU by DPD, the majority of an oral dose of 5-FU is subject to first-pass metabolism reducing its bioavailability. After intravenous administration, 5-FU is rapidly eliminated with a half-life of 8–14 min. Administration protocols that use continuous infusion provide consistent exposure to 5-FU and continuous inhibition of the target enzyme TS [72] with a lower frequency of severe hematotoxicity.

Strategies to modulate the anabolic and catabolic metabolism of 5-FU have been developed. These treatment strategies fall into three main categories:

1. 5-FU prodrugs
2. 5-FU combined with a DPD inhibitor
3. 5-FU prodrugs combined with a DPD inhibitor

These approaches allow oral dosing, as 5-FU is then eliminated almost entirely by renal excretion and plasma concentrations may be more consistent than with intravenous 5-FU [73, 74]. 5-FU prodrugs

include Ftorafur ([R,S-1-1(tetrahydrofuran-2-yl)-5-FU)]) and capecitabine (n4-pentyloxycarbonyl-5′-deoxy-5-fluorocytidine). DPD inhibitors include eniluracil or 5-ethynyluracil (5-EU), a uracil analogue, which increases the bioavailability of 5-FU to ~100 % [75–77].

Ftorafur is converted to 5-FU by hepatic microsomal and cytosolic enzymes; however, clinical use has been limited due a narrow therapeutic window [74]. S-1 is a combination of Ftorafur and two 5-FU modulators, 3-cyano-2,6-dihydroxypyridine (CDHP) and oxonic acid in a molar ratio1:0.4:1. CDHP is a competitive, reversible DPD inhibitor that prolongs the half-life of 5-FU. Oxonic acid is a pyrimidine phosphoribosyltransferase inhibitor that is intended to mitigate 5-FU-related gastrointestinal toxicity by preventing the phosphorylation of 5-FU in the digestive tract. Pharmacokinetic studies with S-1 have shown consistent release of 5-FU [78, 79], and clinical activity has been demonstrated [80]. S-1 is currently used clinically in Japan for the treatment of gastric cancer.

UFT also combines Ftorafur with a DPD inhibitor, uracil, in a molar ration of 1:4, which also produces consistent 5-FU concentrations in plasma [81, 82]. The efficacy of oral UFT is comparable to IV 5-FU in the treatment of stage II, stage 3, and metastatic colorectal cancer [83, 84] and is less toxic than 5-FU in the treatment of metastatic disease [83].

Capecitabine is an orally bioavailable prodrug of 5-FU that requires three sequential enzyme catalyzed modifications to generate 5-FU. Initially hepatic carboxylesterase catalyses the removal of the pentyloxycarbonyl group to generate 5′DFCR. 5′DFCR is oxidatively deaminated to 5′DFUR by cytidine deaminase and is in turn converted to 5-FU by thymidine phosphorylase (TP) [85]. Of these enzymes, TP is preferentially expressed in tumors compared to normal tissue [85] and the 5-FU concentration in colorectal tumors exceeds that in healthy tissue [86]. The toxicity profile more closely resembles long-term infusion of 5-FU rather than bolus injections, with less hematotoxicity and greater incidence of hand-and-foot syndrome [87, 88]. There are indications that DPYD genotypes associated with low DPD activity are also associated with a hematotoxic response to capecitabine [89, 90], and DPD genotyping may be predictive of capecitabine-induced hematotoxicity.

The fluorinated pyrimidines have played a major role in the treatment of many common tumors since the introduction of 5-FU over 40 years ago. Understanding of the pharmacogenetics and enzymology of DPD has permitted the development of strategies to improve the efficacy of 5-FU. These have included the use of biochemical modulators such as folinic acid and the use of either administration of oral inactive 5-FU prodrugs or the administration of 5-FU with inhibitors of the enzyme DPD.

2.3 Thiopurine Methyltransferase

Thiopurine methyltransferase (TPMT) (EC 2.1.1.67) is an enzyme that is found in red blood cell (RBC) lysate, lymphocytes, kidney, liver, lung, and intestine [91]. TPMT catalyses the methylation of aromatic heterocyclic sulfhydryl compounds including the thiopurine drugs, 6-thioguanine (6-TG), 6-mercaptopurine (6-MP), and its prodrug azathioprine, which are commonly used cytotoxic agents and immunosuppressants [92, 93]. TPMT shows a trimodal activity pattern with about one in 300 individuals having no TPMT activity at all. Deficiency of TPMT does not have any impact in a healthy individual, which makes its normal function hard to discern.

The importance of understanding the role of TPMT in drug metabolism is that 6-MP has, since the early 1950s, been used extensively in the continuing treatment of childhood leukemia. 6-MP and 6-TG were first synthesized by Gertrude Elion and George Hitchings who found that the substitution of oxygen by sulfur at the 6-position of guanine and hypoxanthine produced inhibitors of purine utilization. 6-MP and 6-TG were found to be active against a wide spectrum of rodent tumors and in children with acute lymphoblastic leukemia (ALL). At that time, children with ALL had a life expectancy of only a few months. 6-MP could produce temporary complete remission of ALL, leading the US Food and Drug Administration to approve the drug for use in 1953. 6-TG and 6-MP are still used

today in the treatment of leukemia, and azathioprine is still used as an immunosuppressant in transplant therapy and treatment of inflammatory bowel disease.

TPMT activity was first described in 1963 [93], but it was not until 1980 that the first report on the trimodal distribution pattern of red blood cell TPMT activity was published [94]. TPMT activity has since been shown to be the same in both RBC lysate and lymphoblasts [95–97]. Kidney TPMT levels also reflect RBC lysate activity [98, 99]. Adult liver TPMT activity (105 ± 57 pmol/min/mg of protein) is twofold higher than in the intestine and the kidney, threefold higher than in the lungs and about fivefold higher than in the brain [91]. In the human fetus, liver TPMT activity has been found to be about one third that of adult liver and is similar to that of fetal kidney, lung, and intestine [91]. TPMT activity in RBC in neonates is about 50 % higher than that of adults, although the trimodal distribution pattern seen in adults is still apparent [100]. From the age of 2, children have TPMT activities very similar to those of adults [101].

The TPMT gene, which is situated on chromosome 6p22.3, encodes a 245 amino acid protein with a predicted molecular mass of 35 kDa. The gene was originally reported to be approximately 34 kb in length [102]; this has since been modified to 25 kb with minor sequence differences [103]. The underlying genetic reason for the variation in enzyme activity is the presence of single nucleotide polymorphisms (SNPs) in the TPMT gene of which 36 alleles have been described to date [104].

The frequency of SNPs is related to ethnicity, with the most common being TPMT*3A in Caucasians. TPMT*3A (460G > A, 719A > G) results in a substitution of alanine for tyrosine at amino acid 154 and a substitution of tyrosine to cysteine at position 240 [102, 105]. Individuals who are heterozygous for TPMT*3A have intermediate TPMT activity, but if base changes are present on both alleles, no TPMT activity is detected (reviewed in [106]). Polymorphisms have also been identified within the 5′ flanking promoter region of the TPMT gene due to a variable number of tandem repeats (VNTR*3-*8) [102, 103, 107].

In addition to S-methylation by TPMT to form inactive metabolites methylmercaptopurine (meMP) and methylthioguanine (meTG), 6-MP and 6-TG are also metabolized by XO, AO, and hypoxanthine–guanine phosphoribosyltransferase (HGPRT). XO activity is high in the intestinal mucosa and liver where inactive thiouric acid is formed. AO converts meMP [108] and 6-TG [109] into their hydroxylated metabolites. While HGPRT forms thioinosine monophosphate (TIMP), which is further metabolized by a series of kinases and reductases to produce deoxythioguanosine triphosphate (dG^s). Incorporation of dG^s into DNA has been shown to trigger cell cycle arrest and apoptosis by a process that involves the mismatch repair pathway [110] (Fig. 6a, b).

TPMT is subject to noncompetitive inhibition by sulfasalazine and its metabolite 5-aminosalicylic acid (ASA), olsalazine, and olsalazine-*O*-sulphate, drugs that are used in the treatment of inflammatory bowel disease [111]. Therefore, interaction of these drugs has to be taken into account when treating patients with thiopurine drugs.

As discussed above, TPMT shows a high degree of variation in activity and therefore has a direct impact on the cytotoxicity of these drugs. Thioguanine nucleotides (TGN) and their subsequent incorporation into DNA and RNA have been thought to be the main cause of cytotoxicity in patients treated with 6-MP and 6-TG [112]. Formation of free thioguanine nucleotides (TGNs) in the RBC of patients treated with 6-MP is related to myelosuppression [113], and the level of TGN production is inversely proportional to TPMT activity [101, 114, 115]. In children with ALL, the TPMT activity in RBC lysate at diagnosis reflects that of the lymphoblasts [95, 96] and is increased during treatment, reverting to pretreatment levels after 6-MP therapy is stopped [101, 115]. As yet the significance and mechanism by which this occurs is not known. Patients with low TPMT activity may be treated using individualized doses of 6-MP, for example, at 10 % of the normal dose on alternate days [116–120]. Continuity of treatment, even at low doses, is most important for treatment outcome [121].

Recently, evidence has emerged to suggest that dG^s incorporation into the DNA is not the sole cause of cell death after 6-MP. A role for inhibition of de novo purine synthesis (DNPS) has been demonstrated as higher TPMT levels were associated with a greater degree of cytotoxicity [122, 123].

With 6-TG, the chief mode of cytotoxic action was found to be incorporation of dG^s into DNA. Thus, the modes of cell death with 6-MP and 6-TG are not entirely the same and may be dependent on an individual's TPMT activity. This hypothesis is supported by other observations on the cellular pharmacology of 6-MP and 6-TG [117, 124–126]; however, the clinical importance of this mechanism has not been confirmed in a clinical setting.

In most cases the thiopurine drugs are extremely well tolerated, although it is frequently difficult to maintain patients on a stable dose. This is partly because the normal route of administration is by mouth, and this introduces the variables of compliance and absorption [127, 128]. The pharmacogenetics of TPMT introduces a further source of variability and indicates a need for dose individualization. Many centers now routinely assess RBC TPMT activity, and pretreatment assessment has become mandatory in some instances. Elsewhere, physicians have preferred to rely on the monitoring of the white cell count after initiation of therapy [129]. However, the onset of neutropenia can be very swift and life-threatening in patients homozygous for inactivating alleles of TPMT. Assessment of TPMT status prior to treatment could save unnecessary toxicity, which would otherwise compromise successful treatment of leukemia.

2.4 Reductases

2.4.1 Carbonyl Reductase

The major pathway of metabolism for the anthracycline class of topoisomerase II poisons is via reduction of the keto group on carbon 13 (Fig. 5). This reaction is mediated by carbonyl reductase (CBR) members of the short-chain dehydrogenase/reductase (SDR) family of enzymes. CBR1 is a monomeric, cytosolic protein, expressed in a variety of tissues, including liver, gastrointestinal tract, epidermis, CNS, kidney, and cardiac muscle [130], and appears to be the major hepatic enzyme that catalyses the 13 keto reduction of doxorubicin [131]. A variant form, from the same chromosomal location (21q22.13), is CBR3 [132]. CBR3 is expressed at lower levels than CBR1 in most tissues [133] and, in contrast to CBR1, appears to have less than 1000th of the activity against doxorubicin in vitro [131].

Doxorubicinol, the alcohol metabolite of doxorubicin, is relatively inactive compared to the parent compound. Nevertheless, doxorubicinol concentrations in plasma can exceed those of doxorubicin, and the metabolite has a half-life similar to that of the parent [134]. The situation is similar for daunorubicin [135]. Epirubicin is a substrate for CBR [136, 137], but epirubicinol is rapidly glucuronidated. The concentrations of idarubicinol after administration of idarubicin are relatively high [138], and idarubicinol retains equivalent or greater pharmacological potency than the parent [139].

Tumor activity of CBR has been associated with resistance to anthracyclines treatment [126–128]. Conversely, formation of alcohol metabolites of anthracyclines has been associated with anthracycline-induced cardiotoxicity in both loss and gain of function mouse models [140, 141]. Single nucleotide polymorphisms in both CBR1 and CBR3 have been described, but a comprehensive investigation into the impact of these SNPS on anthracycline efficacy or toxicity has yet to be carried out. Pharmacogenetic studies published to date have involved small cohorts of patients [142–145] or have investigated a limited number of SNPs [146].

2.4.2 NQO1

Also known as DT-diaphorase, NQO1 is an obligate two-electron reductase that can use either NADH or NADPH as cofactor. The gene encoding NQO1 is situated on chromosome 16 and a polymorphism

resulting in a proline to serine amino acid substitution has been described [147]. This results in a protein that is more rapidly degraded via polyubiquitination [148].

In reducing quinones to hydroquinones, NQO1 primarily acts to inactivate potential carcinogens. However, some hydroquinones are reactive, alkylating nucleophilic sites such as those on DNA [149]. The bioreductive class of alkylating agents exploits this mechanism to deliver alkylating species to tissues, such as tumor cells, where hypoxia and expression of NQO1 favor this pathway. Mitomycin C (Fig. 4) is the prototype drug of this class [150]. Other reductive enzymes, including XOR, and CYP reductase [151] have been suggested to play a role in mitomycin C activation, but NQO1 appears to play a dominant role. Other drugs which have been designed specifically to be activated by bioreduction include EO9 [151–153] and RH1 [154].

In terms of the pharmacokinetics of bioreductive agents, the rapid elimination of EO9 in humans [155] may be related to metabolism by NQO1. The pharmacokinetics of mitomycin C have not been extensively investigated after systemic administration. More information is available for intravesical administration of mitomycin C [156], but the influence of NQO1 expression is uncertain. A genetic polymorphism has been described for NQO1, with those individuals homozygous for the variant form having low or absent NQO1 activity due to more rapid protein degradation [157]. Whether this pharmacogenetic influence affects the systemic pharmacology of mitomycin C is unknown; however, the activity of NQO1 in peritoneal tumors was lower in individuals heterozygous or homozygous mutant for this polymorphism [158]. This reduction in tumor enzyme activity resulted in worse response to hyperthermic intraperitoneal mitomycin C treatment [158]. In contrast NQO1 genotype had no effect on response of superficial bladder cancer to intravesical administration of mitomycin C [159].

NQO1 has been found to influence the systemic and cellular pharmacology of 17-acetylamino-17-demethoxygeldanomycin (17AAG) [160], which binds to HSP90 and thus promotes the misfolding of HSP90 substrate proteins including PI3K and AKT. In vitro the hydroquinone metabolite of NQO1 catalyzed reduction ($17AAGH_2$) has been shown to be a more potent inhibitor of HSP90 ATPase activity [161]. NQO1 genotype had no impact on 17-AAG metabolism or toxicity in a phase I clinical trial [162].

Recently the rare NQO1 variant has been associated with a poor response to FEC therapy of breast cancer, [163] which is consistent with the sensitization to doxorubicin and epirubicin in NQO1 overexpressing cell line models [163, 164]. In contrast there is no association between NQO1 genotype and survival following FAC therapy [146]. The mechanism by which NQO1 influences cellular response to anthracyclines is unclear as doxorubicin is not a substrate for NQO1 [165, 166]. However, both basal and induced expression of topoisomerase I in PBMCs may be greater in individuals who are homozygous wild type for NQO1 C609T, compared with those who are homozygous for the rare allele [167].

2.5 Esterases

Esterases are ubiquitous enzymes that hydrolyze ester linkages. The main relevance of this class of enzymes for chemotherapeutic drugs is in the release of active agents from ester prodrugs [168]. The evaluation of the contribution of ester hydrolysis to overall metabolism is complicated by the higher activity of these enzymes in rodent plasma compared to that in man.

Esterases are implicated in the metabolic activation of two anticancer drugs. As described earlier (see Sect. 2.2) the initial reaction in the metabolic pathway that releases 5-FU from capecitabine is catalyzed by hepatic carboxylesterase activity. The second drug metabolized by carboxylesterases, irinotecan, comprises a camptothecin, topoisomerase I-binding moiety (SN-38), coupled to a piperidinopiperidine via an ester link. This ester is a substrate for carboxylesterase enzymes present in the

plasma in rodents [169] and in the liver in humans [170]. The microsomal carboxylesterase CES2 has most activity towards irinotecan [171, 172] and is present in normal and tumor tissues [172]. CES2 activity is believed to be the rate-limiting factor in irinotecan activation and expression of CES2 correlates with SN-38 production in human liver microsomes [172]. However, it is still not known if variation in expression in liver or tumor impacts on toxicity or efficacy. Polymorphisms in CES2 have no effect on metabolism of irinotecan [173, 174]. In contrast, a polymorphism in UGT 1A1 is predictive of severe diarrhea and neutropenia [175].

2.6 Cytidine Deaminase

Cytidine deaminase (CDA) catalyses the conversion of cytidine to uridine, by the oxidative replacement of the four amine with a carbonyl, as part of the pyrimidine salvage pathway. Therapeutic pyrimidine analogues are also substrates for CDA [176]. However, depending on the substrate, the CDA-catalyzed reaction can be a prodrug activation or a detoxification.

CDA catalyses the second enzymatic reaction of the sequential activation of capecitabine to 5-FU [85]. The impact of CDA activity on the efficacy or toxicity of capecitabine is uncertain, though a recent case study has reported severe hematotoxicity in a patient treated with capecitabine who had a high level of serum CDA activity [177].

In contrast CDA-catalyzed conversion of gemcitabine and cytarabine to 2′,2′- difluorodeoxyuridine and uracil arabinoside, respectively, is a detoxification reaction. CDA overexpression in isogenic cell line models confers resistance to both gemcitabine and cytarabine [178]. Clinically expression of CDA is associated with resistance to gemcitabine [179], and those patients who have a severe toxic response to gemcitabine have lower CDA activity compared to those with no toxicity [180]. The impact of non-synonymous SNPs on CDA activity and the efficacy of gemcitabine is uncertain. CDA containing an A79C transversion, coding for a substitution of a lysine with a glutamine, has two thirds of the activity of the wild-type enzyme when expressed in COS-1 cells [181]. In contrast, in RBC lysates from patients with NSCLC, CDA activity of homozygous wild-type patients was 60 % of the activity of those individuals carrying at least one minor allele. Median time to progression and overall survival was longer, and incidence of hematotoxicity was greater, in those individuals who were homozygous wild type than in those individuals who had at least one minor allele [182]. In another study, the A79C SNP has no effect on gemcitabine pharmacokinetics [183]. Homozygotes for the G208A SNP exhibit severe hematotoxicity when treated with gemcitabine presumably due to low serum CDA activity and subsequently low clearance of gemcitabine [183, 184].

3 Phase II

3.1 Glucuronidation

Glucuronidation may occur at any suitable hydroxyl, carboxyl, or primary or secondary amine [185]. N-glucuronides are less common than O-glucuronides but are more stable to enzymatic hydrolysis. The increase in molecular weight and in hydrophilicity following glucuronidation aids the elimination of xenobiotics by either biliary or renal excretion. Cleavage of glucuronides in the intestine, often by bacterial glucuronidase enzymes, may result in reabsorption of free drug and enterohepatic recycling. Because glucuronide conjugates are cleared from the body so rapidly, plasma concentrations are often undetectable.

In terms of chemotherapeutic agents, any drug with a suitable hydroxy, amine, or carboxylic acid function or a drug metabolized to introduce such a function may be a substrate for glucuronidation. Examples include epirubicin [186, 187], flavopiridol [188], hydroxy-metabolites of tamoxifen [189, 190], SN38 (activated form of irinotecan) [191], topotecan, retinoic acids [192], perillyl alcohol [193], and DMXAA [194].

A superfamily of genes encoding for UDP-glucuronosyltransferase enzymes has been characterized [195]. Individual isoforms associated with the glucuronidation of specific substrates have been identified. For instance, SN38 is glucuronidated by UGT1A1 [191], and genotypic variation in UGT activity relates to toxicity following irinotecan administration [196]. UGT2B7 plays a role in the glucuronidation of retinoic acid metabolites [192] and also contributes to the formation of morphine glucuronide [197] and that of epirubicin [187]. Polymorphisms have been identified in a number of these genes [198], including UGT1A1 [191], UGT2B7 [199], and UGT1A8 [200].

The UGT1A1*28 allele contains a seven TA repeat TATA box in the promoter region in contrast to a six TA repeat in the wild-type promoter (UGT1A1*1). Individuals homozygous for the *28 allele have an elevated serum bilirubin concentration and a predisposition to Gilberts' syndrome [201]. The minor allele is also associated with an increased risk of irinotecan-induced hematotoxicity [202], and a warning of this increased risk, together with advice that homozygous individuals are started on a lower dose, has been introduced on the label. However, a recent meta-analysis indicated that the impact of the UGT1A1*28 polymorphism may be restricted to high-dose irinotecan therapy [203], and consensus on the clinical implication of UGT1A1*28 testing has not been reached.

3.2 Sulfation

The conjugation of aryl drugs and their primary oxidation metabolites with sulfate is catalyzed by the sulfotransferase enzymes. SULT1A1 is the predominant form, a cytoplasmic enzyme that uses 3′-phosphoadenylsulfate as a sulfate donor. SULT1A1 is mainly expressed in the liver, lung, and kidney and catalyzes the sulfation of tamoxifen metabolites [189] and the putative chemoprevention agent curcumin [204].

3.3 Glutathione S-Transferases

Glutathione *S*-transferases (GSTs) are a family of soluble, dimeric enzymes (EC 2.5.1.18), which play an important role in the cellular detoxification system and are thought to have evolved to protect cells against reactive oxygen metabolites.

The GSTs comprise of two distinct supergene families that catalyze the conjugation of the tripeptide glutathione (γ-glu-cys-gly) (GSH) to a variety of electrophiles including arene oxides, unsaturated carbonyls, organic halides, and other substrates. A wide variety of endogenous (e.g., by-products of reactive oxygen species action) and exogenous (e.g., polycyclic aromatic hydrocarbons) electrophilic substrates have been identified.

Both soluble and microsomal associated GST have been described. The genetic loci encoding the soluble GSTs are located on 8 chromosomes, and to date 17 different isoforms have been identified in humans [205–213]. An additional six microsomal GST proteins classified in three families have also been characterized [214] (Table 1). Based on their substrate specificity, chemical affinity, amino acid sequence, kinetic behavior, and structural properties, the soluble human GSTs are categorized into eight main classes: alpha, mu, pi, sigma, theta, omega (or chi) [206, 210], and zeta in the cytosol and

Table 1 Classification of human glutathione S-transferase enzymes

Family	Genes	Chromosome	References
Soluble			
Alpha	GSTA1-GSTA4	6p12.2	[207]
Mu	GSTM1-GSTM5	1p13.3	[208, 213]
Pi	GSTP1	11q13	[209, 211]
Sigma	GSTS	4q21-22	[215]
Theta	GSTT1-GSTT2	22q11.2	[212]
Omega or chi	GSTO1-GSTO2	10q.	[206, 210, 216]
Zeta	GSTZ1	14q24.3	[205]
Kappa	GSTK	7q34	[217]
Membrane			
MAPEG I	FLAP	13q2	[214, 218]
	LTC4S	5q35	
	MGST2	4q28.3	
MAPEG II	MGST3	1q23	
MAPEG IV	MGST1	12p12	
	PGES1	9q34	

kappa in the mitochondria [219–221]. SNPs have been described in many genes in these families; however, most emphasis focuses on polymorphisms in the mu, theta, and pi families [220, 222].

GST proteins are expressed at high levels in mammalian liver and comprise up to 4 % of the total soluble proteins [223]. Detailed patterns of expression of the GSTs in fetal and adult tissue have been extensively investigated. GSTA1, GSTA2, GSTM1, and GSTP1 have been detected in fetal tissues ([224] and reviewed in [221]), hematopoietic cell lines [225], hematopoietic cells [226], and adult brain [224]. GSTO1-1 expression has been reported in a wide range of adult, fetal, and placental tissue [227].

Expression of GST proteins are controlled by regulatory elements such as the glucocorticoid response element (GRE), antioxidant (or electrophile) response element (ARE), and the xenobiotic response element (XRE). However, GST expression also seems to be induced by compounds such as the isothiocyanates and alpha-beta unsaturated ketones ([228] and references therein).

The GSTs, which exist mainly as dimers, catalyze the nucleophilic attack of GSH on electrophilic substrates, thus forming an important line of defense, protecting various cell components from reactive molecules [223, 229]. There are many examples of chemotherapeutic agents that undergo GSH conjugation, including the electrophilic alkylating agents such as busulfan [223, 228], melphalan, and chlorambucil [230, 231]. Detoxification involves the binding of GSH to electrophilic chemicals and the export of the resulting GSH S-conjugates from the cell.

Glutathione conjugates are excreted immediately via the bile or transported to the kidney where the γ-glutamyl moiety is split off via γ-glutamyl transpeptidase, the glycine via a dipeptidase, and the remaining cysteine is *N*-acetylated to be excreted as a mercapturic acid. Instead of *N*-acetylation, the cysteine conjugate can undergo several other metabolic reactions that can lead to bioactivation [232].

The influence of GST genotype on susceptibility to particular diseases, prognosis, and drug resistance has been extensively investigated over the past 30 years. However, although some issues seem clear, the role of GSTs is still not completely understood. Drug treatments used in cancer vary considerably, such that a particular genotype may be an advantage in one instance and not in another. It is important to emphasize the different influence of particular GST genotype on individual disease types and how the interindividual differences between people may affect regulation of GST expression [233].

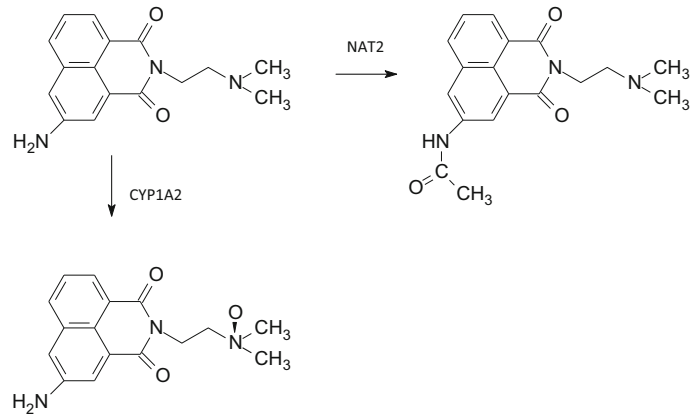


Fig. 9 Metabolism of amonafide by NAT2 and by CYP1A2. The N-acetyl metabolite of amonafide is also a substrate for CYP1A2 and inhibits the metabolism of the parent compound

3.4 N-*Acetyltransferase (NAT)*

The acetylation of aromatic and heterocyclic amines is mediated by two enzymes, NAT1 and NAT2. NAT1 is expressed in a variety of tissues, whereas NAT2 is confined to the liver. A genetic polymorphism in NAT2 was initially characterized as slow and fast acetylators of isoniazid. Polymorphisms in NAT1 have also now been identified, with functional significance in terms of lower enzyme activity [234]. The pharmacogenetics of NAT1 and NAT2 have been extensively investigated with regard to their role in the metabolism of carcinogenic arylamines [235].

In terms of the pharmacology of chemotherapeutic agents, amonafide is the best example of a drug subject to N-acetylation (Fig. 9). With regard to antitumor effect and toxicity, the interpretation of the influence of N-acetylation on clinical outcome is complicated by the fact that the metabolite inhibits the oxidative inactivation of the parent compound [236]. Thus, plasma clearance of amonafide was lower and hematological toxicity was significantly greater in fast acetylators. Subsequent studies of this compound used a dosing scheme based on caffeine acetylation phenotype [237], and NAT status was incorporated into pharmacokinetic and pharmacodynamic models [238].

4 Conclusions

Although the study of drug metabolism focuses on oxidation reactions mediated by cytochrome P450 enzymes, there is a significant role for other pathways of metabolism for many drugs used in cancer chemotherapy. In part this is inherent in the way that many antimetabolites mimic endogenous substrates, which have their own anabolic and catabolic pathways. Non-CYP-mediated metabolism dominates the pharmacology of a number of these drugs (e.g., 6-mercaptopurine and 5-FU), such that understanding of the genetics and enzymology of the enzymes involved (TPMT and DPD, respectively) is essential for the safe use of the drug. In other examples, metabolism may be involved in inactivation of a reactive intermediate or activation of a prodrug, which may have implications for tumor sensitivity or host toxicity. As our knowledge of the genetics of the enzymes involved in these reactions increases, the classification of enzymes is changing. Identification of an enzyme by its substrate or cofactor specificity is being replaced by a classification system based on gene sequence homology. This process has been applied successfully to the CYP family of enzymes and now extends

to ALDH, UGT, NAT, and other non-CYP enzyme superfamilies. The genetic tools that accompany this evolution of nomenclature should also provide techniques for the further understanding and characterization of enzymes and their role in the pharmacology of chemotherapeutic agents.

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Pharmacogenomics and Cancer Therapy: Somatic and Germline Polymorphisms

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Abstract Pharmacogenomics has the potential to not only impact the pharmacokinetics of an anticancer drug but also the tumor response, or pharmacodynamics. This chapter focuses on the most up-to-date clinical trials involving pharmacogenomics and anticancer therapy. A brief introduction of drug development and the difference between somatic and germline DNA mutations sets up the chapter for understanding the progress which has been made in regard to individualized cancer therapy. Although researchers and healthcare practitioners have realized the potential of pharmacogenomics for several years, it has only been until recently that genotype-guided, prospective clinical trials have been done. Validating these biomarkers and genetic associations is vital to translating pharmacogenomics into clinical practice. This chapter highlights the advancements that have been made with key examples such as tamoxifen and CYP2D6, erlotinib and EGFR, vemurafenib and BRAF, and many others.

Keywords Pharmacogenetics • Pharmacogenomics • Somatic • Germline • DNA • Mutation • Response • Toxicity

1 Introduction

Pharmacogenomics, the study of how genetic inheritance influences responses to drugs, is an innovative advancement in pharmacological treatment, with the hope of personalizing cancer therapy [1]. Although there is currently a breadth of treatment available to combat a variety of cancers, the interpatient response to these anticancer drugs tends to fluctuate greatly. The reason for this large heterogeneity among efficacy and toxicity between patients can be explained by genetics [2]. With marginal efficacy, dose-limiting toxicities, and high costs, it is essential that practitioners be able to classify which patients are most likely to respond to treatment and which are most likely to experience toxicity. Pharmacogenetics has the potential to effect all phases of pharmacokinetics, often through germline DNA mutations. In addition, pharmacogenetics can influence tumor response, or pharmacodynamics,

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as mutations can occur within the tumor itself, also known as somatic mutations. The discovery of these mutations through genome-wide association and candidate gene studies is possible through a variety of novel genotyping methods. Validation of these variants through prospective clinical trials will contribute to providing tailored chemotherapy to cancer patients based on the molecular profile of the patient.

2 Drug Development

Traditionally, oncology drug development and approval is similar to that of non-oncology drugs. A potential agent must go through preclinical phase I, II, and III trials before being approved by the Food and Drug Administration (FDA). During preclinical trials, toxicology testing is done in animals to determine the starting dose for human phase I trials. This is usually done using xenografts, tumors obtained from patients at initial diagnosis and grown in mice. The starting dose for phase I trials is defined as 1/10th the dose (based on BSA) that is lethal or causes severe toxicity to 10 % of rodents studied. At this point, an investigational new drug (IND) application is filed with the FDA. Once the IND is approved, human phase I studies can begin. The primary goal for a phase I study is to determine the maximum tolerated dose (MTD) and dose-limiting toxicities (DLTs) of the agent; secondary goals include obtaining pharmacokinetic and pharmacodynamic information. Using the Fibonacci method, the MTD is determined by escalating the dose until a DLT occurs in $\geq$33 % of patients (i.e., 1 out of 3). If three patients are entered on the first dose level and none of the patients experience a DLT, then the dose is escalated and three patients are entered on the next dose level. If 1 out of 3 patients has a DLT, then 3 more patients are entered at the same dose level. If 2 out of 6 patients have a DLT, then this is the MTD and the dose recommended for phase II trials is one dose level lower. The primary goal in a phase II study is to determine the time to disease progression and objective response rates (i.e., efficacy). Finally, phase III trials are done to evaluate the time to disease progression and objective response rates, in comparison to the standard of care (or placebo [best supportive care], if appropriate).

Phase III trials are continuously increasing in size, duration, and unfortunately expense, especially for orphan indications. However, the failure rate of oncology drugs in phase III trials remains high. Only 34 % of these agents, with results announced from 2003 to 2010, achieved statistical significance in their primary end points [3]. On the other hand, the development of targeted therapies has been promoted to the "front" of the queue due to promising results in early phase trials. For example, crizotinib, a tyrosine kinase inhibitor targeting anaplastic lymphoma kinase (ALK), demonstrated significant increases in response rates and progression-free survival (PFS) in phase I trials. With these results, and preliminary findings of phase II trials, the FDA granted accelerated approval of crizotinib with a companion diagnostic test for the ALK rearrangement. Although crizotinib is already approved, the FDA still requires post-approval phase III clinical trials validating the effectiveness compared to the standard of care. The expectation is that new molecularly targeted agents will be more effective and less toxic than the previous generations of anticancer drugs [4]. Important advances in drug discovery technologies, such as high-throughput screening and structure-based design strategies, have made the approach to small molecule targets possible [4].

Pharmacogenomics has the potential to impact pharmaceutical research and drug development by increasing the probability of successfully developing a new drug and/or lowering the costs. Using pharmacogenomic tests to create an enhanced population of responders can allow the drug development process to become more time-efficient and cost-efficient. Pharmacogenomics also has the potential to salvage products that would have otherwise failed in development due to adverse drug reactions in a subset of the target population. Recognizing genomic markers that may make patients prone to adverse events could allow the drug to be made available to patients who only test negative for the genetic variants [5]. Identifying molecular biomarkers with diagnostic and prognostic power may

Table 1 Anticancer drugs approved by the Food and Drug Administration that require labeling regarding pharmacogenomic biomarkers

Somatic		Germline	
Drug	Biomarker	Drug	Biomarker
Arsenic trioxide	PML-RAR alpha	5-fluorouracil/ capecitabine	Dihydropyrimidine dehydrogenase (DPYD) to drug and biomarker, respectively
Cetuximab Panitumumab Erlotinib	Epidermal growth factor receptor (EGFR)	6-Mercaptopurine Thioguanine	Thiopurine-S-methyltransferase (TPMT)
Cetuximab Panitumumab	KRAS	Irinotecan Nilotinib	Uridine diphosphate glucuronosyltransferase (UGT) 1A1
Imatinib Dasatinib Nilotinib	BCR-ABL	Tamoxifen	Estrogen receptor, CYP2D6
Trastuzumab Lapatinib Pertuzumab Ado-trastuzumab emtansine	HER-2/neu		
Imatinib	C-kit		
Vemurafenib Dabrafenib Trametinib	BRAF V600E		
Crizotinib	Anaplastic lymphoma kinase (ALK)		

help, not only to accelerate drug approval but also to manage post-approval risks [6]. On the other hand, the development of a pharmacogenetic test in parallel with a drug may provide additional costs and complexity to the already inherent risky process of drug development [5]. Realizing the potential, most, if not all, pharmaceutical companies are integrating pharmacogenomics into drug development. Regulatory agencies are encouraging companies to explore and apply toxicogenomic and pharmacogenomic technologies in drug development [7].

3 Somatic Versus Germline DNA

One challenge in genotyping patients is whether to analyze germline DNA, somatic mutations, or both. DNA analysis for pharmacogenetic purposes is usually performed with germline DNA attained by blood samples. However, for anticancer drugs, DNA is also analyzed in tumor tissue, referred to as somatic mutation analysis. The major difference between these two methods is that germline polymorphisms are inherited and transmitted to the offspring, whereas somatic mutations are not and are present in the tumor. Analyzing germline DNA can often help to predict pharmacokinetic and pharmacodynamic responses; however, in oncology, analyzing tumor tissue, or somatic DNA, is especially useful in evaluating pharmacodynamic effects, such as tumor response [8]. A prime example is the somatic mutation involved in KRAS activation. This mutation is associated with an increased risk of nonresponse to the epidermal growth factor receptor (EGFR) inhibitors, panitumumab and cetuximab. Patients who harbor wild-type KRAS tumors are almost exclusively likely to respond to these agents. Another key example of a somatic mutation influencing the pharmacodynamic response is in the kinase domain of EGFR seen in non-small-cell lung cancer (NSCLC) patients. Tumors expressing mutated EGFR have been shown to have an increased response to the small molecule tyrosine kinase

inhibitor, erlotinib [8]. Table 1 illustrates the anticancer drugs approved by the FDA and mandated to have pharmacogenetic information in the labeling. By analyzing both germline and somatic DNA, researchers are able to predict both pharmacokinetic and pharmacodynamic responses.

Studies have established that germline genotype is highly conserved in DNA from fresh/frozen tumor tissue. Trials comparing genotype using paraffin-embedded tumor samples and germline samples show that concordance between germline and tumor DNA genotypes is virtually 100 % [9]. Investigators conclude that paraffin-embedded adjacent normal tissue can be used for normal tissue genotyping, providing accurate and useful genetic material. Although these percentages are high, there is the possibility of discordance. For example, in a study by Marsh et al., examination of the tandem repeat sequence in the thymidylate synthase gene (TYMS), one out of 45 colorectal cancer patients genotype was not identical between paired samples of colorectal tumor and normal tissue [9]. This can happen with loss of heterozygosity (LOH), which occurs when an individual inherits a gene with one inactivated allele and subsequent inactivation of the second allele by a mutation in the tumor leads to loss of function. An example would be the inactivation of tumor-suppressor genes in oncology (i.e., p53), resulting in tumorigenesis. Therefore, in the presence of LOH, germline DNA would have a higher frequency of heterozygotes than the corresponding somatic DNA. While germline DNA is easily obtained, more readily available, and sufficient for genetic analysis, somatic DNA may provide unique information that could have significant implications on using targeted therapy [10].

4 Anticancer Drugs and Related Pharmacogenomics

4.1 Tamoxifen and CYP2D6

Tamoxifen is a selective estrogen receptor modulator used in the treatment of hormone-positive breast cancer in both pre- and postmenopausal women. It significantly reduces the recurrence rate of breast cancer, mainly through its primary metabolite, endoxifen [11]. Endoxifen has roughly a 50-fold higher affinity for the estrogen receptor than tamoxifen [12]. The enzyme responsible for this reaction is cytochrome P450 (CYP) 2D6. Anywhere from 35 to 50 % of patients taking tamoxifen may not receive the maximum benefit secondary to genetic differences that limit its metabolism [12]. Previous studies estimate that approximately half of all women are extensive metabolizers (EM, active CYP2D6), 40 % are intermediate metabolizers (IM, reduced function CYP2D6), and 10 % are poor metabolizers (PM, inactive CYP2D6). The most common allele resulting in loss of CYP2D6 activity is *4. Other common alleles which lead to reduced activity include *10 and *17. A wide heterogeneity exist among populations, with roughly 20 % of Caucasians, 8 % of African Americans, and <1 % of Japanese carrying the *4 allele [13].

A prospective study of 1,370 breast cancer patients investigated the correlation between endoxifen concentrations and recurrence rates in ultra-extensive metabolizers (UEM), EMs, IMs, and PMs [14]. A threshold was identified with women in the upper four quintiles of endoxifen concentration appearing to have a 26 % lower recurrence rate than women in the bottom quintile (hazard ratio [HR] = 0.74; 95 % confidence interval [CI], 0.55–1.00). The predictors of this higher-risk bottom quintile were PM/IM genotype, higher body mass index (BMI), and lower endoxifen concentrations as compared with the mean for the cohort as a whole. UEMs, EMs, IMs, and PMs had average endoxifen levels (ng/mL) of 22.8, 15.9, 8.1, and 5.6, respectively [14].

In a multicenter prospective study, investigators examined the feasibility of using CYP2D6 genotype to guide tamoxifen dosing [12]. One hundred and nineteen patients taking tamoxifen for at least 4 months were genotyped. Patients determined to be EMs continued treatment with tamoxifen 20 mg once daily. Patients determined to be IMs or PMs had their tamoxifen dose doubled to 40 mg daily.

After 4 months ± 4 weeks of genotype-directed therapy, tamoxifen metabolite concentrations were repeated. The median endoxifen concentrations (ng/mL) at baseline were 34.3, 18.5, and 4.2 in EM, IM, and PMs, respectively. The median endoxifen concentrations 4 months later were 29.2, 21.8, and 12.9 in EM, IM, and PMs, respectively. The median intra-patient change from baseline was −1.5, +7.6, and +6.1 in EM, IM, and PMs, respectively. An increase in dose for the PMs resulted in a significant pattern of change ($P = 0.0035$) in median endoxifen concentration when compared to EMs, but not between IMs and PMs. After dose adjustment, the endoxifen concentrations between IMs and EMs were no longer significantly different [12].

Although the FDA required a change in labeling for tamoxifen regarding pharmacogenomics and CYP2D6, the clinical utility is still highly debated. Two large studies have demonstrated a lack of association with regards to CYP2D6 status and breast cancer recurrence [15, 16]; however, major flaws in study design were noted, such as the use of tumor DNA. Prospective data evaluating the impact of dose adjustment in PMs/IMs on recurrence rates compared to recurrence rates in EMs is needed to define the clinical utility of preemptive CYP2D6 pharmacogenetic testing in practice.

4.2 Irinotecan and UGT1A1

Irinotecan, a camptothecin derivative, prevents the religation of cleaved DNA strands by inhibiting topoisomerase I. Uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) conjugates irinotecan to a glucuronide inactive metabolite. This metabolite can undergo enterohepatic recirculation or is eventually excreted in the bile and urine. Polymorphisms of UGT1A1 can lead to a significant reduction in conjugation, thus leading to an increase in the active metabolite, SN-38 [17]. The increase in exposure to SN-38 can increase the risk of severe neutropenia. More than 60 polymorphisms have been identified to date which cause TA repeats in the promoter region. The wild-type allele is UGT1A1*1, while the most common polymorphic variants are *28, *93, *60, and *6; however, UGT1A1*28 is the most common variant and leads to a 70 % reduction in expression of the gene. The frequency of the *28 allele is 39 % in Caucasians, 16 % in Asians, and 43 % in Africans. Roughly 10–20 % of Caucasians and African Americans are homozygous for *28, and less than 5 % of Asians are homozygous for *28 [17].

A meta-analysis demonstrated that there was no difference in toxicity between homozygous wild-typed and heterozygous versus homozygous mutant (*28/*28) at low doses (100–125 mg/m^2); however, there was a significant increase in toxicity for the *28/*28 patients at medium and high doses (odd ratio [OR] 3.22 and 27.8, respectively) [18]. The toxicity was significant for neutropenia, while there was no difference in diarrhea. In a study of 250 metastatic colorectal cancer patients, the hematologic effects and tumor responses were higher for *28/*28 patients ($P = 0.03$). UGT1A1 *28/*28 was specifically associated with a higher risk of grade 3–4 neutropenia (OR 8.63; 95 % CI 1.31–56.55), which was only relevant for the first cycle [18].

A recent study analyzed the maximum tolerated dose in *1/*1, *1/*28, and *28/*28 patients and the impact on overall response rate (ORR) [19]. The starting dose of biweekly irinotecan was 180 mg/m^2 for the *1/*1, 110 mg/m^2 for the *1/*28, and 90 mg/m^2 for the *28/*28 genotypes. The dose of irinotecan was escalated to 450 mg/m^2 in patients with *1/*1 genotype, to 390 mg/m^2 in *1/*28 genotype, and to 150 mg/m^2 in *28/*28 genotype. Neutropenia and diarrhea were the most common grade 3 or 4 toxicities. In all, 56 patients were assessable for tumor response with an overall response rate (ORR) of 46 % ($n = 25$). The ORR was 60 % in patients with *1/*1 genotype, 39 % in those with *1/*28 genotype, and 13 % in *28/*28 ($P = 0.049$). To evaluate the relationship between dose and response, investigators grouped patients into two cohorts: 27 patients treated with less than 260 mg/m^2 and 29 patients treated with greater than or equal to 260 mg/m^2. In all, 67 % of patients treated with greater than or equal to 260 mg/m^2 of irinotecan achieved a complete or partial response in comparison with only 24 % of patients treated with <260 mg/m^2 ($P = 0.001$) [19].

Not only can screening for the TA repeat polymorphism select patients who are likely to experience toxicity on irinotecan, recent data suggests that with the MTD being much lower in *28/*28 patients, the efficacy may also be compromised. Furthermore, *1/*1 and *1/*28 patients were found to tolerate significantly higher doses compared to the recommended 180 mg/m^2 provided to patients currently. Preemptive UGT1A1 testing may allow for higher, more effective doses to be administered to these patients, while limiting the dose and toxicities seen in *28/*28 patients.

4.3 6-Mercaptopurine and TPMT

6-Mercaptopurine (6-MP) is one of the backbone agents used to treat acute lymphoblastic leukemia (ALL) in children. It inhibits glutamine-5-phosphoribosylpyrophosphate amidotransferase, the first enzyme unique to the de novo pathway for purine ribonucleotide synthesis. Thiopurine-S-methyltransferase (TPMT) is a cytosolic enzyme ubiquitously expressed in the human body and catalyzes the S-methylation of thiopurines into inactive compounds. The TPMT locus is subject to genetic polymorphisms with roughly 6–11 % of the population being heterozygous and 0.2–0.6 % being homozygous for the variant allele. TPMT*2 and *3 account for 95 % of defective TPMT activity. Myelotoxicity during 6-MP is elevated in patients who, because of a TPMT deficiency, have increased thioguanine nucleotides and therefore increased cytotoxicity [20].

In a study of 180 children with ALL treated with a 6-MP regimen, patients were genotyped and phenotyped for TPMT activity. A statistically significant ($P<0.01$) inverse relationship between concentration of thioguanine nucleotides and TPMT was seen, with an average concentration (pmol/8×10^8 erythrocytes) of 417, 963, and 3,565 in TPMT homozygous wild type ($n=161$), heterozygous ($n=17$), and homozygous-deficient ($n=2$) patients, respectively. The percentage of wild-type, heterozygous, and homozygous-deficient patients who were able to tolerate the full dose throughout treatment was 84 %, 65 %, and 7 %, respectively. All homozygous-deficient patients required a decrease in dose, while 35 % and 7 % of heterozygous and wild-type patients required a decrease in dose, respectively [21]. Another study evaluated the association of TPMT genotype with minimal residual disease load before and after treatment with 6-MP in 814 children with ALL. Patients ($n=55$) heterozygous for allelic variants of TPMT had significantly lower rate of minimal residual disease positivity (9.1 %) compared with homozygote wild-typed patients ($n=755$) (22.8 %) ($P=0.02$), yielding a 2.9-fold reduction in risk for heterozygous patients. All patients ($n=4$) homozygous for the mutant TPMT allele were treated with an approximate 10-fold reduced dose of 6MP, while dose adjustments were not performed for heterozygote patients [20].

It is evident that homozygosity of the TPMT allele necessitates an approximate 10-fold reduction in initial dose due to a significantly increased risk of myelosuppression. However, patients who are heterozygous may be at an increased risk for toxicity, but may also have increased chance of efficacy. The Clinical Pharmacogenetics Implementation Consortium (CPIC) recommends specific dosing guidelines for homozygous mutant, heterozygous, and homozygous wild-type patients [22].

4.4 Fluorouracil and TYMS, MTHFR, DPD

4.4.1 Thymidylate Synthase

Fluorouracil is one of the backbone agents used in several pharmacotherapy regimens to treat colorectal cancer and gastric cancer. Thymidylate synthase (TS) is a critical enzyme involved in DNA synthesis and serves as the primary target of fluorouracil [23]. Single nucleotide polymorphisms (SNPs)

and variable number of tandem repeat (VNTR) polymorphisms are common genetic variants that may occur in specific regions of the human genome. One such polymorphism is that of the thymidylate synthase gene (TYMS). There has been increasing evidence supporting the role of TYMS for determining sensitivity to fluorouracil. TYMS contains a tandem repeat consisting of 28-base pair (bp) repeat units found in the 5′ untranslated region, acting as an enhancer to the TYMS promoter (TS enhancer region [TSER]). Since fluorouracil is involved with the inhibition of TS, a low TYMS-mRNA expression would increase the cytotoxicity of fluorouracil, whereas a high TYMS-mRNA expression would decrease the cytotoxicity of fluorouracil. Polymorphisms which confer low mRNA expressing alleles include the 6-bp deletion in the TYMS 3′-untranslated region (3′-UTR) and a VNTR sequence in the TYMS 5′-untranslated region (5′-UTR), TSER *2/*2. Polymorphisms which confer high mRNA expressing alleles include the 6-bp addition in the 3′-UTR region and the 5′-UTR, TSER *3/*3 [11]. TSER *3/*3 seems to be associated with a lower response to neoadjuvant fluorouracil-based chemoradiotherapy (CRT) for patients with rectal cancer [23]. Approximately 25 % of colorectal cancer patients are homozygous for TSER *3/*3, 20 % are homozygous for *2/*2, and 55 % are heterozygous for *2/*3. In a study of 65 patients with rectal cancer treated with fluorouracil-based preoperative CRT, patients harboring TSER *3/*3 achieved a 22 % downstaging rate, whereas *2/*2 patients achieved a 60 % downstaging rate [24].

A prospective, single-institution, phase 2 study was completed using TYMS genotyping to direct neoadjuvant CRT for patients with rectal cancer. Overall, 135 patients were enrolled and genotyped [25]. Investigators labeled those patients with TYMS *3/*3 or *3/*4 as the poor-risk group (27.4 %, those least likely to respond to conventional fluorouracil-based treatment) and those with *2/*2, *2/*3, and *2/*4 as the good-risk group (72.6 %, those most likely to respond). The poor-risk group received CRT with fluorouracil plus weekly IV irinotecan. The good-risk group was treated with standard CRT using infusional fluorouracil. The primary end points of downstaging and complete tumor response rates reached 64.4 % and 20 % for good risk and 64.5 % and 42 % for poor-risk patients, respectively. Prior studies demonstrated that the downstaging rates for unselected patients and those homozygous for the TYMS *3 allele with rectal cancer treated with CRT were 45 % and 22 %, respectively. One-year, 2-year, and 3-year overall survival were 96.9 %, 80.6 %, and 78.2 %, respectively, in the good-risk group. One-year, 2-year, and 3-year overall survival were 94.3 %, 94.3 %, and 83.6 %, respectively, in the poor-risk group [25].

4.4.2 Methylenetetrahydrofolate

Methylenetetrahydrofolate (MTHFR) is a key enzyme for the intracellular folate homeostasis and metabolism. It catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate (MTHF) to 5-methyltetrahydrofolate, the primary methyl donor for the re-methylation of homocysteine to methionine [26]. A ternary complex is formed with FdUMP (active metabolite of fluorouracil), TS, and 5,10-MTHF, which halts pyrimidine and DNA synthesis. A reduction in enzyme activity of MTHFR would therefore increase 5,10-MTHF and increase fluorouracil cytotoxicity. The two polymorphic variants described are 677C>T and 1298A>C, with the 677C>T variant being the most common and more highly associated with clinical outcome [26].

In a study of 75 gastric carcinoma patients treated with fluorouracil-based therapy, all patients were genotyped for the 677C>T variant [27]. The results showed that the 677TT genotype had significantly greater response rates than patients with CC or CT genotypes (83 % vs. 8.3 % and 15.2 %, respectively, $P<0.001$). The adverse effects were greater in the TT genotypes (which were mainly gastrointestinal side effects) [27]. Other studies have shown the 677TT genotype to be associated with a significantly increased time to progression, while no relevant effects were noticed on overall survival [26]. The 1298CC genotype, in a group of advanced colorectal cancer patients, was correlated with an increased risk of developing severe adverse events after fluorouracil-based chemotherapy [26]. In a study

of 132 advanced gastric cancer patients taking fluorouracil-based therapy, patients were genotyped for TS polymorphisms, as well as MTHFR polymorphisms. Results showed that patients who had MTHFR 677TT also had significantly better survival compared with patients with CT or CC (HR 0.57 $P = 0.039$) [28].

4.4.3 Dihydropyrimidine Dehydrogenase

Dihydropyrimidine dehydrogenase (DPD), the rate-limiting enzyme involved in the metabolism of fluorouracil to 5,6-dihydrofluorouracil, has been shown to exhibit polymorphic potential [29]. It has been reported that roughly 80 % of administered fluorouracil is catabolized by DPD. A reduction in enzyme activity would dramatically increase fluorouracil levels in the body, enhancing its cytotoxicity. An estimated 3 % of patients exhibit a deficiency in DPD translating to an approximate 20-fold increase in half-life of fluorouracil. To date, 31 variants of DPD have been identified [30]. The specific polymorphic variant most commonly associated with DPD is a splice-site mutation, IV14+1G>A (located on exon 14-flanking region and causing skipping of 165 bp), leading to decreased enzyme activity [29].

In a study of 60 patients who had experienced grade 3 or 4 toxicity after administration of 5-FU therapy, all patients were genotyped for the splice-site mutation [30]. Results showed that 28 % of all patients were heterozygous or homozygous for the mutation and overall decreased DPD activity was seen in about 60 % of all cases. Out of the patients who had low DPD activity, 42 % were genotyped as heterozygous and 3 % were homozygous. Of the patients with normal DPD activity, only 4 % had the detected mutation [30]. In a separate study of 122 colorectal cancer patients treated with fluorouracil-based chemotherapy, patients were genotyped for TS, MTHFR, and DPD polymorphisms [31]. Results showed that patients with the genetic variant IVS14+1 G/A or c1896 C/T in the DPD gene had a statistically significant increased risk of experiencing toxicity (RR 2.0 and 6.0, respectively), both having a high specificity (0.97 and 0.98, respectively) and low sensitivity (0.04 and 0.13, respectively) [31].

With fluorouracil being such a key agent in the treatment of colorectal and gastric carcinomas, identification of likely responders is vital. Polymorphisms affecting TS expression have been fairly consistent with regard to clinical outcome and/or toxicity. However, the correlation of MTHFR and DPD mutations with outcome and/or toxicity is not as well established. It should be noted that in the majority of studies demonstrating no correlation between MTHFR and outcome, fluorouracil was used in association with other antineoplastic agents, while in the analyses that showed positive correlations, fluorouracil was generally employed alone or with leucovorin [26]. With such a low prevalence of patients with a DPD deficiency, it is difficult to ascertain enough power to establish a clear association.

4.5 Methotrexate and MTHFR

MTHFR is not only involved with the pharmacogenomics of fluorouracil but methotrexate as well. Methotrexate (MTX) is utilized in a variety of regimens to treat leukemias, lymphomas, head/neck cancers, and sarcomas. Inherited changes in MTHFR activity can cause a change in reduced folate pools, thus having an impact on the response of malignant and nonmalignant cells to antifolate drugs such as MTX [26]. As mentioned previously, the C677T and A1298C variants have been associated with decreased activity of MTHFR, an increased level of homocysteine, and an altered distribution of folate. These variants occur frequently among Caucasian, Asian, Hispanic, and Latino/Mexico-American populations, with a prevalence of roughly 25–45 %. About 15–23 % of Caucasians are heterozygous for the variant alleles [26].

In a study of 110 adult patients with high-grade non-Hodgkin's lymphoma taking MTX-containing combination chemotherapy, an association between 677TT genotype and increased risk of developing

toxicity was found [30]. The 677TT genotype was significantly overrepresented among cases with mucositis (OR = 4.85; $P = 0.009$) and those with hepatic toxicity (OR = 3.43; $P = 0.052$). Worse prognosis in terms of event-free survival was also seen with the 677T allele [32]. Several studies have shown the same outcomes; however, other studies in which leucovorin was given as rescue therapy along with the MTX-containing regimen showed no effect on toxicity. In fact, one study of 186 pediatric acute lymphocytic leukemia patients taking MTX (high-dose)-containing therapy plus leucovorin actually showed lower rates of hematologic toxicity in patients carrying the 677T allele [26].

Salazar and colleagues investigated the usefulness of the MTHFR genotype to guide the MTX dosage in the consolidation phase in 141 ALL children [31]. Investigators analyzed the C677T and A1298T mutations in the MTHFR gene. Patients with an unfavorable MTHFR genotype (homozygous 677T, homozygous 1298C, and compound heterozygous patients) associated with a decreased enzymatic activity were given 3 g/m^2 MTX in a 24-h infusion, whereas patients with a favorable MTHFR genotype (heterozygous and wild-type patients), associated with a normal enzymatic activity, were given an increased dose of 5 g/m^2 MTX. Patients with a favorable MTHFR genotype had a significantly lower risk of suffering an event than patients with an unfavorable MTHFR genotype ($P = 0.012$). Patients with a MTHFR genotype associated with decreased enzyme activity were 4.3 times more at risk to suffer an event than those with a genotype associated with normal activity. Those with decreased MTHFR activity had a significant decrease in the number of platelets (34.3 % vs. 14.3 %; $P = 0.014$) and a significant increase in serum creatinine with the same genotype (18.2 % vs. 3.9 %; $P = 0.013$). Investigators identified that the favorable group had similar, and in some cases less toxicity, and an increased event-free survival with the increased dosage [33].

Although there is data to suggest mutations within the MTHFR gene may impact outcomes and/or toxicity with MTX, this association is not as evident when patients are given leucovorin as rescue therapy. However, one prospective, genotype-guided study was able to increase the dose of MTX in patients demonstrating a favorable genetic profile in order to maximize response.

4.6 Platinums and GSTs, NERs

4.6.1 Glutathione-S-Transferase

Platinum compounds, including cisplatin, carboplatin, and oxaliplatin, are used as alkylating agents to treat a variety of tumors including lung cancer, ovarian cancer, gastric cancer, and several others. Platinum complexes are inactivated by sulfhydryl groups, which are then covalently bound to glutathione and thiosulfate via glutathione-S-transferase (GST). These GSTs are crucial for the cells defense system as it is involved in the detoxification of platinum compounds [34]. Polymorphic variants of different GSTs have led to diminished enzyme activity, therefore, increasing the chance of platinum toxicity. GSTP is the predominant GST in the majority of tumors; however, its concentration was found to be significantly increased in lung, colon, and stomach cancer tissues and lower in lymphoma and breast cancer. In in vitro analyses of cancer cell lines, the GSTM1 null genotype was dominant in small cell lung, kidney, breast, and ovarian carcinoma cells, whereas the GSTT1 null genotype was dominant in cervical and endometrial carcinoma cells. Moreover, GSTP seems to be predominantly correlated with the detoxification of platinum compounds as opposed to GSTT and GSTM variants. The specific polymorphic variants associated with GSTP1 include A1404G (Ile105Val) and C2294T (Ala114Val). The occurrence of A1404G, roughly 19 % in African Americans, 7 % in European Americans, and 9 % in Australian Europeans, is much more common than the C2294T variant. GSTT1 and GSTM1 are homozygous deletions that lead to absence of enzyme activity and occur in roughly 50 % of Egyptians and 15 % of North Americans [34].

In a study of 175 patients taking fluorouracil and cisplatin for advanced gastric cancer treatment, patients were genotyped for GSTP1, GSTT1, and GSTM1 [35]. There were no significant differences

in response for GSTT1 and GSTM1 variants. However, 33 % of patients expressing GSTP1 105 Val/Val were nonresponders, while 77 % of patients expressing 105 Ile/Ile were nonresponders ($P<0.001$) [35]. In another study, DNA was isolated from 139 patients with locally advanced gastric cancer before chemotherapy [36]. One hundred ten patients were analyzed for GSTT1, 112 patients for GSTM1, and 132 patients for GSTP1. GSTT1, GSTM1, and GSTP1 genotypes were not correlated with response to chemotherapy ($P=0.57$, $P=0.38$, $P=0.33$). However, in tumor-resected patients, an improved survival for patients with the GSTM1-present genotype compared to patients with the GSTM1 null genotype was found ($P=0.017$). Moreover, the GSTM1-present genotype showed a significantly better tumor-related ($P=0.017$) and disease-free survival ($P=0.029$) [36].

4.6.2 Nucleotide Excision Repair Enzymes

Platinum–DNA adducts are repaired by the nucleotide excision repair (NER) pathway. Suboptimal NER enzymes can render cancers more sensitive to cisplatin treatment; therefore, SNPs within the NER pathway can alter the DNA repair capacity [37]. Studies have shown that the variant alleles of the xeroderma pigmentosum group D-complementing gene (XPD) polymorphisms, Asp312Asn and Lys751Gln, are associated with decreased mRNA levels compared with the wild-type allele. Excision repair cross-complementing 1 (ERCC1), another NER enzyme, has specific variants of 118CT, which has shown to be associated with cisplatin sensitivity in ovarian cell lines, and ERCC1 8092CA which may be associated with altered mRNA stability. An Arg399Gln substitution in the XRCC1 gene has been correlated to increased levels of markers of DNA damage [37].

One study enrolled 156 patients onto a phase III study comparing fluorouracil, leucovorin, and oxaliplatin with fluorouracil, leucovorin, and cisplatin [38]. Each patient was genotyped for TS, MTHFR, XPD, ERCC1, XRCC1, GSTP1, GSTT1, and GSTM1. The TS-3R/+6 haplotype (HR 0.61, $P=0.004$), GSTT1 deletion (HR 1.94, $P=0.015$), and XRCC1-399Gln/Gln (HR1.99, $P=0.023$) could be identified as independent predictors of overall survival. The presence of the ERCC1-118C/8092C haplotype (wild type) was significantly associated with response with an odds ratio of 2.55 for response to treatment ($P=0.023$). Grade 3 and 4 neutropenia was directly associated with GSTP1-105Ile/Ile (OR 4.45, $P=0.02$) and ERCC1-118T/8092C-haplotype (OR 2.68, $P=0.042$). GSTP1-105Ile/Ile was also associated with neurotoxicity (OR 5.8, $P=0.028$), while XPD-Asn312/751Gln-haplotype was associated with nephrotoxicity (OR 2.27, $P=0.005$) [38].

The true overall impact of GST and NER enzyme variants remains unknown. Of the three GSTs, GSTP appears to be the most common variant observed with a modest impact on toxicity. The key trials evaluating these mutations enrolled approximately 130–160 patients. Larger studies, with higher power to detect differences, are underway in order to validate these findings.

4.7 Vemurafenib and BRAF V600E

For a long period of time, dacarbazine was the only FDA approved agent for the treatment of metastatic melanoma. Phase 3 studies have demonstrated a response rate of 7–12 % and a median overall survival of 5.6–7.8 months after the initiation of treatment with dacarbazine in metastatic melanoma patients. However, it has been shown that approximately 40–60 % of cutaneous melanomas carry mutations in BRAF that lead to constitutive activation of downstream signaling through the MAPK pathway. Roughly 90 % of these mutations result in the substitution of glutamic acid for valine at codon 600 (BRAF V600E). Vemurafenib is a potent inhibitor of mutated BRAF and has marked antitumor effects against melanoma cell lines with the BRAF V600E mutation, but not against cells with wild-type BRAF [39].

In a phase 3 clinical trial, 675 untreated metastatic melanoma patients harboring BRAF V600E mutation were randomly assigned to treatment with either dacarbazine 1 g/m^2 every 3 weeks or vemurafenib 960 mg orally twice daily [39]. Primary end points included overall and progression-free survival. The final analysis was planned after 196 deaths, and an interim analysis was planned after 50 % of the projected deaths had occurred. A total of 118 patients had died at the time of the interim analysis. The data and safety monitoring board made the recommendation to allow all dacarbazine patients to crossover and receive vemurafenib due to significant increases in OS and PFS with vemurafenib. The hazard ratio for death in the vemurafenib group was 0.37 (95 % CI, 0.26–0.55; $P<0.001$). At 6 months, OS was 84 % (95 % CI, 78–89 %) in the vemurafenib group and 64 % (95 % CI, 56–73 %) in the dacarbazine group. The estimated median PFS was 5.3 months in the vemurafenib group and 1.6 months in the dacarbazine group. Adverse events led to dose modification or interruption in 129 of 336 patients (38 %) in the vemurafenib group and in 44 of 282 patients (16 %) in the dacarbazine group [39].

The discovery of vemurafenib has significantly increased survival and response rates in patients harboring the BRAF mutation. Vemurafenib is a key example of how targeted therapy is used to attack key somatic mutations.

4.8 Crizotinib and ALK

Activating mutations or translocations of the anaplastic lymphoma kinase (ALK) gene have been identified in several types of cancers, including anaplastic large-cell lymphoma, neuroblastoma, inflammatory myofibroblastic tumor, and non-small-cell lung cancer (NSCLC). In NSCLC, EML4-ALK is an abnormal fusion gene that encodes a cytoplasmic chimeric protein with constitutive kinase activity. EML4-ALK is relatively uncommon, occurring in roughly 2–7 % of all NSCLC cases [40]. Other fusion partners have been identified (i.e., KIF5B, TFG); thus, ALK rearrangements as a whole may define a molecular subgroup of tumors that is susceptible to targeted therapy. Crizotinib is an ALK inhibitor and has been shown to reduce the proliferation of cells carrying genetic alterations in ALK in phase I and II clinical trials [40].

Kwak et al. conducted an open-label, multicenter, two-step, phase 1 trial of crizotinib to evaluate the safety profile and efficacy in a cohort of 82 NSCLC patients harboring ALK rearrangements [40]. In the first step, doses were escalated based on toxicities, with a starting dose of 50 mg twice a day titrated up to 300 mg twice a day. Once the MTD was identified, the expanded cohort with FISH-positive results for ALK rearrangement received 250 mg twice daily. An overall response rate of 57 % was observed with 33 % having stable disease. The disease-control rate at 8 weeks was 87 %. No patients with ALK rearrangement had a concurrent mutation in EGFR. This is an interesting finding given the clinical similarities in patients harboring an EGFR mutation or ALK rearrangement (i.e., adenocarcinoma, nonsmoking history). Although the trial was not designed to measure PFS as an end point, investigators predicted the probability of PFS at 6 months to be approximately 72 % (95 % CI 61–83 %) [40].

The accelerated approval of crizotinib is yet another key example of how targeted therapies are advancing the field of oncology and drug development. As with vemurafenib, crizotinib was approved with a companion diagnostic test.

4.9 Erlotinib and EGFR

Erlotinib is an oral tyrosine kinase inhibitor that specifically binds to the epidermal growth factor receptor (EGFR). It was approved for use in the second-line setting; however, as a result of large phase 3 trials, it has become the mainstay of treatment for mutated EGFR NSCLC patients. A deletion of

exon 19 of EGFR and mutation at exon 21 L858R are the most common EGFR mutations and predict higher response to EGFR tyrosine kinase inhibitors [41]. Accumulating data show clinical differences in both response and survival between these two EGFR mutations. A retrospective study of 87 patients with NSCLC was done to investigate the clinical impact of EGFR exon 19 deletion and L858R mutation. Patients with exon 19 deletion had significantly longer PFS, compared with patients with L858R mutation (9.3 vs. 6.9 months, $P=0.02$). In a multivariate Cox regression model, EGFR exon 19 deletion was independently predictive of longer PFS ($P=0.02$). However, no significant differences in response rates (64 % vs. 62 %, $P=0.83$) and OS (17.7 vs. 20.5 months, $P=0.65$) were observed between these two mutations [42]. Notably, EGFR mutations occur with greater frequency in Asian patients compared with white patients, with typical mutation rates of around 30 % and 8 %, respectively. Clinicopathologic features tend to be very similar among patients harboring an EGFR mutation: younger age, adenocarcinoma, and nonsmokers [41].

In Caucasian patients harboring mutated EGFR kinase domains, outcomes of a 70 % response rate, 14-month PFS, and 27-month median survival have been attained with erlotinib. Although current markers are available that help practitioners predict the length of PFS in these patients, a secondary mutation in EGFR, the T790M "acquired resistance mutation," has been observed in 50 % of cases resistant to erlotinib. Whether this mutation occurs secondary to treatment or it exists prior to treatment remains unknown [43].

Zhou and colleagues published results from a multicenter, open-label, randomized, phase 3 study evaluating erlotinib versus chemotherapy as first-line treatment for patients with advanced NSCLC harboring a mutated EGFR [44]. One hundred sixty-five patients were randomized to receive erlotinib 150 mg/day or gemcitabine plus carboplatin. Median PFS was significantly longer in erlotinib-treated patients than in those on chemotherapy (13.1 months vs. 4.6 months; HR 0.16, $P<0.0001$). The PFS benefit seemed to be consistent across all subgroups irrespective of age, sex, performance status, disease stage, tumor histology, or smoking status. This suggests that EGFR mutations are the most important factor in predicting PFS benefit. The overall response rate was 83 % and 36 % for erlotinib and chemotherapy, respectively. In contrast to previous studies, there was an association between reduced PFS and the presence of the L858R mutation as compared with a deletion at exon 19 (HR 1.92, $P=0.02$) [44].

Response to the oral tyrosine kinase inhibitor, erlotinib, has extensive data demonstrating a clear benefit in patients harboring mutated EGFR. These patients tend to be younger, Asian, nonsmokers with adenocarcinoma of the lung.

4.10 Cetuximab/Panitumumab and KRAS, BRAF

4.10.1 KRAS

Cetuximab and panitumumab are monoclonal antibodies that bind to the extracellular EGFR domain and are commonly used in the treatment of metastatic colorectal cancer. Mutation of the KRAS oncogene has emerged as a powerful negative predictive biomarker to identify patients with metastatic colorectal cancer who do not benefit from EGFR-inhibitor therapy [45]. KRAS is a member of the Ras family of small G proteins involved in intracellular signaling. Activating mutations in KRAS results in the constitutive activation of downstream signaling pathways and confers resistance to inhibition of cell surface receptor tyrosine kinases, including EGFR. Multiple retrospective analyses have demonstrated that clinical benefit from treatment with EGFR inhibitors is limited to patients with tumors harboring the wild-type KRAS oncogene. Roughly 60–65 % of the population has the wild-type KRAS oncogene, whereas 35–40 % of the population has mutations and may not respond to treatment with cetuximab or panitumumab [45].

Van Cutsem et al. prospectively evaluated fluorouracil, leucovorin, and irinotecan (FOLFIRI) alone versus FOLFIRI plus cetuximab in patients evaluable for KRAS status [46]. The addition of cetuximab to FOLFIRI in patients with KRAS wild-type disease resulted in significant improvements in overall survival (median, 23.5 vs. 20.0 months; HR 0.796; $P=0.0093$), progression-free survival (median, 9.9 vs. 8.4 months; HR 0.696; $P=0.0012$), and response rate (57.3 % vs. 39.7 %; OR 2.069; $P=0.001$) compared with FOLFIRI alone. In patients whose tumors carried mutations in KRAS, there was no evidence of a benefit associated with the addition of cetuximab to FOLFIRI in relation to PFS, overall survival, or best overall response. Patients were also analyzed for the BRAF mutation, which was a strong indicator of poor prognosis [46].

Guidelines now recommend for metastatic colorectal cancer patients to be tested for the KRAS mutation prior to beginning therapy with cetuximab or panitumumab. Patients expressing KRAS mutations are not likely to respond to these agents, as evident through multiple phase II/III studies.

4.10.2 BRAF

In addition to KRAS, BRAF mutations can increase the predictive ability of response to EGFR inhibitors in patients with WT KRAS tumors. The serine–threonine kinase BRAF is the principal effector of KRAS. A retrospective study analyzed objective tumor response, time to progression, overall survival, and the mutational status of KRAS and BRAF in 113 tumors from cetuximab/panitumumab-treated metastatic colorectal cancer patients. The BRAF V600E mutation was detected in 11 of 79 patients who had wild-type KRAS. None of the BRAF-mutated patients responded to treatment, whereas none of the responders carried BRAF mutations ($P=0.029$). BRAF-mutated patients had significantly shorter progression-free survival ($P=0.011$) and OS ($P<0.0001$) than wild-type patients. Treatment with the BRAF inhibitor sorafenib restored sensitivity to panitumumab or cetuximab of colorectal cancer cells carrying the V600E allele [47].

4.11 Trastuzumab and HER2/neu

Trastuzumab, a humanized monoclonal antibody targeting the human epidermal growth factor receptor type 2 (HER2), has shown high efficacy in breast cancer. HER2 protein overexpression occurs in approximately 15–20 % of all breast cancers. Before the development of trastuzumab, patients with HER2-positive breast cancer diagnosis often experienced more aggressive tumor progression and an inferior prognosis. After the development of targeted therapies, such as trastuzumab, and subsequently lapatinib, the response rates increased dramatically. Lapatinib is the only therapy, other than trastuzumab, approved for HER2-positive breast cancer and works intracellularly to battle resistance to trastuzumab [48].

In one study, patients with operable or locally advanced, HER2-positive tumors were treated preoperatively with four cycles of epirubicin/cyclophosphamide followed by four cycles of docetaxel with or without capecitabine (EC-T[X]) and trastuzumab [49]. Patients with HER2-negative tumors treated in the same study with the same chemotherapy but without trastuzumab were used as a reference group. Of 1,509 participants, 445 had HER2-positive tumors treated with trastuzumab and chemotherapy. Pathologic complete response (pCR, defined as no invasive or in situ residual tumors in the breast) rate was 31.7 %, which was 16 % higher than that in the reference group (15.7 %). HER2-positive patients without response to the first four cycles of EC showed an unexpectedly high pCR rate of 16.6 % (3.3 % in the reference group). Breast conservation rate was 63.1 % and comparable to that of the reference group (64.7 %) [49].

Evidence since the 1980s shows that some gastric cancer tumors overexpress the HER2 receptor [50]. However, since then, researchers have demonstrated conflicting data on whether this

overexpression leads to poor outcomes and aggressive disease. In 2010, Bang et al. revealed the results of the ToGA trial, a phase 3 open-label, multicenter, randomized controlled clinical trial investigating the outcome of trastuzumab plus chemotherapy versus chemotherapy alone in HER2-positive advanced gastric or gastroesophageal junction cancer patients [50]. Five hundred ninety-four patients expressing HER2-positive tumors were randomly assigned to receive capecitabine plus cisplatin or fluorouracil plus cisplatin in combination with trastuzumab. Median overall survival was 13.8 months in those assigned to trastuzumab plus chemotherapy compared with 11.1 months in those assigned to chemotherapy alone (HR 0.74; 95 % CI 0.60–0.91; $P=0.0046$). There was no significant difference in the subset of patients with locally advanced ($n=20$) disease or patients diagnosed with gastroesophageal junction cancer, while the difference remained significant in those with metastatic disease or stomach cancer. These inconsistencies in overall survival were seen between gender, age, chemotherapy regimen, ECOG performance status, ethnicity, gastric cancer type, previous chemotherapy, previous gastrectomy, and number of metastatic sites/lesions [50].

Trastuzumab has long been the targeted agent of choice in treating HER2-positive breast cancers. Based on the results from the ToGA trial, trastuzumab is the first biological to show a survival benefit in advanced adenocarcinoma of the stomach, and it may be a new treatment option for patients with metastatic HER2-positive gastric cancer. However, these findings must be taken cautiously as there were significant differences in overall survival between subsets of patients, indicating that a very narrow population of gastric cancer patients may actually benefit from the addition of trastuzumab.

4.12 Imatinib and BCR-ABL, C-Kit

4.12.1 BCR-ABL

The development of targeted treatment was made promising by landmark innovations that identified the chromosomal abnormality and molecular mechanisms responsible for chronic myeloid leukemia (CML). In 1960, Nowell and Hungerford published their findings that characterized the Philadelphia chromosome and its association with chronic granulocytic leukemia [51]. Subsequent work demonstrated that this chromosome abnormality was formed by a translocation between chromosomes 9 and 22, resulting in a fusion between the breakpoint cluster region (BCR) and the c-ABL oncogene [52]. It was further validated that this chimeric BCR-ABL tyrosine kinase is constitutively active, triggering numerous signal transduction pathways associated with cell survival, proliferation, and resistance to apoptosis [52]. Imatinib was discovered in a high-throughput screening assay designed to identify small molecules which were able to inhibit a panel of various kinases [53].

In phase I trials, 53 of 54 (98 %) chronic phase CML patients who had failed therapy with interferon alfa achieved a complete hematologic response, and with 1 year of follow-up, only one of these patients relapsed [54]. In myeloid blast crisis, 55 % of patients responded. In phase II trials, 95 % and 60 % of chronic phase patients who had failed interferon-alfa therapy achieved a complete hematologic response and a major cytogenetic response, respectively [54].

The pivotal trial, IRIS, included 5 years of follow-up and was initiated in June 2000 for patients newly diagnosed with CML in the chronic phase [55]. Five hundred fifty-three patients were randomized to each of the two treatments, imatinib at 400 mg/day or interferon alfa plus Ara-C. With a median follow-up of 19 months, patients randomized to imatinib had significantly better results than patients treated with interferon alfa plus Ara-C in all parameters measured, including rates of complete hematologic response (97 % vs. 56 %, $P<0.001$), major and complete cytogenetic responses (85 % and 74 % vs. 22 % and 8 %, respectively, $P<0.001$), discontinuation of assigned therapy due to intolerance (3 % vs. 31 %), and progression to accelerated phase or blast crisis (3 % vs. 8 %, $P<0.001$) [55].

4.12.2 C-Kit

Imatinib is not only a selective inhibitor targeting BCR-ABL but also c-kit and the platelet-derived growth factor receptor. C-kit mutations are likely the most common genetic mutations in Asians, and the investigation of c-kit inhibitors is a high priority in this population.

Progression-free survival, overall response rates, and overall survival were measured in patients with metastatic melanoma harboring a c-kit mutation in a phase II, open-label, single-arm trial [56]. Forty-three patients received continuous therapy with imatinib (400 mg/day) unless intolerable toxicities or disease progression occurred. The response rate of early disease control was 53.5 %, while 18 patients (41.9 %) demonstrated regression of tumor mass. For 15 patients who demonstrated progression of disease on 400 mg/day and had their dose escalated to 600 or 800 mg/day, only one patient achieved stable disease for 4 months, indicating that an increase in dose from 400 to 600 mg/day or 800 mg/day does not restore disease control. The 6-month PFS rate was 36.6 %, and the median PFS was 3.5 months. The 1-year OS rate was 51.0 %, and the median OS time was 14.0 months. For the ten patients with progressive disease, nine of them harbored mutations in exon 11 or exon 13. This trial suggests that application of imatinib in the treatment of patients with metastatic melanoma is preferred for patients showing genetic aberrations in c-kit gene. However, the unexpected clustering of responses among those patients whose tumors harbored exon 11 or exon 13 mutations suggests that even more refined genetic selection strategies may be appropriate in subsequent trials [56].

5 Conclusion

Currently, approximately 25 % of all outpatients receive one or more drugs that have pharmacogenomic information on the labeling [57]. Anticancer drug response has the potential to be heavily influenced by pharmacogenomics due to its unique pathology. Genome-wide association and candidate gene studies have allowed researchers to identify hundreds of polymorphisms within genes that have the potential to influence a patient's response to chemotherapy. Over the past several years, researchers have been able to validate some of these findings in genotype-guided, prospective clinical trials. Although pharmacogenomics has proven to be useful in tailoring therapy, several challenges exist before this practice becomes mainstream, including infrastructural necessities, ethical considerations, sufficient evidence for intervention, and costs. While the expanding use of high-throughput technology will help speed up the identification of drug pathways and DNA repair mechanisms, novel genetic tests such as PCR-based mutation-directed assays and drug-metabolizing enzymes and transporters (DMET) microarrays have led to time and cost-efficient techniques for genotyping patients. The integration of germline and somatic mutations, along with clinicopathologic criteria, will help to establish personalized cancer medicine in the future.

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Cytochrome P450

Yuichi Ando

Abstract Cytochrome P450 (CYP, CYP450, or P450) represents the enzyme that metabolizes drugs with various manners of oxidation as the Phase I reaction. A variety of anticancer drugs are metabolized by P450, including tegafur, cyclophosphamide, ifosfamide, *vinca alkaloids*, tamoxifen, etoposide, docetaxel, paclitaxel, and molecular-targeting drugs. The variation in drug metabolism causes pharmacokinetic variability and may influence drug efficacy and toxicity. Drug metabolism depends on both genetic and environmental factors, which include genetic polymorphism and drug interactions (induction or inhibition of P450 activity). CYP3A4 is the major human P450 isoform with a remarkable interindividual variation in its activity. With regard to CYP3A4 and CYP3A5, environmental factors appear to influence the CYP3A enzymatic activity more than genetic status. The challenges have been made to control the phenotypic CYP3A4 activity and to reduce pharmacokinetic variability of the relevant drugs. However, the clinical advantages obtained from these efforts should be carefully evaluated in view of clinical practice.

Keywords Cytochrome P450 • CYP3A4

1 Overview

1.1 Role of P450 in Drug Metabolism

Drug metabolism is an enzymatic biotransformation of drugs. The early stage of drug metabolism generally consists of Phase I reactions such as oxidation, reduction, and hydrolysis, which are achieved by introducing a polar group into the parent molecule. The Phase I reactions are typically followed by conjugations with hydrophilic compounds such as glucuronic acid and glutathione to yield more hydrophilic metabolites (Phase II reaction). Cytochrome P450 (CYP, CYP450, or P450) represents the enzyme that metabolizes drugs with various manners of oxidation as the Phase I reaction. P450 is comprised of a large superfamily of heme-containing membrane-binding proteins that are classified into families and subfamilies. Most of the P450 related to drug metabolisms in human belong to

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CYP1, CYP2, or CYP3 families that are known as “drug-metabolizing enzymes.” Two or more P450 isoforms are frequently involved in the metabolism of the same drug, suggesting broad substrate specificity. P450 exists mainly in the liver but may also exist in various organs including the brain, lung, gastrointestinal tract, kidneys, and gonads. CYP3A4 is the most abundant isoform, accounting for approximately 30 % of the total P450 amount in the human liver [1]. Because many therapeutic drugs are metabolized by CYP3A4 and CYP3A5, drug interactions related to the isoforms and interindividual variation of CYP3A activity are sometimes clinically significant via pharmacokinetic and pharmacodynamic actions. Furthermore, when a CYP3A substrate is administered orally, the CYP3A activity in the intestines has a clinically significant effect on the bioavailability of the drug.

1.2 Nomenclature of P450

When a cytochrome P450 gene is described, cytochrome P450 is abbreviated as *CYP* or *Cyp* (*cy*tochrome *p*450), where all the letters are italicized. If all the letters are capitalized as in *CYP*, it represents a human gene, whereas if the latter two are lowercase *Cyp*, then it represents an animal model (mouse and *Drosophila*). The *CYP* or *Cyp* are usually followed by an Arabic number that designates the P450 family (amino acid homology >40 %), a letter indicating the subfamily (homology >55 %), and an Arabic numeral representing the individual gene. *P* (*ps* in mouse and *Drosophila*) after the gene number denotes a pseudogene. The cDNAs, mRNAs, and enzymes in all species (including mouse) should include all capital letters. Recommendations for naming P450 have been published by the Nomenclature Committee [2]. Updated information on the P450 nomenclatures is available through the Web (http://www.cypalleles.ki.se/ and http://drnelson.uthsc.edu/CytochromeP450.html).

1.3 Factors Affecting P450 Activity

Several factors can cause interindividual variations of P450 activity. These factors include age; disease state; intake of foods, drugs, or alcohol; environmental factors such as smoking; and genetic polymorphism. There is a decline in P450 activity with aging, which varies in different isoforms [3]. Cigarette smoking increases P450 activity by enzyme induction, most notably CYP1A1 and CYP1A2 [4]. The plasma concentrations of erlotinib, a small molecule inhibitor of tyrosine kinase and a substrate of CYP1A, are reduced in current smokers partly by the CYP1A induction [5]. In addition, cancer patients frequently have various comorbidities (i.e., malnutrition, liver damage secondary to metastasis, and toxicity of chemotherapy), all of which may alter P450 activity. P450 activity is decreased in patients with chronic liver diseases, especially with severe cirrhosis [6]. This topic is discussed further in chapter “Organ Dysfunction Trials: Background, Historical Barriers, Progress in Overcoming Barriers, and Suggestions for Future Trials.”

Some P450 genetic polymorphisms have been known to cause phenotypic variability in the enzymatic process that could change the in vivo pharmacokinetics and be the reason for the variable susceptibility to a drug. Since a drug’s effect is the sum of gene–environmental interactions, other factors may affect the phenotypic activity more than the genetic status, thereby masking pharmacogenetic consequences.

1.4 Inhibition and Induction of P450

Concomitant drug combinations may affect drug metabolism, which may change the pharmacokinetics of the drugs and their clinical effects (Table 1). Since induction and inhibition of drug-metabolizing

Table 1 Representative inhibitors of P450 isoforms

CYP1A2	Ciprofloxacin (quinolones)	Fluvoxamine	Imipramine	
CYP2C8	Gemfibrozil	Pioglitazone	Montelukast	
CYP2C9	Fluvoxamine	Fluvastatin	Amiodarone	
CYP2C19	Fluvoxamine	Fluoxetine	Omeprazole	Indomethacin
CYP2D6	Cimetidine	Paroxetine	Fluoxetine	Imipramine
	Quinidine	Haloperidol		
CYP2E1		Isoniazid		
CYP3A4/5	Ketoconazole (antifungal azoles)	Cimetidine	Erythromycin	Clarithromycin
	Fluvoxamine	Ethinyl estradiol	Cyclosporine	Ritonavir
	Indinavir	Diltiazem	Verapamil	Grapefruit juice
Nonspecific	Ketoconazole (antifungal azoles)	Cimetidine		

Table 2 Representative inducers of P450 isoforms

CYP1A	Carbamazepine	Omeprazole	Ritonavir	
	Cigarette smoking	Charcoal-broiled food		
CYP2A6	Phenobarbital (barbiturates)			
CYP2B6	Phenobarbital (barbiturates)	Phenytoin		
CYP2C	Phenobarbital (barbiturates)	Carbamazepine	Rifampicin	Phenytoin
	Dexamethasone			
CYP2E	Ethanol	Isoniazid		
CYP3A	Phenobarbital (barbiturates)	Carbamazepine	Phenytoin	
	Rifampicin	Rifabutin	Dexamethasone	Nelfinavir
	St. John's wort			

enzymes have been regarded as the major mechanisms that may cause for drug interactions, elucidation of the metabolic pathway is crucial to predict and avoid unwanted drug interactions. A series of in vitro metabolism experiments using human liver microsomes coupled with recombinant human liver P450 isoforms are powerful tools to characterize the metabolic pathway and to identify the specific isoforms involved in the metabolism of each drug. When drugs having different affinity to a single P450 isoform are concurrently administered to a patient, the drug with the strong affinity will competitively inhibit the metabolism of the drug with the weaker affinity. As a result, the pharmacological effects of the latter drug would be potentiated by increasing the amount of drug exposure. Some drugs are known to inhibit P450 activity nonspecifically, such as cimetidine [7] and ketoconazole [8], which may increase exposure and pharmacological activity of other drugs that are metabolized by some of the P450 isoforms. Satraplatin (JM216), the first oral platinum-containing anticancer drug, inhibits prototype reactions by P450 isoforms nonspecifically in vitro [9]. The mechanism of the inhibition remains to be elucidated, but satraplatin may interact with the heme moiety of P450, which is critical to the activation of molecular oxygen to oxidize substrates. When satraplatin was given with etoposide (a substrate of CYP3A4) in mice with murine tumors, a significant dosage reduction was required when compared to monotherapy and theorize to be due to CYP3A4 inhibition [10]. One could speculate that satraplatin might inhibit the in vivo metabolism of etoposide, thereby enhancing toxicity through increased exposure to the drug.

Drugs can induce P450 activity to enhance metabolism and reduce plasma concentrations of the same (autoinduction) or another drug (Table 2). CYP3A is most sensitive to the enzymatic induction which occurs via nuclear receptors such as steroid and xenobiotic-sensing nuclear receptor (SXR) or retinoid X receptor (RXR). Hyperforin, a constituent of the herbal antidepressant St. John's wort, can induce CYP3A as a result of activating SXR and therefore decrease effective concentrations of a CYP3A substrate [11].

2 Anticancer Drugs Metabolized by P450

A variety of anticancer drugs are metabolized by P450 (Table 3). Knowledge of the specific P450 isoforms involved in the metabolism of a drug can lessen the impact of drug interactions and trigger clinicians to assess genetic polymorphisms. For example, a patient who is taking a therapeutic drug that is inactivated by CYP3A4 should be recommended not to take medications with strong CYP3A4 inhibitors such as ketoconazole and grapefruit juice.

2.1 Tegafur

Tegafur is a prodrug for cytotoxic 5-fluorouracil (5-FU), which exerts its cytotoxic effects through the inhibition of thymidylate synthase and/or by its incorporation into RNA. 5-FU is then further biotransformed to inactive molecules by dihydropyrimidine dehydrogenase (DPD). Tegafur is converted to 5-FU mainly by the liver via an unstable metabolic intermediate, 5′-hydroxytegafur. The 5′-hydroxylation of tegafur is mediated primarily by CYP2A6, followed by spontaneous decomposition of 5′-hydroxytegafur to 5-FU. In an in vitro study using a panel of human liver microsomes [12], formation rates of 5-FU showed a significant correlation with activities of coumarin 7-hydroxylation, a prototype reaction of CYP2A6. The activity of 5-FU formation by recombinant CYP2A6 isoform was the highest among ten other expressed P450 isoforms. Furthermore, specific chemical inhibitors and antiserum against CYP2A6 inhibited 5-FU formation. Therefore, it can be speculated that CYP2A6 may have an effect on the in vivo activation of tegafur. For instance, a patient who has lower activity of CYP2A6 may have little benefit from tegafur because of an insufficient exposure to 5-FU. As genetic polymorphism of the *CYP2A6* gene has been known to cause poor or absent activity of CYP2A6 [13], a patient having the variant allele would also be included in those who benefit little from tegafur.

Tegafur is now only formulated in combination with gimeracil and potassium oxonate to be administered orally (S-1). S-1 is widely prescribed for treatment of stomach and colorectal cancers in Japan. Gimeracil competitively inhibits DPD thus preventing degradation of 5-FU. In a pharmacogenetic study of *CYP2A6* genetic polymorphisms (**4A*, **7*, and **9*) with deficient or reduced activity in 54 Japanese patients who were treated with S-1, tegafur clearance was associated with the *CYP2A6*

Table 3 Anticancer drugs metabolized by P450 isoforms

CYP1A1/1A2	**Dacarbazine**	Flutamide	Bortezomib	Erlotinib
	Pazopanib			
CYP2A6	**Tegafur**	Letrozole		
CYP2B6	**Cyclophosphamide**	Ifosfamide		
CYP 2C8	**Paclitaxel**	Lapatinib	Pazopanib	
CYP 2C19	Lapatinib	Bortezomib		**Thalidomide**
CYP 2D6	**Tamoxifen**			
CYP 3A4/3A5	Cyclophosphamide	**Ifosfamide**	**Docetaxel**	Paclitaxel
	Vincristine	**Vinblastine**	**Vindesine**	**Vinorelbine**
	Ixabepilone	Irinotecan	**Etoposide**	Tamoxifen
	Letrozole	Exemestane	**Medroxyprogesterone**	**Erlotinib**
	Gefitinib	**Lapatinib**	Sorafenib	**Sunitinib**
	Pazopanib	**Imatinib**	**Dasatinib**	**Nilotinib**
	Bortezomib	**Temsirolimus**	**Everolimus**	Depsipeptide

Drugs in bold indicate a major or clinically important drug metabolism
Information available on package inserts

genotypes while 5-FU exposure was correlated with gimeracil exposure [14]. Therefore, pharmacokinetic variability of tegafur is caused by *CYP2A6* genetic polymorphisms, but the key determinant of 5-FU exposure is the DPD inhibition by gimeracil.

2.2 *Oxazaphosphorine*

Cyclophosphamide and ifosfamide are oxazaphosphorine-alkylating agents that require metabolic activation to exert their pharmacological activity. The metabolism and activation of these agents have been reviewed elsewhere [15]. There are two distinct metabolic pathways of these drugs: 4-hydroxylation as an activating pathway and *N*-dechloroethylation as an inactivating one. The active metabolites, 4-hydroxycyclophosphamide and 4-hydroxyifosfamide, are produced in the human body mainly by CYP2B6 and CYP3A4, respectively [16, 17]. CYP2C9 has also been reported in 4-hydroxylation of cyclophosphamide to a minor extent [18]. The pathway of *N*-dechloroethylation is catalyzed by CYP3A4 for cyclophosphamide and by both CYP3A4 and CYP2B6 for ifosfamide [19]. As an important difference in drug metabolism between cyclophosphamide and ifosfamide, the *N*-dechloroethylation accounts for approximately 50 % of the total administered dose of ifosfamide but only 10 % of cyclophosphamide [20]. Therefore, patients treated with ifosfamide are more exposed to toxic chloroacetaldehyde and are more likely to experience nephrotoxicity or neurotoxicity than those treated with cyclophosphamide.

P450 activity may alter the balance between the activating (4-hydroxylation) and inactivating (*N*-dechloroethylation) metabolic pathways, leading to variations in the pharmacokinetics and pharmacodynamics profile of oxazaphosphorine. In a clinical trial with 11 patients, cyclophosphamide was administered weekly as single intravenous doses of 500 mg/kg [21]. The patients were pretreated with 200 mg of phenobarbital as a P450 inducer for 3 consecutive days prior to the second administration of the drug. Pharmacokinetic analysis showed that blood levels of the parent cyclophosphamide were decreased and mustard-like metabolites were increased. This finding suggested that phenobarbital induced CYP2B6 and CYP3A4 activities, resulting in an enhanced metabolism of cyclophosphamide. With regard to ifosfamide, CYP2B6 is the dominant isoform in inactivating the *N*-dechloroethylation pathway and, furthermore, it plays a minor role in activating the 4-hydroxylation pathway. Thus, selective inhibition of CYP2B6 could improve therapeutic efficacy of ifosfamide theoretically, albeit definite clinical evidence has not been demonstrated. In animal models, retrovirus-mediated expressions of CYP2B6 in tumor cells have been reported to enhance the efficacy of cyclophosphamide by increasing metabolic activation [22].

The oxazaphosphorines contain a chiral phosphorus atom, and therefore they are usually used as racemic mixtures of (+)-*R* and (−)-*S* enantiomers. According to an in vitro study on stereoselective metabolism of ifosfamide, *R*-ifosfamide has more favorable pharmacological properties than *S*-ifosfamide with respect to less extensive *N*-dechloroethylation and more rapid 4-hydroxylation [23]. It has been reported that phenytoin would induce the enantioselective metabolism of cyclophosphamide, in which inactivating *N*-dechloroethylation was induced to a greater extent in the *S*-enantiomer than the *R*-enantiomer [24]. However, the clinical consequence of these stereoselective differences in drug metabolism remains unclear.

Cyclophosphamide is occasionally used in high-dose chemotherapy with bone marrow supports. Oncologists have a plausible concern that the drug metabolism by CYP2B6 might be saturated at high doses because CYP2B6 is one of the minor P450 isoforms. The saturation may cause a greater proportion of the inactive or toxic metabolites generated by *N*-dechloroethylation pathway by CYP3A4. Therefore, a continuous infusion or divided doses over several days are usually preferred in high-dose cyclophosphamide to avoid saturation of the drug metabolism [25]. In addition, cyclophosphamide and ifosfamide can induce their own metabolism (autoinduction) with prolonged use of the drugs.

A study of 15 patients receiving 1.5 mg/m^2 of intravenous ifosfamide over 30 min every day for 5 consecutive days demonstrated a time-dependent increase of the metabolism, where clearance of ifosfamide increased from 66 mL/min on day 1 to 115 mL/min on day 5 [26]. The clinical significance of the prolonged use of cyclophosphamide and ifosfamide has not been well investigated.

2.3 Tamoxifen and Aromatase Inhibitors

Tamoxifen, a synthetic antiestrogen, has been used for many years to treat breast cancer. This drug requires metabolic activation by the P450 system to generate adducts of tamoxifen with DNA and protein [27]. The major metabolites of tamoxifen are *N*-desmethyltamoxifen that is formed by CYP3A, which undergoes further metabolism to form a 4-hydroxy-*N*-desmethyltamoxifen (a.k.a. endoxifen) by CYP2D6, 4-hydroxytamoxifen by CYP2D6, and tamoxifen *N*-oxide by flavin-containing monooxygenase [27, 28]. The *N*-desmethyl and 4-hydroxy derivatives have equivalent and 25–50 times more affinity for the estrogen receptor α and β, respectively, compared with the parent drug [29]. Endoxifen is the most abundant and potent metabolite that plays a decisive role in therapeutic efficacy of tamoxifen [30].

Since endoxifen is formed by CYP2D6, it is reasonable to hypothesize that genetic polymorphism and concurrent use of inhibitors CYP2D6 could theoretically diminish the therapeutic efficacy of tamoxifen secondary to endoxifen. Some drugs including phenobarbital, rifampicin, aminoglutethimide, medroxyprogesterone, and bromocriptine have been reported to alter plasma concentrations of tamoxifen and *N*-desmethyltamoxifen, but their clinical importance is not known. A pharmacogenetic study demonstrated that plasma endoxifen concentrations after 4 months of tamoxifen therapy were lower in patients having variant *CYP2D6* alleles and in patients with the wild-type genotype taking CYP2D6 inhibitors than in those with the wild-type genotype [31]. In a retrospective adjuvant breast cancer study in which the relationship between *CYP2D6* genotype and disease outcome was investigated, the patients with a poor metabolizing genotype of *CYP2D6*4/*4* had a higher risk of disease relapse and less frequent toxicity of hot flashes [32]. These findings underscore the notion that patients who have decreased CYP2D6 metabolism, caused by either the genetic polymorphism or the inhibitors, would have less benefit from tamoxifen therapy.

The third-generation aromatase inhibitors (anastrozole, letrozole, and exemestane) are administered orally in the endocrine treatment of postmenopausal patients with hormone-receptor-positive breast cancer. These drugs inhibit and inactivate aromatase, an enzyme produced by the *CYP19* gene that is responsible for the synthesis of estrogens from androgens, to lower plasma estrogen levels. Anastrozole and letrozole are nonsteroidal aromatase inhibitors that competitively and reversibly bind to the heme of the enzyme. Anastrozole inhibits CYP1A2, CYP2C9, and CYP3A4 at concentrations higher than achieved with clinical use [33]. Letrozole is metabolized by CYP3A4 and CYP2A6 and inhibits CYP2A6 and CYP2C19. Plasma concentrations of anastrozole and letrozole are reportedly reduced when they are administered concomitantly with tamoxifen [34, 35]. The steroidal analogue of androgens, exemestane, binds to the aromatase irreversibly. Although exemestane is extensively metabolized by CYP3A4 in vitro [36], it has been reported in the package insert that ketoconazole has no significant effect on exemestane pharmacokinetics.

2.4 Vinca Alkaloids

Vinca alkaloids (vincristine, vinblastine, vindesine, and vinorelbine) are antimitotic and anti-microtubule agents that are metabolized by CYP3A4 into unidentified metabolites [37]. There are

several clinical reports of drug interactions with vinca alkaloids. Among 14 patients with acute lymphoblastic leukemia (ALL) receiving induction chemotherapy consisting of vincristine and itraconazole, four patients experienced severe neuropathy (paresthesia, muscle weakness, and paralytic ileus) after the first or second dose of vincristine [38]. Increased neurotoxicity of vinca alkaloid-containing chemotherapy has also been reported in patients who simultaneously received cyclosporine [39, 40] and in combination with erythromycin [41]. As regards to anticancer agents, etoposide and teniposide reportedly enhance vincristine-induced neurotoxicity, albeit other studies found no evidence of the interaction [42, 43]. The exact mechanism(s) of the drug interactions remains unclear. However, drugs that increase the toxicity of vinca alkaloids have been known to inhibit CYP3A4-mediated metabolism in a competitive or noncompetitive manner. Thus, it is possible that the inhibition of the detoxifying pathway by CYP3A4 would cause an increase in exposure to the drug, leading to unexpected toxicity. In addition, because cyclosporine and itraconazole also inhibit P-glycoprotein, the modulation of the P-glycoprotein would be another mechanism of the drug interaction.

Vinorelbine, a semisynthetic drug used to treat non-small-cell cancer and breast cancer, has a higher therapeutic index with less neurotoxicity than other vinca alkaloids [44]. Drug metabolism of vinorelbine by CYP3A4 appears to cause large interpatient pharmacokinetic variability. According to an in vitro study using human liver microsomes, 50 % inhibitory concentration (IC_{50}) of vinorelbine for testosterone 6-β-hydroxylase activity, which is catalyzed by CYP3A4, was estimated to be 155 μM [37]. Although plasma concentrations of vinorelbine are much lower than the IC_{50} value, drug combinations that could inhibit or induce CYP3A4 activity may alter drug metabolism and pharmacological effects of vinorelbine.

2.5 *Thalidomide*

Thalidomide was originally developed as a sedative but was eventually removed from the market because of significant teratogenic effects [45]. Recently, it has been reintroduced for the treatment of erythema nodosum leprosum and multiple myeloma [46]. While the true mechanism of action still remains controversial, it has been suggested that thalidomide requires CYP450-catalyzed biotransformation to exert its pharmacological activities [46–48]. Indeed, the main transformation of thalidomide is considered as a spontaneous hydrolysis; however, these breakdown products are not responsible for this activity [48].

At least two hydroxylated metabolites have been found in patients' plasma or urine: 5-hydroxythalidomide and 5′-hydroxythalidomide, which are generated by the CYP2C19 metabolism [49–51]. The interindividual variation of the CYP2C19 activity caused by its genetic polymorphism may attribute to the efficacy and toxicity profile of thalidomide. In a case-control study in 63 prostate cancer patients who received thalidomide therapy, plasma concentrations of the metabolites were below quantification in the two patients who were homozygous for the poor metabolizing phenotype variant *CYP2C19*2* [52]. The clinical effects of the *CYP2C19* genotype on clinical consequences of thalidomide therapy requires further investigation.

2.6 *Irinotecan*

Irinotecan is a prodrug that is hydrolyzed by carboxylesterase into an active metabolite, SN-38, which is subsequently conjugated and inactivated by UDP-glucuronosyltransferase (UGT). CYP3A transforms irinotecan to less active metabolites. Concurrent use of St. John's wort with irinotecan decreases the plasma concentration of SN-38, most likely due to CYP3A induction [53]. Glioma patients who

regularly take enzyme-inducing anticonvulsants require more than the standard dose of irinotecan [54]. According to a dose escalation study in glioma patients with anticonvulsant use, the standard dose of 350 mg/m^2 every 3 weeks could be increased to 800 mg/m^2 with the recommended dose determined to be 750 mg/m^2 [54]. Pharmacokinetic analysis showed that the AUC of irinotecan at the dose of 750 mg/m^2 in the patients receiving anticonvulsants was equivalent to that at the dose of 350 mg/m^2 in those receiving no anticonvulsants. These findings indicated that enzymatic induction of CYP3A would enhance conversion of irinotecan to the less active metabolites, leading to decreased exposure to irinotecan and active metabolite SN-38.

2.7 Emerging Molecular-Targeting Drugs

Small molecule agents targeting one or multiple key molecular pathways of cancer cells have emerged as a key strategy in cancer chemotherapy. Most are substrates of P450 isoforms. Since most of these drugs are administered orally chronically, the likelihood of drug interactions is great.

Erlotinib, an oral drug which inhibits the intracellular phosphorylation of tyrosine kinase, is indicated for the non-small-cell lung cancer and pancreatic cancer. Erlotinib is metabolized primarily by CYP3A4/5 and, to a lesser extent, by CYP1A1/2 [55]. Erlotinib exposure increases by 39 % (AUC) and 17 % (C_{max}), respectively, when administered with the CYP3A or CYP1A inhibitor ciprofloxacin. The concurrent use of omeprazole with erlotinib decreases the plasma concentrations of erlotinib due to decrease bioavailability of erlotinib by diminishing drug solubility. A former oral EGFR inhibitor, gefitinib, is also metabolized by CYP3A and CYP1A isoforms, showing the similar CYP3A-related drug interactions [56].

Lapatinib, an oral kinase inhibitor of the intracellular tyrosine kinase, is indicated in HER2-overexpressing breast cancer. Lapatinib is metabolized extensively by CYP3A4. Besides CYP3A, lapatinib inhibits in vitro activity of CYP2C8 and P-glycoprotein, having potential drug interactions with medications that are substrates of CYP2C8 and P-glycoprotein in addition to CYP3A4. Sorafenib, an oral drug that targets multiple receptor kinases, is indication for renal cell carcinoma and hepatocellular carcinoma in the USA. Since less than 5 % of the dose is metabolized via CYP3A primarily to the *N*-oxide metabolite with 50 % of the dose excreted unchanged in the feces, the clinically relevant drug interactions of sorafenib with CYP3A inhibitors are unlikely to occur. However, concurrent use of strong CYP3A inducers with sorafenib may reduce therapeutic efficacy of sorafenib due to decreased plasma concentrations of the drug. Although, there is evidence mounting suggesting increased variability in exposure to the active *N*-oxide metabolite which may be related to drug metabolism [57].

Sunitinib and pazopanib, both oral multi-kinase inhibitors, are metabolized primarily by CYP3A4. In the USA, at this time sunitinib has indications for advanced renal cell carcinoma and gastrointestinal stromal tumor (GIST), and pazopanib for advanced renal cell carcinoma. The primary metabolite of sunitinib has activity similar to the parent drug and is further metabolized by CYP3A4. In addition to the CYP3A metabolism, pazopanib is metabolized, to a minor extent, by CYP1A2 and CYP2C8.

Imatinib is an oral BCR-ABL and also a multi-kinase inhibitor used in the treatment of hematological malignancies including Philadelphia chromosome-positive chronic myeloid leukemia (Ph+ CML) and ALL and GIST. Imatinib is a substrate of CYP3A4 and, to a minor extent, of CYP1A2, CYP2D6, CYP2C9, and CYP2C19. Imatinib inhibits CYP2D6 and CYP3A4 activity in vitro. Drug interactions have been noted with CYP3A4 inducers when administered as a single dose with St. John's wort [58] and chronically with enzyme-inducing anticonvulsants [59]. However, at steady state, imatinib is insensitive to CYP3A4 inhibition by ritonavir [60].

Dasatinib and nilotinib, oral multi-kinase inhibitors that are used for imatinib-resistant or imatinib-intolerant CML and Ph+ ALL (dasatinib), are also metabolized by CYP3A4. Nilotinib competitively inhibits CYP2C8, CYP2C9, and CYP2D6 and may induce CYP2B6, CYP2C8, and CYP2C9 in vitro.

In a study in healthy volunteers, significant drug interactions were noted with the CYP3A4 inhibitor ketoconazole and CYP3A4 inducer rifampicin [61].

Bortezomib is a proteasome inhibitor indicated for multiple myeloma and mantle cell lymphoma, which is metabolized by CYP3A4, CYP2C19, and CYP1A2. CYP3A4 inhibition by ketoconazole resulted in a 35 % increase in bortezomib exposure in cancer patients [62]. However, there was no clinically significant drug interaction noted in patients receiving the concomitant CYP2C19 inhibitor omeprazole [63].

Temsirolimus, an intravenous infusion drug that inhibits mTOR (mammalian target of rapamycin), is used for advanced renal cell carcinoma. Temsirolimus is metabolized by CYP3A4/5 to the active metabolite sirolimus, which is also metabolized by CYP3A4/5. Drug interactions with temsirolimus have been observed in patients taking enzyme-inducing anticonvulsants and in healthy volunteer receiving rifampicin [64]. Similar interactions have been noted in healthy volunteer receiving rifampicin and everolimus, an oral mTOR inhibitor [65].

3 Interindividual Variation of CYP3A Activity

CYP3A isoforms (CYP3A4 and, to a lesser extent, CYP3A5) exist most abundantly among human P450 isoforms in adults. The enzymatic activity of CYP3A4 exhibits a remarkable interindividual variation as high as 20-fold [1], which can be induced by glucocorticoids, rifampicin, and phenobarbital. Antifungal azole derivatives, such as ketoconazole and itraconazole, inhibit CYP3A4, and 14-member macrolides also inhibit the isoform irreversibly by its active metabolites binding to the heme portion of P450 (mechanism-based inhibition). Furthermore, CYP3A is abundant in the liver and the intestinal wall, which plays a major role in bioavailability of orally administered drugs.

With traditional cytotoxic drugs, Phase I studies have determined the "maximum-tolerated dose" that is utilized during drug development. Thus, anticancer drugs which are known to have narrow therapeutic windows are administered at a dose close to the toxic level with a high potential for drug interactions. To date, extensive attempts have been made to minimize pharmacokinetic and pharmacodynamic variability of the drugs that are metabolized by CYP3A. However, the clinical significance of these attempts should be carefully evaluated in view of clinical practice.

3.1 Genetic Polymorphism of CYP3A Genes

Despite attempts to explore genetic variations causing wide variability of CYP3A4, identifying genetic variants have minimal contribution to the interindividual variability [66]. Although typical polymorphic P450 isoforms, such as CYP2D6 and CYP2C19, show bimodal or multimodal phenotypic distributions, CYP3A activity is essentially unimodal. In a study that explored *CYP3A4* genetic variations using 213 genomic DNA samples from Caucasian subjects, a total of 7.5 % of the population studied had one of these variants heterozygously, and four of the eight protein variants exhibited some alteration in the enzymatic activity in their in vitro expression systems [66]. However, most (15/18) of the variants had allele frequencies below 1 %, and obviously the variants in the coding regions did not fully explain the observed large variability of CYP3A4 expression and activity.

A variant allele *CYP3A4*1B*, which has a -392A>G transition within the promoter region of *CYP3A4* gene, has been reported to be associated with disease risks, such as prostate and lung cancers [67, 68]. Subsequent investigators, however, have for the most part demonstrated that *CYP3A4*1B* changes neither functions of the *CYP3A4* gene nor the phenotypic enzymatic activity. This points to a linkage disequilibrium between *CYP3A4*1B* and distinct genes that have more evident effects.

*CYP3A5*3*, which contains a 6986A>G transition within intron 3 of the *CYP3A5* gene, severely decreases the enzymatic activity to yield a polymorphic distribution of CYP3A5 expression in the liver and the intestine. Despite the remarkable ethnic differences in allele frequency (~90 % in Caucasians, ~70 % in Asians, and ~50 % in African-Americans), clinical relevance has not been elucidated. In a pharmacogenetic study of 92 Caucasian patients who received docetaxel for several cancers, the patients who had at least one *CYP3A4*1B* allele and at least one *CYP3A5*1* simultaneously showed a 64 % higher clearance of docetaxel than the others [69]. However, *CYP3A4*1B* and *CYP3A5*1* are acknowledged to be in linkage disequilibrium in Caucasians which may confound the clinical results [70].

3.2 Phenotyping of CYP3A Activity: Docetaxel

Docetaxel is primarily metabolized by CYP3A4 and CYP3A5 in humans, which causes a large interindividual variability in the pharmacokinetic/pharmacodynamic profile [71, 72]. Body surface area has been used for dosing of anticancer agents including docetaxel. However, because of the large variability in CYP3A activity, guidance by CYP3A activity appears to be a better way to determine a dose of docetaxel. Attempts have been made to quantify a phenotypic activity of CYP3A4 and to explore a correlation between the activity and the pharmacokinetic parameters of docetaxel. In a study with 21 sarcoma patients who were treated with docetaxel at a dose of 100 mg/m^2, [^{14}C-*N*-methyl]-erythromycin breath test (14C-ERMBT) accounted for 67 % of the variation in docetaxel clearance, with severe toxicity noted in the patients with the lowest 14C-ERMBT values [73]. In another study, 6-β-hydroxycortisol in urine was measured in 29 patients with non-small-cell lung cancer after receiving 300 mg of hydrocortisone intravenously, followed by docetaxel treatment of 60 mg/m^2 [74]. Multivariate analysis revealed that the total amount of 24-h urinary 6-β-hydroxycortisol was the strongest significant factor to predict docetaxel clearance. These studies suggested that CYP3A4 activity would be a helpful indicator to predict in vivo docetaxel clearance and potentially its toxicity; thus, the CYP3A4-guided dosing would be a promising method for docetaxel dosing. Besides the enzymatic activity, expression levels of the CYP3A4 mRNA in peripheral mononuclear cells may be a marker for docetaxel metabolism in the total body if the level of gene expression correlates between peripheral mononuclear cells and hepatocytes [75].

3.3 Modulation of CYP3A Activity

When a drug is mainly metabolized and detoxified by CYP3A, the elimination pathway of the drug is slowed down and its pharmacological effects are potentiated by combination with strong CYP3A inhibitors. Particularly, inhibition of the CYP3A4 enzyme in the intestinal wall and liver may boost bioavailability of orally administered drugs that are substrates of CYP3A, by diminishing the first-pass metabolism. CYP3A and P-glycoprotein (ABCB1), a multidrug transporter encoded by the *MDR1* gene, have many common substrates and in turn inhibitors. The relative contributions of CYP3A and P-glycoprotein to the pharmacokinetic and pharmacodynamic alterations would be too complex to assess separately.

3.3.1 Etoposide and Ketoconazole

Etoposide is a substrate of CYP3A4 and its pharmacokinetic parameters have a large interpatient variability [76, 77]. A wide variability in oral etoposide bioavailability has also been known [78].

In a pharmacokinetic study of oral etoposide with ketoconazole, 13 patients received etoposide at a dose of 50 mg every other day or daily over 21 days [79]. When etoposide was administered with ketoconazole, a median increase of AUC values was 44 % (range, 14–50 %) as compared to AUC without ketoconazole. Although it was unclear whether the variability in pharmacokinetics was decreased, the results implied that equivalent therapeutic efficacy could be expected with smaller doses of oral etoposide by combined use with ketoconazole. Unfortunately, the clinical significance of this foresighted study is limited owing to lack of an advantage of oral etoposide over conventional intravenous administration [80].

3.3.2 Docetaxel and Cyclosporine

In a pharmacokinetic study of 14 patients with solid tumor, who received oral docetaxel at a dose of 75 mg/m^2 with or without oral cyclosporine of 15 mg/kg, the AUC of oral docetaxel was remarkably increased by the coadministration of cyclosporine from 0.37 ± 0.33 mg h/L to 2.71 ± 1.81 mg h/L (mean ±standard deviation) [81]. The AUC of oral docetaxel with cyclosporine was equivalent to 90 % ± 44 % of AUC after intravenous docetaxel normalized to the same dose level. Interestingly, metabolites of docetaxel were detected in plasma only when docetaxel was administered with cyclosporine. The mechanism of emergence of the metabolites was unclear; however, it would be possible that the elimination of the metabolites was delayed by the use of cyclosporine. The data suggested that oral formulation of docetaxel, together with cyclosporine, might be a possible dosing route in the future for cancer chemotherapy.

3.3.3 Paclitaxel and Valspodar

The cyclosporine derivative valspodar (PSC833) is a non-nephrotoxic and non-immunosuppressive P-glycoprotein antagonist. Biotransformation of valspodar is CYP3A dependent. A Phase I study of paclitaxel over 4 d and oral valspodar (5 mg/kg administered every 6 h over 7 days) was conducted in patients with refractory cancer, where valspodar was primarily used to reverse multidrug resistance [82]. When patients received paclitaxel doses of 13.1 or 17.5 mg/m^2/day with valspodar, the mean steady-state concentrations and AUC of paclitaxel were similar to those when given at a dose of 35 mg/m^2/day. Inhibition of rhodamine efflux from CD56+ cells was used as a surrogate marker for P-glycoprotein inhibition in this study. Despite complete inhibition of P-glycoprotein in the surrogate assay, the large variability in paclitaxel pharmacokinetics was still observed, suggesting that it may be due to the variation in P450 activity. The use of valspodar increased not only plasma concentrations of paclitaxel but also metabolites of the drug, similar to the case of docetaxel and cyclosporine [83]. Plasma concentrations of 6-α-hydroxypaclitaxel, a major metabolite of paclitaxel [84], were increased to measurable levels in 21 of 22 patients in this trial. The metabolite was not detectable in plasma when paclitaxel was administered without valspodar in the same patients. There are several possible explanations for the clinical presence of the metabolite, albeit none of which are mutually exclusive. First, paclitaxel also undergoes 3′-*p*-hydroxylation by CYP3A4. Thus, it is possible that the pathway of 3′-*p*-hydroxylation may be inhibited competitively by valspodar, and, consequently, the metabolism of paclitaxel might shift from producing 3′-*p*-hydroxypaclitaxel to producing 6-α-hydroxypaclitaxel. Second, reabsorption of the excreted metabolites is increased through the inhibition of intestinal P-glycoprotein. Finally, valspodar might promote cholestasis and enhance the enterohepatic circulation, increasing plasma concentrations of the metabolite. Although the clinical significance of this metabolite remains unclear, one must emphasize that the concurrent use of an agent such as valspodar could considerably alter the drug metabolism and disposition.

3.4 Dose Adaptation During Protracted Chemotherapy

When relationships between plasma drug concentration and clinical effects are recognized in a protracted use of a drug, therapeutic drug monitoring (TDM) may be a potential approach to improve the therapeutic efficacy and toxicity. Chronic use of an oral agent is the best candidate due to its wide range of bioavailability.

3.4.1 Dose Adaptation During Protracted Chemotherapy: Etoposide

Etoposide undergoes CYP3A4-mediated metabolism of *O*-demethylation, forming a catechol [76]. Thus, in vivo clearance of oral etoposide depends on CYP3A4 activity in the liver and in the intestine, which would be one of the reasons for the large pharmacokinetic variability. Indeed, the variability of oral etoposide bioavailability is wide [78], and concomitant use of CYP3A inducers increases the clearance of etoposide [85]. A pharmacokinetic study of the protracted use of intravenous etoposide suggested that maintaining plasma levels (>1 μg/mL) would enhance the antitumor effect, whereas high peak levels (>2 μg/mL) may cause severe myelotoxicity [77]. A subsequent study was conducted to utilize TDM for the protracted etoposide schedule in patients with lung cancer [86]. As a starting dose, a 25-mg capsule of etoposide was taken orally three times daily (75 mg/day). The target range of plasma concentration was determined as 1.0–1.5 μg/mL. The dose was adapted to either 50, 75, or 100 mg/day on and after day 5 to achieve the target range, according to the average concentration obtained on days 3 and 4. This study demonstrated that TDM would be applicable to reduce the pharmacokinetic variability.

3.4.2 Dose Adaptation During Protracted Chemotherapy: Imatinib

Imatinib, an oral multi-kinase inhibitor, has dramatically changed the standard treatments of CML and GIST. At this time, imatinib would be the most suitable candidate for TDM in cancer chemotherapy because the drug is primarily metabolized by CYP3A4 and has large interpatient variability in the pharmacokinetics with relationships between pharmacokinetics and efficacy. In 68 patients with CML, imatinib trough levels were associated with the better cytogenetic and major molecular responses, with a plasma threshold of 1,002 ng/mL [87]. In another study of 73 patients with advanced GIST, the trough levels at steady state below 1,100 ng/mL were associated with a worse objective response and shorter time to progression [88]. By introducing the TDM during the imatinib therapy, the patients would be able to take full advantage of their treatments by minimizing variability in the pharmacokinetic and pharmacodynamic effects. The impact of the TDM on clinical outcomes, such as reduced incidence of adverse effects or improved survival, requires further studies.

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Polymorphisms in Genes of Drug Targets and Metabolism

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Abstract It is well known that patients do not respond uniformly to anticancer therapies nor develop identical toxicities when drug dose is adjusted to weight and height. Several mechanisms have been incriminated including genetic background. Polymorphisms in genes of drug targets and metabolism, whom many have been shown to be functional, represent attractive candidates that could provide insights for divergences in outcome to a given treatment. They are increasingly recognized as an important field of study and a promising tool for tailored therapies in cancer patients. In comparison to classic chemotherapeutic drugs having multiple distinct targets, target polymorphisms of recently emerged "targeted therapies" may have even more impact on outcome and be able to select patients benefiting from treatment as well as patients at risk for toxicities. In this chapter, we will discuss the most important studies that have evaluated the importance of genetic polymorphisms in drug targets and metabolism in patients with solid tumors and their impact on daily clinical practice.

Keywords Genetic polymorphisms • Drug targets • Metabolism • VEGFR

1 Introduction

It is a common phenomenon that drug response or host toxicity varies considerably among patients. Potential causes for such variability include tumor histology and differentiation, stage of disease, drug interactions, patient's age, and comorbidities. Despite important implication of those clinical variables in the heterogeneity of drug response, it is recognized that differences in drug metabolism and targets may have even a great influence. A large part of those dissimilarities are inherited as opposed to somatic mutations in the tumor. Functional genetic polymorphisms may therefore determine which specific patient may respond to a given therapy or be at risk for increased toxicity. With the development of targeted therapy, as opposed to common chemotherapy drugs with multiple targets, it is likely that a single or few functional polymorphisms may significantly influence outcome.

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This chapter will discuss the association of functional polymorphisms of drug targets and metabolism with outcomes (Table 1), with an emphasis on gastrointestinal malignancies, as most of our work has been generated in those tumors.

Since the use of predictive and prognostic markers is completely different, a definition of terms is critical. A predictive factor is a marker that allows the identification of individuals who will or will not benefit from the use of a particular therapy. A prognostic factor is a marker that gives information concerning the natural history of the disease regardless of the therapy given. In fact, predictive markers can be used to decide with what to treat, what dose to use, and what combination of therapies will increase efficacy. Prognostic markers may be used to identify patients at high risk of tumor recurrence. However, predictive markers can also be prognostic. A good example is high estrogen expression by immunohistochemistry (IHC) in breast cancer. Its expression predicts response to endocrine therapies, but also indicates a good prognosis independently of any treatment.

2 Polymorphisms in Genes of Drug Targets

2.1 Thymidylate Synthase Polymorphisms

2.1.1 Background

Thymidylate synthase (TS) represents an important chemotherapeutic target for 5-FU. The active metabolite of 5-FU, 5-fluorodeoxyuridine (FdUMP) binds to TS and blocks the generation of thymidine, rapidly shutting off DNA synthesis and repair, triggering apoptosis. TS expression is an important indicator of fluoropyrimidines sensitivity in vitro and in vivo, with its overexpression leading to 5-FU resistance [22, 23].

Several gene polymorphisms are known to influence TS expression. A variable number of 28-bp tandem repeats (VNTR), either double or triple, has been located in the TS gene 5′-untranslated region (5′-UTR) also referred as the promoter enhancer region. Alleles containing 4 (4R), 5 (5R), and 9 (9R) copies of the tandem repeat have also been identified, although the effect of these rare alleles remains unclear [24]. The allele containing the triple repeat (3R) is associated with increased transcription and with 3–4-fold translational efficiency, compared with the double repeat allele (2R) [1, 2]. More recently, a second TS promoter enhancer region (TSER) polymorphism has been identified in the second 28-bp repeat of 3R allele, consisting of a G>C base change at the twelfth nucleotide (3RG>3RC). This single nucleotide polymorphism (SNP) makes the transcriptional activity of the 3R allele as low as the 2R allele. It was shown that repeats one and two of 3R and repeat one of 2R contain upstream stimulating factors (USF) consensus elements, whereas the last repeat in either construct contains a variant consensus sequence due to a G/C base change. Ligand (USF-1 and USF-2) binding leads to enhance transcriptional activity. It was demonstrated that the G>C base change in the second repeat of the 3R allele leads to decreased ability of USF to bind within the repeat and therefore results in decreased transcriptional activity of the 3R TS gene variant [3]. In fact, the number of theoretical USF binding sites defined by the TSER genotype may define groups according to their functional significance. A third polymorphism of the TS gene is a 6-bp deletion that may occur in the 3′-untranslated region (3′-UTR). The 6-bp deletion has been associated with decreased mRNA stability and lower TS protein expression in vitro [4] (Fig. 1). Thus, those three polymorphisms may be used as surrogates for intratumoral TS protein and mRNA levels.

TS gene is localized to the short arm of the chromosome 18. Chromosome 18 is a site of frequent deletions in colorectal cancer tissues [25, 26]. Those deletions may result in loss of heterozygosity (LOH) at the TS locus in cancer tissues, which leads to modification of the TS genotype in the tumor. The occurrence of LOH in individuals who have a heterozygous VNTR 2R/3R genotype in their

Table 1 Characteristics of discussed gene polymorphisms of drug targets and metabolism

Genes	rs number	Polymorphisms	Localization	Functional significance	References
Drug targets					
TS 5′-UTR	–	Double (2R) or triple repeats (3R) of 28 bp	5′-UTR	Increased TS translational efficiency in 3R allele carriers	[1, 2]
TS 5′ G>C SNP	rs2853542	G>C synonymous SNP at the 12 nucleotide (3RG>3RC) in the second 28-bp repeat of 3R allele	5′-UTR	Decreased transcriptional activity in 3RC allele carriers	[3]
TS 3′-UTR	rs16430	6-bp deletion/insertion	3′-UTR	Decreased TS mRNA stability in 6-bp deletion carriers	[4]
EGFR $(CA)_n$ repeat	rs45608036	Variable number of CA dinucleotides	Intron 1	Increased EGFR gene and protein expression levels in carriers of longer CA repeat	[5–8]
EGFR +497	rs2227983	G>A nonsynonymous SNP leading to AA exchange from arginine to lysine	Exon 13	Reduced affinity to EGFR ligand binding with consequent attenuated growth in A/A genotype carriers	[9]
EGFR −216	rs712829	G>T synonymous SNP	Promoter region	EGFR promoter activity increased in T allele carriers	[10]
VEGF −1154	rs1570360	G>A synonymous SNP	Promoter region	Reduced VEGF transcription in A allele carriers	[11]
VEGF −2578	rs699947	C>A synonymous SNP	Promoter region	Increased VEGF production in blood mononuclear cells in C/C genotype carriers	[12]
VEGF −634	rs2010963	G>C synonymous SNP	5′-UTR	Reduced posttranscriptional VEGF level in G allele carriers	[11]
VEGF +936	rs3025039	C>T synonymous SNP	3′-UTR	Lower VEGF plasma level in T allele carriers	[13–15]
VEGF −1498	rs833061	G>A synonymous SNP	Promoter region	No functionality demonstrated	–
HER2 +655	rs1136201	A>G nonsynonymous SNP leading to AA exchange from isoleucine to valine	Exon 20	Reduced HER2 protein kinase activity in A allele carriers	[16]
Drug metabolism					
MTHFR 677	rs1801133	C>T nonsynonymous SNP leading to AA exchange from alanine to valine	Exon 4	Reduced enzymatic activity and thermolability in T allele carriers	[17]
MTHFR 1298	rs1801131	A>C nonsynonymous SNP leading to AA exchange from glutamic acid to alanine	Exon 7	Reduced enzymatic activity in C allele carriers	[18]
DPD IVS14+1G>A	rs3918290	G>A substitution resulting in exon 14 deletion	Intron 14	A allele carriers have a truncated protein with virtually absent catalytic activity	[19]
UGT1A1 *28	rs8175347	Two-base pair insertion resulting in a $(TA)_7TAA$ sequence (UGT1A1*28)	Promoter region (TATA box)	Reduced enzymatic activity in UGT1A1*28 Allele carriers	[20, 21]

SNP single nucleotide polymorphism, *AA* amino acid

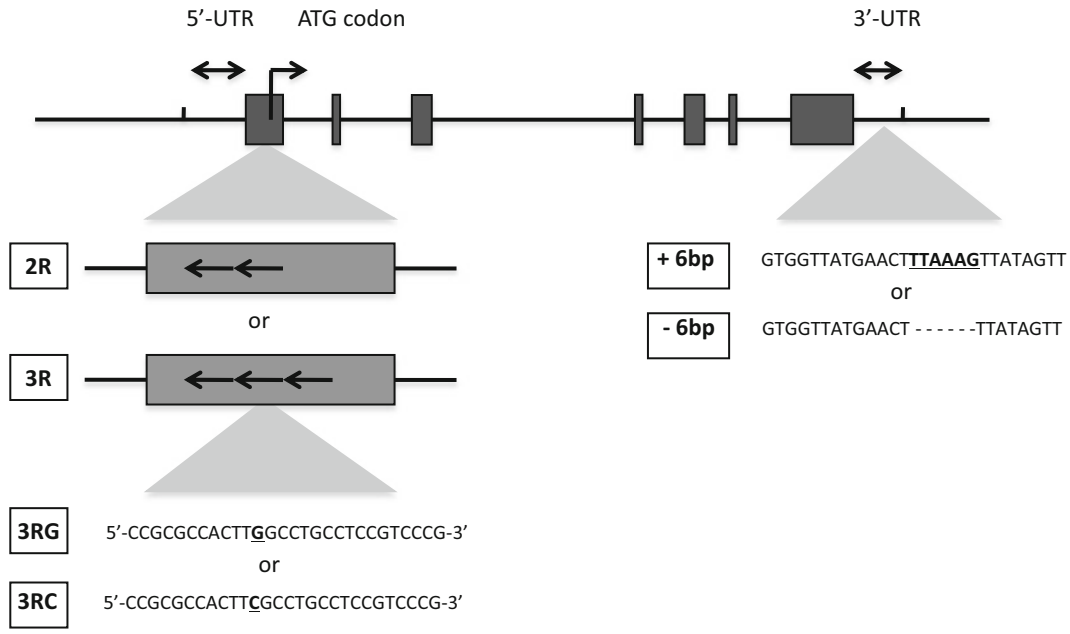

Fig. 1 Functional polymorphism within the thymidylate synthase gene (TYMS). Thymidylate synthase enhancer region (TSER) 2R/3R polymorphism is a tandem repeat upstream of the translational start site containing either double (2R) or triple (3R) repeats of 28-bp sequences. Additional G to C single nucleotide polymorphism (SNP) has been identified within the second repeat of the 3R allele. The third polymorphism is a 6-bp deletion within the 3′-UTR untranslated region

normal tissue may result in a tumor with either a 2R/loss or the 3R/loss TSER genotype. Uchida et al. have shown in 30 patients with metastatic colorectal cancer that LOH (occurring in 77 % of heterozygotes) can significantly modulate intratumoral TS mRNA expression. Patients with 2R/2R or 2R/loss genotype in the cancer tissue had TS mRNA of 2.45 (range, 0.6–4.14), 2.97 (range, 1.63–19.23) for patients with 2R/3R genotype, and 3.68 (range, 1.64–11.97) for patients with 3R/loss or 3R/3R genotype ($P=0.026$) [27]. Others also reported that LOH is common (between 38 and 62 %) in patients with colorectal cancer [28–30] Allelic imbalance is also frequent (44 %) in esophageal adenocarcinoma [31]. The frequency of LOH in other tumors has not been determined yet. This difference between tumor and normal tissue TS genotype due to LOH hints performing TS genotyping in laser-captured microdissected tumor tissue for correlation with efficacy outcomes. However, for evaluation of toxicity, germline DNA analysis provides probably better prediction.

2.1.2 Colon Cancer

Analysis of germline TS polymorphism may predict not only response or survival but also toxicity for 5-FU. It has been suggested that high TS expression levels in normal tissue may protect the cell against damage by 5-FU treatment due to the low efficacy of TS inhibition. The resulting low cell death may lead to low toxicity. Comparison between studies is difficult, as major differences exist between trials. Important factors can account for the discrepant results and should therefore be critically analyzed in those studies (type of analyzed tissue [tumor vs. germline], population [Caucasian vs. Asian], mode of 5-FU administration [bolus, infusion, or both], route of administration [oral vs. intravenous], and concomitant drugs [oxaliplatin or irinotecan]). Since most work has been done in colorectal cancer patients, we will discuss the association of TS polymorphisms with toxicity in this section (Table 2).

Table 2 Predictive value of TS polymorphisms for toxicity

First author	Fluoropyrimidine	Other drugs	Trial type	Cancer type	Toxicity (3–4 grade)	No. of patients	Toxicity (%)					*P* value
							3R/3R	2R/3R	2R/2R	G/C SNP	TS 3′	
Pullarkat et al. [2]	N/A	None	Retrospective	Metastatic CR	Any	50	27	32	63	N/A	N/A	0.008
Schwab et al. [32]	Infusion 5-FU (50 %) or bolus (50 %)	Other drugs (13 %)	Prospective trial, prospective analysis	CR (87 %)	Any	680	13.3	14.2	22.5	N/A	N/A	<0.05
Lecomte et al. [30]	Infusion 5-FU (80 %) or bolus (20 %)	Oxaliplatin (30 %); irinotecan (5 %)	Retrospective	CR (2/3 metastatic)	Any	86	4	18	43	No association	No association	0.02
Capitain et al. [33]	Bolus 5-FU (59 %) or infusion (41 %)	None	Retrospective	Metastatic CR	Any	76	No association			No association	N/A	>0.05
Braun et al. [34]	Infusion and bolus 5-FU	None Irinotecan oxaliplatin	Phase III FOCUS trial, prospective analysis	Metastatic CR	Any	661 256 281	51 54 72	48 65 70	47 60 72	N/A	No association	0.771 0.461 0.957
Ruzzo et al. [35]	Infusion and bolus 5-FU	Oxaliplatin	Prospective trial and analysis	Metastatic CR	Any	166	No association			No association	No association	>0.05
Ruzzo et al. [36]	Infusion and bolus 5-FU	Irinotecan	Prospective trial and analysis	Metastatic CR	Any	146	No association			No association	No association	>0.05
Ichikawa t al. [37]	Bolus 5-FU	None	Retrospective	Stage II-III CR	Diarhea Neutropenia	65	3.9 9.8	27.2 36.4	66.7 66.7	N/A	N/A	0.0005 0.007
Hitre et al. [38]	Infusion 5-FU (58 %) or bolus (42 %)	None	Retrospective	Stage II-III CR	Not specified	166	30 in low expression TS vs 20 in high expression TS*			N/A	*	0.034
Gusella et al. [39]	Bolus 5-FU	None	Retrospective	Stage II-III CR	Any	130	26.7	35.4	40	34.7 in low activity vs 34.5 in high activity**	No association	>0.05
Largillier et al. [40]	Capecitabine	None	Retrospective	Advanced breast	Any	105	No association			50 in high vs 19.4 in intermediate and 13 in low activity***	No association	0.064

5-FU 5-fluorouracil; CR, Colorectal, *N/A* not availabl

*Low expression: 2R/3R with -6-bp/+6-bp or -6-bp/-6-bp and 2R/2R with +6-bp/+6-bp or -6-bp/+6-bp; High expression:TS, homozygous 3R with any 3'-UTR genotype and 2R/3R with +6-bp/+6-bp.

**Low activity: 2R/2R, 2R/3RC, 3RC/3RC; High activity: 2R/3RG, 3RC/3RG, 3RG/3RG

***Low expression: 2R/2R, 2R/3RC or 3RC/3RC; intermediate expression: 2R/3RG, 3RC/3RG; high expression: 3RG/3RG

Pullarkat et al. have been the first to report an association between TS polymorphism and 5-FU-related toxicity. They saw a significant inverse association between the number of 28-bp tandem repeats in the 5′-untranslated region of the TS gene in the tumor and the severity of toxicity ($P=0.008$) in 50 patients with metastatic colorectal cancer treated with 5-FU (regimen not specified). In 63 % (5/8) of patients with 2R/2R genotypes, a toxicity grade 3 ("severe") was observed compared to 27 % (6/22) in the 3R/3R group [2]. Those results were supported by Lecomte et al., who also observed an inverse relation between number of 28-bp tandem repeats and the severity of toxicity in 86 patients with colorectal cancer (two-thirds had metastatic disease) treated with infusion 5-FU-based chemotherapy (30 % and 5 % had concomitant oxaliplatin and irinotecan). In 43 % (6 of 14) of patients with germline 2R/2R genotype, a toxicity grade 3 or 4 was observed compared with 18 % (8 of 44) in the 2R/3R group and 4 % (1 of 28) in the 3R/3R group ($P=0.02$). There was no evidence for a role of the TSER single nucleotide G>C polymorphism in the occurrence of grade 3–4 toxicity. No association was observed between TS 3′-UTR polymorphisms and the severity of toxicity [30]. Schwab et al. confirmed prospectively that germline TS VNTR polymorphism was associated with toxicity in 683 patients with cancer (87 % of colorectal cancer) treated with 5-FU monotherapy (half of patients received infusion 5-FU). VNTR 2R/2R genotype increased the risk for overall toxicity (leukopenia, diarrhea, and mucositis) 1.56-fold (95 % CI 1.08–2.27; $P=0.018$). Moreover, multivariate analysis of individual toxicity suggested that VNTR polymorphism affected diarrhea only. Treatment setting (metastatic or adjuvant) was not specified, nor was the G>C SNP evaluated [32].

However, other authors did not confirm those associations. Ruzzo et al. did not find germline TS polymorphisms (TS 3-UTR, TS 5-UTR VNTR, or G>C substitution) to be associated with toxicity in a prospective clinical trial that evaluated 166 patients with metastatic colorectal cancer treated with first-line 5-FU and oxaliplatin (FOLFOX-4) [35]. The same group did not find a relation of any germline TS polymorphisms with toxicity in 146 patients with colorectal cancer treated with 5-FU and irinotecan (FOLFIRI) [36]. Captain et al. did not observe an association of TS germline VNTR polymorphism with grade 3–4 toxicity in 76 patients with advanced colorectal cancer treated with 5-FU and leucovorin (59 % had bolus 5-FU regimen) [33]. Braun et al. did not find tumor TS VNTR polymorphism to be associated with toxicity in 1,036 patients with metastatic colorectal cancer enrolled in a large phase III trial (FOCUS trial). Two toxicity outcomes were used, primary a delay and/or dose reduction as a result of chemotherapy toxicity within 12 weeks of starting treatment and secondary any grade 3 toxicity. Patients were treated with first- or second-line 5-FU-based chemotherapy (688 patients treated with bolus and infusion 5-FU alone; the remaining patients were treated with concomitant irinotecan or oxaliplatin). TS polymorphisms were not associated with any of the toxicity outcomes, when analyzed in patients receiving the same treatment or between-treatment analysis [34]. The last two studies did not evaluated the TSER G>C SNP.

Largillier et al. have evaluated prospectively the effect of germline TS polymorphisms (TS 3-UTR 6-bp insert–deletion, TS 5-UTR VNTR, and G>C substitution) on toxicity in a phase II study with 105 advanced breast cancer patients treated with capecitabine monotherapy. TS 5′-UTR genotype revealed a trend toward higher grade 3–4 global toxicity at first capecitabine cycle. However, in contrast to other studies, toxicity rate was higher in patients with 3RG/3RG genotype compared to 2R/3RG, 3RC/3RG, and 2R/2R, 2R/3RC, or 3RC/3RC genotypes (50 % vs. 19.4 % vs. 13.0 % toxicity rate; $P=0.064$). When considering only the 28-bp tandem repeats, TS genotype was not related to toxicity [40].

In the adjuvant setting, two studies suggested that TS polymorphisms could predict toxicity. Ichikawa et al. showed that germline TS VNTR polymorphism was related to grade 3–4 neutropenia and diarrhea in 65 Japanese patients with colorectal cancer treated with adjuvant bolus 5-FU. Grade 3–4 neutropenia occurred in 66.7 %, 27.2 %, and 3.9 % in the 2R/2R, 2R/3R, and 3R/3R genotype ($P=0.0005$). Grade 3–4 diarrhea occurred in 66.7 %, 36.4 %, and 9.8 % in the 2R/2R, 2R/3R, and 3R/3R genotype ($P=0.007$). The multivariate analysis confirmed 2R/2R TS polymorphism as an independent risk factor for grade 3–4 neutropenia (OR: 19.2; 95 % CI 2.2–334.4; $P=0.016$) and for

grade 3–4 diarrhea (OR = 11.1; 95 % CI 1.6–117.0; $P = 0.022$) [37]. Hitre et al. have evaluated the influence of germline TS polymorphisms (TS 5-UTR VNTR and 3′-UTR 6-bp insert–deletion) on side effects of patients with colorectal cancer treated with adjuvant 5-FU bolus (42 %) or continuous infusion 5-FU chemotherapy (58 %). They included 166 patients with stage II or III colorectal cancer. Half of the patients had rectal cancer and receive also preoperative radiotherapy. They demonstrated that patients with low expression TS (2R/3R with -6-bp/+6-bp or -6-bp/-6-bp and 2R/2R with +6-bp/+6-bp or -6-bp/+6-bp) had more frequent toxic side effects (30 % vs. 22 %; $P = 0.034$) when compared to patients with high expression TS (homozygous 3R with any 3′-UTR genotype and 2R/3R with +6-bp/+6-bp) [38]. However, Gusella et al. did not find any of the germline TS polymorphisms (TS 3-UTR 6-bp insert–deletion, TS 5-UTR VNTR, and G>C substitution) to be related with bolus 5-FU toxicity in 130 stage II and III colorectal cancer patients, either analyzed separately or in combination according to expression groups [39].

In summary, there are controversial data about the association of low expression TS VNTR polymorphism (2R/2R) with any 5-FU-related grade 3–4 toxicity, in metastatic or in adjuvant setting. It should be noticed that the largest negative trial has performed tumor genotyping, which may have interfered significantly with the results as LOH has been frequently reported in colorectal cancer. There is currently no evidence for a role of the TSER single nucleotide G>C polymorphism nor for TS 3′-UTR polymorphism in the occurrence of toxicity. The latter has been linked with side effects in only one study, in the combined genotype analysis (TS 5-UTR VNTR and 3′-UTR 6-bp insert–deletion) [38]. Most studies enrolled patients treated with 5-FU irrespectively of its mode of administration (bolus vs. infusion), making difficult to evaluate an eventual specific association with TS polymorphisms. It seems even more unlikely to be ever reported, at least in colorectal cancer, as most current regimens include both bolus and infusion 5-FU. It appears that TS VNTR polymorphism does not predict toxicity in patients treated with 5-FU in association with other drugs (irinotecan or oxaliplatin) further suggesting its limited clinical usefulness, as most patients are currently treated with combination regimens. As suggested by Schwab et al., further work should focus on specific toxicities, especially gastrointestinal toxicities [32].

It has been shown that levels of TS enzyme may vary between metastasis and the primary tumor or other metastatic sites [41, 42]. Therefore, studies that looked if TS polymorphisms were associated with outcome in the metastatic setting should be analyzed separately from those that recruited patients treated with adjuvant 5-FU-based chemotherapies. One of the major sources of discrepant results in the literature may be the mode of administration of 5-FU. Fluoropyrimidines may have different antitumor effects according to the mode of administration. Bolus 5-FU may exert its major effect through incorporation into RNA, whereas continuous infusion may have a preferential effect on TS inhibition [43]. Another source of discrepancy is the inclusion of patients with rectal cancer. In this regard, it is being increasingly recognized that colon and rectal cancer are distinct disease groups, in terms of treatment, clinical outcome, risk factors, and molecular markers [44–47]. They should therefore be considered separately. Studied populations with different allele distribution can impact on the data. It has been well demonstrated that East Asian populations have significantly higher frequency of 3R/3R genotype compared to Caucasians. As expected, in the following discussed studies, 3R/3R genotype was predominant in East Asian populations when compared to Caucasian populations (69–56 % vs. 35–23 %).

We will focus first on studies that evaluated stage II–III colorectal cancer (Table 3). Only two studies excluded rectal patients from their study population. Suh et al. were the first to report an association of VNTR polymorphism with outcome in the adjuvant setting. They reviewed 121 Korean patients with stage II or III colon cancer treated with adjuvant fluoropyrimidines (the majority was treated with continuous 5-FU). 3R/3R tumor genotype predicted a significant poor 5-year overall survival (OS) (53 % vs. 80 %; $P = 0.048$) compared to patients with 2R/3R or 2R/2R genotype. However, this difference was significant only in patients with stage III colon cancer [48]. Lurje et al. supported those results. They were able to demonstrate in 197 patients (79 % of Caucasian) with

Table 3 Thymidylate synthase (TS) polymorphisms and its predictive value for 5-fluorouracil (5-FU)-based treatments in the adjuvant setting for patients with colorectal cancer

First author	Trial type	Fluoropyrimidine	Other drugs	Genotyping	Population	No. of patients	Stage of disease	Positive results	Negative results/ comments
Suh et al. [48]	Retrospective	Continuous 5-FU (60 %) and doxifluridine (40 %)	None	Tumor	Korean	121	II + III colon cancer	Poor five-year OS in 3R/3R (53 % vs. 80 %; $P = 0.048$) compared to all others	G/C SNP and TS 3′-UTR not analyzed
Lurje et al. [49, 50]	Retrospective	Infusion 5-FU (85 %) or bolus (15 %)	Not specified	Germline	Mainly (79 %) Caucasians	197	II + III colon cancer	Higher recurrence risk for 3RG/3RG (RR = 3.48; $P = 0.013$) compared with 2R/2R, 2R/3RC, or 3RC/3RC Higher recurrence risk for 3RG/3RG irrespective of TS 3′-UTR (RR = 3.41; $P = 0.044$) compared with other combinations Higher recurrence risk for 3RG/+6-bp haplotype (RR = 2.25 ; $P = 0.032$) compared with 2R/+6 bp haplotype	TS VNTR and TS 3′-UTR alone were not associated with TTR
Hitre et al. [38]	Prospective study and analysis	Infusion 5-FU (58 %) or bolus (42 %)	None	Germline	Caucasian	166	II + III colorectal cancer	Higher risk of disease progression (HR 1.79; $P = 0.048$) and death (HR = 3.79; $P = 0.009$) for 2R containing genotype Longer OS in highexpression TS (HR = 0.3; $P = 0.005$) and PFS (HR = 0.46; $P = 0.005$) compared to low expression TS[b]	TS 3′-UTR not related with OS or DFS G/C SNP not analyzed
Dotor et al. [28]	Prospective study, retrospective analysis	Bolus 5-FU	None	Germline and tumor	Caucasian	129	II, III + operated IV colorectal cancer	Reduced risk of death for tumor 3R/3R (HR = 0.38; $P = 0.02$) compared to others Reduced risk of death for tumor and germline -6-bp (for each allele the HR was 0.47; $P = 0.011$) Reduced risk of death for 3R/-6-bp haplotype (HR = 0.42; $P = 0.017$) compared with 2R/+6-bp	Germline TS VNTR polymorphism was not associated with OS G/C SNP added no information
Prall et al. [51]	Retrospective	5-FU	Not specified	Tumor	Caucasian	94	III colorectal cancer	–	TSER polymorphisms did not predict OS

Gusella et al. [39]	Prospective study and analysis	Bolus 5-FU	None	Germline	Caucasian	130	II + III colorectal cancer	–	TSER and TS 3′-UTR polymorphisms were not associated with DFS or OS
Tsuji et al. [52]	Retrospective	Oral fluoropyrimidines	None	Tumor	Japanese	128	I–III colorectal cancer	–	TS VNTR polymorphism not associated with OS
Park et al. [53]	Retrospective	5-FU	None	Tumor	Korean	201	III colorectal cancer		TSER polymorphisms did not predict DFS

[a]Significant only in stage II patients

[b]High expression TS: homozygous 3R with any 3′-UTR genotype and 2R/3R with +6-bp/+6-bp ; low expression TS : 2R/3R with -6-bp/+6-bp or -6-bp/-6-bp and 2R/2R with +6-bp/+6-bp or -6-bp/+6-bp

DFS Disease-free survival, *OS* Overall survival, *PFS* Progression-free survival, *SNP* single nucleotide polymorphism, *TSER* thymidylate synthase enhancer region, *TTP* time to progression, *VNTR* variable number of tandem repeats

high-risk stage II or stage III colon cancer treated with 5-FU-based chemotherapy (85 % infusion 5-FU) that germline TS polymorphisms could separate patients into groups according to their TS expression status. In the joint analysis of the two TSER polymorphisms, patients homozygous for the 3RG allele were at greatest risk of tumor recurrence (RR = 3.48; 95 % CI = 1.61–7.54; $P = 0.013$), compared with patients displaying the 2R/2R, 2R/3RC, or 3RC/3RC genotype. The combination analysis of the two TSER and the 3′-UTR polymorphism showed a significant relationship with time to recurrence. Patients with the 3RG/3RG genotype were at greatest risk to develop tumor recurrence irrespective of the 3′-UTR polymorphism (RR = 3.41; 95 % CI: 1.33–8.75; $P = 0.044$), compared with patients displaying other genotype combinations. The haplotype analysis revealed that patients harboring the 3RG/+6-bp haplotype were at greatest risk to develop tumor recurrence (RR = 2.25; 95 % CI: 1.04–4.85; $P = 0.032$), compared with patients with the most prevalent haplotype, 2R/+6 bp [49, 50].

An abundant literature further accessed TS polymorphisms' predictive value in the adjuvant setting. Unfortunately, the studied populations were heterogeneous, notably with the inclusion of patients with rectal cancer. Dotor et al. also showed that both germline and tumor TS polymorphisms (TS 5-UTR VNTR and 3′-UTR 6-bp insert–deletion) were associated with OS in 129 Caucasian patients with colorectal cancer treated with adjuvant bolus 5-FU-based chemotherapy. This study also included patients with resected stage IV colorectal cancer (unique liver synchronous metastases). However, in contrast to most publications, 3R/3R tumor genotype was predictive of a better OS (HR = 0.38; 95 % CI 0.16–0.93; $P = 0.020$) compared to 2R/2R or 2R/3R genotypes. This association did not reach significance when germline genotypes were accessed. G/C SNP added no information. They also reported that -6-bp allele was associated with reduced risk of death (for each -6-bp allele, the HR was 0.42; 95 % CI 0.22–0.82; $P = 0.0034$). This was true for both tumor and germline genotypes. Moreover, the haplotype analysis revealed an OS benefit for patients harboring 3R/-6-bp haplotype compared to patients with 2R/+6-bp (HR = 0.42; 95 % CI 0.2–0.85; $P = 0.017$). Interestingly, no correlation was detected with TS IHC and any of the three polymorphisms [28]. Hitre et al. evaluated prospectively the influence of germline TS polymorphisms (TS 5-UTR VNTR and 3′-UTR 6-bp insert–deletion) on disease-free survival (DFS) and OS of Caucasian patients with colorectal cancer treated with adjuvant 5-FU bolus (42 %) or continuous infusion 5-FU (58 %) chemotherapy. They included a heterogeneous population of 166 patients with stage II or III colorectal cancer. Half of the patients had rectal cancer and receive also preoperative radiotherapy. They demonstrated that patients with high expression TS (homozygous 3R with any 3′-UTR genotype and 2R/3R with +6-bp/+6-bp) had a longer OS (HR = 0.3; 95 % CI 0.13–0.69; $P = 0.005$) and PFS (HR = 0.46; 95 % CI 0.29–0.79; $P = 0.005$) when compared to patients with low expression TS (2R/3R with -6-bp/+6-bp or -6-bp/-6-bp and 2R/2R with +6-bp/+6-bp or -6-bp/+6-bp) [38]. The interpretation of those two studies is puzzling. The results of the former study may be partially explained by the lack of VNTR polymorphism correlation with the functional G>C SNP. As previously explained, 3RC variant causes a lower transcription rate compared the 3RG variant, comparable with the 2R variant. Both studies are small and studied heterogeneous populations.

Two relatively small studies with Caucasian patients and two studies with East Asian patients did not find TS polymorphisms to be associated with outcome. Prall et al. did not find tumor TSER polymorphisms (TS 5-UTR VNTR and G>C SNP) to predict OS in 94 Caucasian patients with stage III colorectal cancer treated with adjuvant 5-FU-based chemotherapy (regimen not described) [51]. Gusella et al. did not observe any of the germline TS polymorphisms (TS 3-UTR 6-bp insert–deletion, TS 5-UTR VNTR, and G>C SNP) to be related with clinical outcome (DFS or OS) in 130 stage II or III colorectal cancer patients (Caucasians), either analyzed separately or in combination according to expression groups [39]. Tjusi et al. did not find tumor TS VNTR polymorphism to be associated with OS in 128 Japanese patients with stage I–III (11 % of stage I) colorectal cancer treated with oral fluoropyrimidines [52]. Park et al. did not find TS G>C SNP and VNTR in the tumor to predict DFS in 201 Korean patients with stage III colorectal cancer who received adjuvant 5-FU chemotherapy (bolus or infusion 5-FU; proportion not specified) after surgery. This study also included patients with rectal cancer (50 %) that received preoperative or postoperative radiotherapy [53].

In summary, there are contradictory data in the literature about the predictive value for outcome of TS polymorphisms for 5-FU-based chemotherapy in the adjuvant setting. The comparison across studies is difficult because most studies investigated heterogeneous populations treated with different regimen of fluoropyrimidines (bolus 5-FU, infusion 5-FU, or oral fluoropyrimidines). However, in homogeneous patients populations with stage II or III colon cancer treated with infusion 5-FU, high expression TSER variants alone, or in combination with 3′-UTR polymorphism may predict worse outcome. Large prospective studies are needed to validate those results.

An abundant literature also accessed the predictive value of TS polymorphisms for outcome in the metastatic setting (Table 4). All studies have included patients with colon and rectal cancer. Pullarkat et al. reported that the tumor VNTR polymorphism could predict response to protracted infusion of 5-FU in 50 patients with metastatic colorectal cancer. Individuals with 2R/2R genotype had a response rate of 50 % (4/8) when compared to 9 % (2/22) in those with 3R/3R genotype and 15 % (3/20) in those with 2R/3R genotype ($P=0.041$). Patients with the 2R/2R genotype had a longer median survival of 16.2 months when compared to 8.3 months and 8.5 months in those with the 2R/3R and 3R/3R genotypes. However it did not reach statistical significance ($P=0.37$) [2]. Capitain et al. found 3R/3R germline VNTR polymorphism to be associated with shorter OS compared to 2R/3R and 2R/2R ($P=0.007$) in 76 patients with advanced colorectal cancer treated with 5-FU (59 % had bolus 5-FU regimen). The response rate for 3R/3R was decreased by almost 50 % in comparison to other genotypes. This link was however not significant [33]. Graziano et al. associated germline low expression TSER genotypes (2R/2R, 2R/3C, and 3C/3C) with high response (complete or partial) in 80 patients treated with first-line 5-FU-based chemotherapy with either irinotecan or oxaliplatin (54.2 % vs. 25 %; $P=0.01$). The TS VNTR polymorphism alone did not predict outcome, nor did the TS 3′-UTR polymorphism [54, 55]. Lecomte did not find germline or tumor TS polymorphisms (TS 3-UTR 6-bp insert–deletion, TS 5-UTR VNTR, and G>C substitution) to be significantly associated with outcome (response or OS) in 64 patients with colorectal cancer (two-thirds had metastatic cancer) treated with mainly infusion 5-FU-based chemotherapy (30 % and 5 % had concomitant oxaliplatin and irinotecan). However, patients with the 2R/2R genotype had a longer OS of 27 months when compared with 15 months and 21 months in those with the 2R/3R and 3R/3R genotype. Nonetheless, It did not reach significance ($P=0.08$) [30].

Jakobsen et al. found a correlation of germline TS VNTR polymorphism with response in 88 patients with metastatic colorectal (half of patients with rectum cancer) treated with bolus 5-FU. However, in contrast to previous publications, the rate of response was almost double in the 3R/3R (52 % vs. 27 %; $P=0.03$) compared to the two other groups taken together (2R/3R and 2R/2R) [56]. Those results may be partially explained by the lack of correlation with the functional G>C SNP.

Stoehlmacher et al. have examined whether germline TS polymorphism may predict outcome in 106 patients with metastatic colorectal cancer treated with second-line 5-FU and oxaliplatin. TS VNTR polymorphism displayed no association with time to progression (TTP) or OS. However, patients with TS 3′-UTR +6-bp/-6-bp and -6-bp/-6-bp had a lower risk of progression compared to patients with +6-bp/+6-bp genotype (HR=1.76; 95 % CI 1.08–2.86; $P=0.02$). TS 3′-UTR was not significantly associated with OS [57]. Ruzzo et al. looked in 146 patients with metastatic colorectal cancer treated with first-line 5-FU and irinotecan (FOLFIRI). Germline 3′-UTR +6 bp/+6 bp genotype was significantly related to a worse PFS (HR=1.98; 95 % CI 1.23–3.09; $P=0.01$) compared to -6 bp/-6 bp genotype. TSER polymorphisms (TS 5-UTR VNTR and G>C SNP) were not associated with outcome [36].

Others did not observe any of the TS polymorphisms to predict outcome in patients with metastatic colorectal cancer. Etienne-Grimaldi et al. did not find tumor TSER polymorphisms to be associated with response in 93 patients with metastatic colorectal cancer treated with 5-FU-based chemotherapy [59]. TS VNTR tumor polymorphism did not predict response or OS in 103 patients with colorectal cancer and liver metastasis treated with infusion 5-FU-based chemotherapy [58]. Ruzzo et al. did not find germline TS polymorphisms (TS 3-UTR 6-bp insert–deletion, TS 5-UTR VNTR, and G>C SNP) to be associated with PFS or response in a prospective clinical trial that evaluated 166 patients with metastatic colorectal cancer treated with first-line 5-FU and oxaliplatin (FOLFOX-4) [35].

Table 4 Thymidylate synthase (TS) polymorphisms and its predictive value for 5-fluorouracil (5-FU)-based treatments in the metastatic setting for patients with colorectal cancer

First author	Trial type	Fluoropyrimidine	Other drugs	Genotyping	Population	No. of patients	Positive results	Negative results/ comments
Pullarkat et al. [2]	Prospective trials with retrospective analysis	Infusion 5-FU	None	Tumor	Mixte	50	Higher RR for 2R/2R compared to 2R/3R or 3R/3R (50 vs. 15 vs. 9 %; $P=0.041$) Trend for longer OS for 2R/2R compared to 2R/3R or 3R/3R (16.2 vs. 8.3 vs. 8.5months; $P=0.37$)	G/C SNP and TS 3′-UTR polymorphism not analyzed
Capitain et al. [33]	Retrospective	Bolus (59 %) 5-FU or infusion (41 %) 5-FU	None	Germline	Caucasians	76	RR for 3R/3R was decreased by almost 50 % compared to other genotypes (19 vs. 41 %; $P>0.05$) Shorter OS for 3R/3R compared to other genotypes ($P=0.007$)	G/C SNP added no information TS 3′-UTR polymorphism not analyzed
Graziano et al. [54, 55]	Prospective trials with retrospective analysis	Infusion and bolus 5-FU	Oxaliplatin or irinotecan	Germline	Caucasian	80	High RR for low vs. high expression TSER genotypes (54.2 % vs. 25 %; $P=0.01$)	TS 3′-UTR polymorphism not related with RR TS VNTR polymorphism alone not associated with OS
Lecomte et al. [30]	Retrospective	Infusion 5-FU (80 %) or bolus (20 %) 5-FU	Oxaliplatin (30 %) or irinotecan (5 %)	Germline and tumor	Caucasian	55	Trend for a longer OS for 2R/2R vs. 2R/3R and 3R/3R (27 vs. 15 vs. 21 months; $P=0.08$)	G/C SNP added no information TSER and TS 3′-UTR were not associated with RR No significant difference when evaluating tumor or germline polymorphisms
Jakobsen et al. [56]	Prospective trial with retrospective analysis	Bolus 5-FU	None	Germline	Not reported	88	Higher RR for 3R/3R compared to other genotypes (52 % vs. 27 %; $P=0.03$)	TS VNTR polymorphism was not associated with TTP G/C SNP and TS 3′-UTR were not analyzed

Stoehlmacher et al. [57]	Prospective trial with retrospective analysis	Infusion 5-FU	Oxaliplatin	Germline	Majority of Caucasians (75 %)	106	Lower risk of progression for +6 bp/-6 bp and -6 bp/-6 bp compared to +6 bp/+6 bp (HR = 1.76; $P = 0.02$)	TS VNTR polymorphism was not associated with TTP or OS TS 3′-UTR polymorphism not related with OS
Ruzzo et al. [36]	Prospective trial and analysis	Infusion and bolus 5-FU	Irinotecan	Germline	Caucasian	146	Higher risk for progression for +6 bp/+6 bp genotype (HR = 1.98; $P = 0.01$) compared to -6 bp/-6 bp	TSER polymorphisms were not associated with PFS TS 3′-UTR polymorphism not related with RR
Etienne et al. [58]	Retrospective	Infusion 5-FU	None	Tumor	Not reported	103	–	TS VNTR polymorphism was not associated with RR or OS
Ruzzo et al. [35]	Prospective trial and analysis	Infusion and bolus 5-FU	Oxaliplatin	Germline	Caucasian	166	–	TSER and TS 3′-UTR polymorphisms were not associated with RR or PFS
Etienne-Grimaldi [59]	Retrospective	5-FU	None	Tumor	Not reported	93	–	TSER polymorphisms were not associated with RR

OS overall survival, *PFS* progression-free survival, *SNP* single nucleotide polymorphism, *TSER* thymidylate synthase enhancer region, *TTP* time to progression, *VNTR* variable number of tandem repeats

[a]Low expression TSER genotypes: 2R/2R, 2R/3RC and 3RC/3RC; High TSER expression genotypes: 3RG/3RG, 3RC/3RG, 2R/3RG

In summary, there are inconclusive data about TS polymorphisms to predict outcome in patients with metastatic colorectal cancer. In contrast to the adjuvant setting were only TSER polymorphisms seem to be related with outcome, TS 3′-UTR may play a role in the metastatic setting. Unfortunately, few studies correlated the three known functional polymorphisms with outcome. Large prospective clinical marker-embedded trials are needed to validate TS polymorphisms as a predictor of outcome in patients with metastatic colon cancer treated with infusion 5-FU-based chemotherapy. Tumor genotyping should be preferred. Moreover, laser-captured microdissection should be performed to purify tumor cells from adjacent stroma cells, avoiding heterogeneous cell populations with different allele distributions. As mentioned earlier, allelic imbalance at TS locus is frequent in colorectal cancer and may lead to different genotype when compared to normal tissues.

Prognostic Value

There is very little information about the prognostic importance of TS polymorphisms in patients with colon cancer. This lack of literature may be explained by the fact that fluoropyrimidines are the backbone of all past and current chemotherapy regimens. Therefore, currently, only patients with stage I or low-risk stage II colon cancer patients may provide prognostic information as those patients have not been shown to benefit from adjuvant treatments. To our knowledge, a single report, published in a form of letter to the editor, suggested a prognostic value for TS VNTR polymorphism. Dysager et al. looked in 79 patients with stage II colon cancer treated exclusively with surgery and reported that the 5-year OS was higher in TS VNTR homozygous 2R/2R and 3R/3R genotypes versus heterozygous 2R/3R ($P=0.04$). The authors did unfortunately not examine the TSER G>C SNP. In consequence, there is no substantial evidence to support a prognostic value for TS polymorphisms.

2.1.3 Rectal Cancer

Rectal cancer has a unique therapeutic approach compared to colon cancer because it involves preoperative combined modality with radiation and chemotherapy in patients with locally advanced disease. Since 2004, neoadjuvant chemoradiation with 5-fluorouracil (5-FU) has become the standard of care for patients with rectal cancer [60]. Preclinical data have shown that fluoropyrimidines could radio-sensitize cells through the inhibition of TS and thus decreasing the repair of radiation-induced DNA double-strand breaks [61]. It was then postulated that high TS expression could alter the 5-FU sensitization in rectal cancer thus decreasing response to chemoradiation.

No study investigating the prognostic value of TS polymorphisms in rectal cancer patients has been reported. However, the predictive value of TS genotyping for preoperative fluoropyrimidine-based chemoradiation has been investigated in three studies. The high expression genotype of the tumor TS VNTR polymorphism (3R/3R) was associated with poor tumor downstaging (22 % vs. 60 %; $P=0.002$) compared to low expression genotype (2R/2R or 2R/3R) in 65 patients with rectal cancer treated with fluoropyrimidine-based chemoradiation [62]. Spindler et al. found that the low expressing germline 2R/2R VNTR polymorphism was associated with higher pCR than 3R containing genotypes in 60 patients treated with preoperative fluoropyrimidine-based chemoradiation (53 % vs. 22 %; $P=0.048$) [63]. However, those results were questioned by Terrazzino et al. who did not find germinal TS VNTR polymorphism to predict response in 125 patients treated with 5-FU-based chemoradiation [64]. A prospective phase II neoadjuvant trial was initiated based on encouraging previous results. This study randomized patients to different treatment groups according to their genotype. High-risk patients were defined by the presence of high expression genotypes of the TS VNTR polymorphism. This group of patients was treated more aggressively with a combination of 5-FU and irinotecan during the radiotherapy. The low-risk group, with the lower expression genotypes of the TS VNTR

polymorphism, was treated with 5-FU throughout radiotherapy. Results were recently updated at the 2009 annual ASCO meeting and confirmed that the addition of irinotecan could achieve very encouraging high rates of pathological complete response (42 %) in the poor-risk group [65].

Those data suggest that TS polymorphisms may have a predictive value in patients with locally advanced rectal cancer treated with preoperative fluoropyrimidine-based chemoradiation, and thus identify patients unlikely to respond to standard chemoradiation schedules. The results from the latter phase II study need to be validated in a large phase III trial, which should also include the TS G>C SNP.

2.1.4 Gastric Cancer

In contrast to the standard adjuvant 5-FU-based chemoradiation in the USA, many parts of Europe and Asia treated patients with locally advanced gastric cancer with adjuvant fluoropyrimidine-based chemotherapy [66]. Recently, there has been a shift toward 5-FU-based preoperative chemotherapy with or without postoperative chemotherapy, which gives a unique opportunity in the future to access markers for pathological response and their correlation with survival [67].

Ott et al. have found germline TS VNTR polymorphism to be associated with tumor-related survival in 135 Caucasian patients with locally advanced gastric cancer treated with preoperative 5-FU and cisplatin-based chemotherapy. 3R/3R genotype was an independent risk of poor tumor-related survival (RR = 4.57; 95 % CI 1.88–11.14; $P = 0.0008$). TS VNTR polymorphism was not associated with pathological response defined as less than 10 % residual tumor [68]. Kawakami et al. evaluated 90 Caucasian patients with gastric cancer treated with surgery and adjuvant 5-FU-based chemotherapy. Chemotherapy regimens were heterogeneous with 78 % of patients given also cisplatin or mitomycin-C. Carriers of both low germline TS expression genotypes (2R/2R, 2R/3C, 3C/3C and -6-bp/-6-bp, -6-bp/+6-bp) showed the best 3-year DFS (76 % vs. 20 %; $P < 0.001$) and OS (84 % vs. 40 %; $P < 0.001$) compared to patients with both high TS genotypes (3G/3G, 3G/3C, 3G/2R, and +6-bp/+6-bp). The presence of at least one high TS genotype was an independent poor prognostic factor for DFS (HR = 3.5; 95 % CI 2.1–4.9) and OS (HR = 2.9; 95 % CI 1.7–4.1) [69]. Huang et al. investigated the association of TS germline polymorphisms (TS 3-UTR 6-bp insert–deletion, TS 5-UTR VNTR, and G>C SNP) with OS in 116 Chinese patients with gastric cancer treated with surgery and adjuvant 5-FU-based chemotherapy (88 % also were treated with concomitant oxaliplatin). TS 3′-UTR polymorphism was significantly associated with OS. Patients with 6+/6+ genotype had a shorter OS of 20.7 months when compared with 29.8 and 41 months in those with the 6+/6- ($P = 0.022$) and 6-/6- genotype ($P = 0.017$). Moreover, the 6+/6+ genotype was an independent factor of poor prognosis (HR = 2.437, $P = 0.041$). No significant association was found for the VNTR polymorphisms [70, 71].

In summary, high expressing TS polymorphisms are promising to predict worse outcome in patients with locally advanced gastric cancer treated with a combination of 5-FU and platinum. TSER polymorphisms may not be relevant in East Asian populations, perhaps as a result of different allele frequencies when compared to Caucasians populations.

Three studies have evaluated if TS polymorphisms could predict outcome in Caucasian patients with metastatic gastric cancer. Goekkurt et al. showed a trend for superior OS in 52 patients harboring the low expression tumor genotypes (2R/2R, 2R/3RC, 3RC/3RC) in combined analysis of TSER polymorphisms. All patients were treated with 5-FU, folinic acid, and cisplatin. The low expression group experienced a superior median survival time of 10.2 months compared to only 6 months in the high expression group ($P = 0.099$). 3′UTR polymorphism was not associated with outcome [72]. Similarly, Ruzzo et al. showed in 175 Caucasian patients with advanced gastric cancer treated with 5-FU and cisplatin that patients with high expression germline genotype (2R/3RG, 3RC/3RG, 3RG/3RG) had inferior response, PFS, and OS ($P = 0.0003$, $P = 0.0002$, and $P = 0.0002$). 3′UTR

polymorphism was not associated with outcome [73]. Conversely to those two study, in the univariate analysis, the high expression TS VNTR 3R/3R germline genotype was associated with a better PFS and OS compared to patients with the 2R/2R genotype (9.1 vs. 5.8 months; $P=0.006$ and 17.3 vs. 14.8 months; $P=0.022$) in 134 with advanced gastric cancer included in a phase III study comparing weekly 5-FU combined either with biweekly cisplatin (FLP regimen) or oxaliplatin (FLO regimen). The TS G/C SNP did not add significant information. TS 3′UTR polymorphism was not linked with outcome. However, as TS 3′UTR and VNTR polymorphisms were in linkage disequilibrium, meaning that some combination of alleles occurs more or less frequently as would be expected from a random formation of haplotypes, the authors included haplotypes in the multivariate analysis. The presence of at least one 3R/+6 allele was related to superior median PFS (7.1 vs. 5.6 months; $P=0.003$) and OS (13.9 vs. 10.7 months; $P=0.004$) [74]. Those unexpected results have not yet been explained. However, we may incriminate tumor LOH, which was shown to be frequent in gastric cancers [31]. Consistent with this hypothesis, the authors showed that patients with the 3R/3R genotype had lower risk for developing grade 3–4 leukopenia compared with patients harboring the 2R/2R genotype (0 vs. 19 %; $P=0.047$).

Conversely to Caucasian patients, TS 3′-UTR polymorphism has been reported to play a significant role in East Asian patients. Lu et al. suggested that the response rate of 106 Chinese patients with advanced gastric cancer harboring the -6/-6-bp and -6/+6-bp germline genotypes was significantly higher than patients with the +6/+6-bp genotype ($P=0.045$). However, patients included in that study had very heterogeneous 5-FU-based chemotherapies making the interpretation difficult [75]. Keam et al. showed that germline TS 3′-UTR polymorphism could predict outcome in 76 Korean patients with advanced gastric cancer treated with 5-FU, folinic acid, and oxaliplatin (modified FOLFOX-6). Response rate was significantly higher in patients with a -6/-6 genotype (55 % vs. 30 %; $P=0.034$) compared to patients with +6/+6 or +6/-6 genotypes. TS 3′-UTR -6/-6 genotype was also associated with a longer TTP (6.3 vs. 4.7 months; $P=0.014$) and a longer OS (17.8 vs. 10.3; $P=0.032$). However, no correlation with outcome was demonstrated for the TSER polymorphisms (5-UTR VNTR and G>C SNP) [76]. Those data suggest that low expressing TS 3′-UTR genotypes may predict better outcome in patients with advanced gastric cancer treated with 5-FU in combination with platinum, in East Asian populations. In Caucasian population, such association has not been demonstrated. In contrast, in Caucasian patients, TS VNTR polymorphism may be linked to outcome. Once again, those results need to be validated prospectively in large clinical trials.

2.1.5 Esophageal Cancer

Esophageal cancer is a heterogeneous disease constituted of two different histological types that have different treatment modalities, prognosis, and risk factors [77, 78]. They should therefore be analyzed separately. The standard of care for locally advanced squamous cell esophageal cancer is preoperative chemoradiation [79]. Locally advanced esophageal adenocarcinoma may be treated either with preoperative 5-FU-based chemotherapy or chemoradiation, depending on their situation to the gastroesophageal junction [67].

Three small studies evaluated TS polymorphisms and its relation to outcome in patients with esophageal squamous cell carcinoma. None observed a significant association with outcome. Okuno et al. did not find an association of germline VNTR polymorphism or 3′-UTR with pCR or OS in 31 Japanese patients with esophageal squamous cell carcinoma treated with neoadjuvant 5-FU and cisplatin chemoradiation [80]. Sarbia et al. examined the correlation between response, either clinical or pathological, and OS with tumor VNTR polymorphism in 68 patients with locally advanced esophageal cancer treated with induction chemotherapy followed by cisplatin and etoposide chemoradiation either definitive or followed by surgery. The induction chemotherapy consisted of bolus 5-FU, folinic acid, etoposide, and cisplatin (FLEP). No relationship was found with clinical response after induction

chemotherapy, with pCR after chemoradiation (small subgroup of patients; $n=20$) or with OS [81]. Liao et al. tested if germline TS 3′-UTR polymorphism could predict outcome in 146 Caucasian patients with esophageal adenocarcinoma treated with preoperative 5-FU-based chemoradiation. In addition, patients received concurrent platinum or taxanes or both. They did not find a significant correlation between TS 3′-UTR polymorphism and outcome [82].

One small study suggested that TSER polymorphisms might be related to outcome in esophageal adenocarcinoma. Kuramochi et al. examined the predictive value of germline TSER polymorphisms and 3′-UTR polymorphism in 34 Caucasian patients with esophageal adenocarcinoma treated with 5-FU-based chemotherapy (45 % were metastatic). They reported a significant better OS for the non-3RG group of patients ($P=0.018$). Interestingly, they did not report a survival difference between patients with 3RG allele (3RG group) and non-3RG group among 48 patients with esophageal adenocarcinoma treated with surgery alone, suggesting that TS genotype is not a prognostic marker in this disease [31]. Validation of those results is needed in a large clinical trial.

2.1.6 Breast Cancer

Huang et al. investigated 192 Taiwanese patients with operated stage II or III breast cancer treated with a combination of adjuvant cyclophosphamide, epirubicin, and 5-FU (FEC). Germline TS VNTR polymorphism was not associated with tumor relapse [83]. The authors did not evaluate the impact of TSER G>C substitution.

One single study has evaluated the impact of TS polymorphisms on patients with advanced breast cancer treated with fluoropyrimidines. Largillier et al. have evaluated prospectively the effect of germline TS polymorphisms (TS 3-UTR 6-bp insert–deletion, TS 5-UTR VNTR, and G>C SNP) on toxicity (previously discussed) and efficacy in a phase II study with 105 advanced breast cancer patients treated with capecitabine monotherapy. In univariate analysis, TS 3RG/3RG genotype was significantly related to longer response duration compared to all other genotypes ($P=0.037$). However, it lost its significance in the multivariate analysis [40]. Thus, there is limited evidence that TS polymorphisms may predict response to fluoropyrimidine-based treatments in advanced breast cancer patients. The numerous chemotherapy combinations with or without targeted therapies will probably limit, in the future, comparison across studies. No studies evaluated the TS polymorphisms′ prognostic value in breast cancer patients.

2.2 Epidermal Growth Factor Receptor

2.2.1 Background

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase (TK) of the ErbB/HER family. Ligand binding to EGFR induces receptor homo- or heterodimerization with other ErbB family members or with other extracellular receptors (e.g., insulin-like growth factor-1 receptor). Receptor activation signals key downstream pathways that regulate cell proliferation, differentiation, and survival [84]. EGFR is overexpressed in a variety of malignancies including squamous cell carcinoma of the head and neck (SCCHN), colon cancer, and non-small-cell lung cancer (NSCLC) and is associated with tumor progression and poor prognosis [85, 86].

Different approaches to inhibiting EGFR exist, including monoclonal antibodies and small-molecule EGFR-TK inhibitors. Small-molecule EGFR-TK inhibitors (gefitinib or erlotinib) act by blocking the ATP binding site of the EGFR-TK enzyme inside tumor cells. On the basis of this mechanism of action, EGFR-TK inhibitors have the potential to inhibit all mechanisms of EGFR-TK

activation, including constitutively activating mutations. The targeted agent cetuximab is a chimeric monoclonal antibody directed against the extracellular, ligand-binding domain of EGFR that competes with ligand for receptor binding. Inhibition of the EGFR pathway with anti-EGFR monoclonal antibodies or TK inhibitors has been shown to inhibit tumor growth in a variety of tumor models [87–89].

Three functional polymorphisms in the EGFR gene have been described. The first one is a polymorphic simple sequence repeat (SSR) with variable number of CA dinucleotides located close to an enhancer element in intron 1. The distribution of the number of repeats varies according to the ethnicity. In Caucasian, the distribution is trimodal with the most frequent number of repeat being 16 (40 %), followed by 20 (26 %), and 18 (20 %), the others being relatively rare [90]. It has been shown to influence the gene and protein expression levels of EGFR in a way that longer the CA repeat, the lower the gene and protein EGFR expression is. It was true when the reference was the sum of repeat numbers in both alleles [5] or alternatively the smallest repeat number [6–8]. Moreover, longer CA repeat has been shown to predict poor response to EGFR targeting in various cell lines [5, 91, 92]. However, recently the functionality of the EGFR $(CA)_n$ polymorphism was challenged by Liu et al. who did not find the sum of repeat numbers to be associated with EGFR gene or protein expression in NCI60 cell lines [93]. The second one is an SNP (+497G>A) in exon 13 that leads to amino acid exchange from arginine to lysine in the extracellular domain. Moriai et al. showed that the lysine/lysine (A/A) genotype had less affinity to EGFR ligand binding with consequent attenuated growth response than the arginine/arginine (G/G) genotype [9]. Wang et al. confirmed its functionality by showing a marked decrease in tumor EGFR phosphorylation and c-Myc activation in 36 patients with colon or rectal cancer containing the +497A allele [94]. The third polymorphism is also an SNP (−216G>T) situated in the promoter region at one recognition site of the SP1 transcription factor. Liu et al. showed that the T allele increased promoter activity and EGFR gene and protein expression [10, 93].

2.2.2 Predictive Value for Cetuximab

Klinghammer et al. evaluated the association of two tumor EGFR polymorphisms with skin rash and outcome in 51 patients with recurrent or metastatic SCCHN treated with cetuximab and docetaxel in a phase II multicentric clinical trial. The A containing EGFR +497G>A genotype was associated with a lower incidence of skin rash grade >1 compared with the G/G genotype (25.9 % vs. 58.3 %; $P=0.024$). Furthermore, patients with the A containing EGFR +497G>A genotype showed a trend to shorter PFS (HR = 0.55; 95 % CI 0.28–1.08; $P=0.08$) with no influence on OS. The EGFR $(CA)_n$ polymorphism was not associated with toxicity or outcome when a length of ≤16 CA repeats was used as a cutoff [95]. As there is no commonly accepted predictor of cetuximab efficacy in SCCHN, those results seem interesting. Patients with SCCHN that do not possess A alleles may bind monoclonal antibodies with higher affinity resulting in downstream signaling abolition. However, those results need to be validated in a larger group of patients.

Three studies were reported in patients with metastatic colon or rectal cancer with divergent results. Lurje et al. analyzed 130 patients with metastatic colon or rectal cancer. Those patients were part of a phase II multicenter study (IMC 0144) of third-line treatment with cetuximab. Tumor EGFR +497A/A genotype was associated with a shorter PFS compared to other genotypes (1.2 months vs. 1.3 months in G/G and 1.8 months in G/A; $P=0.017$). The EGFR +497G>A polymorphism remained significant in the multivariate model also adjusted for K-ras status (RR = 3.04; 95 % CI 1.38–6.72; $P=0.01$). In this study, the EGFR $(CA)_n$ polymorphism was not associated with outcome when patients where subdivided into two groups: both alleles <20 CA repeats and any alleles ≥20 CA repeats. Garm Spindler et al. did not find the EGFR +497G>A or −216G>T tumor polymorphisms to be associated with outcome in 71 patients with metastatic colon or rectal cancer treated with third-line cetuximab and irinotecan [96]. The third study published by Graziano et al. showed in 110 patients with

metastatic colon or rectal cancer treated with third-line cetuximab with irinotecan that germline EGFR $(CA)_n$ repeat polymorphism was associated with PFS and OS. Patients with homozygous short (S) allele, defined as less than 17 CA repeats, had a favorable median OS of 13 months compared to 9.8 months in S/L and 5.6 months in L/L carriers ($P=0.01$). Moreover, patients with S/S genotype were more prone to grade 2–3 toxicity compared to L/L carriers (65 % vs. 17 %; $P=0.0017$). EGFR −216G>T and EGFR +497G>A polymorphisms were not associated with outcome or toxicity [54, 55].

The comparison of those studies is difficult because of methodological divergences. Conversely to the two other studies that evaluated a combination of irinotecan and cetuximab, Lurje et al. evaluated patients treated with cetuximab monotherapy; the cutoff for the number of CA repeats defining high and low EGFR expression variants varied between studies; and importantly, all studies did not adjust for K-ras status which has recently been shown to be a strong negative predictive factor for response to monoclonal antibodies targeting EGFR in metastatic colorectal cancer [97–99]. K-ras mutations, commonly at codons 12 and 13, cause constitutive activation of K-ras-associated signaling, and thus EGFR expression level is unlikely to predict response to EGFR-targeted therapies in K-ras mutated patients. Patients with colon and rectal cancers should be studied separately as there are growing data showing molecular differences between rectal and colon cancers [47, 100–102]. Further work is needed, preferably in wild-type K-ras patients.

2.2.3 Predictive Value for Gefitinib-Related Efficacy

Two studies evaluated if EGFR polymorphisms were related with outcome in Caucasian patients with NSCLC treated with gefitinib. Tiseo et al. evaluated prospectively a panel of molecular markers including germline EGFR $(CA)_n$ polymorphism in 91 Caucasian patients with advanced NSCLC treated with gefitinib. They showed that patients bearing at least one $(CA)_{16}$ allele showed a significantly longer survival (12 vs. 4 months; $P=0.044$). EGFR $(CA)_n$ polymorphism was the only molecular marker retained as a strong prognostic factor in the multivariate analysis (HR = 1.95; 95 % CI 1.12–3.7; $P=0.02$). Interestingly, EGFR $(CA)_n$ polymorphism did not predict response to treatment, conversely to EGFR mutation (82 % vs. 10 % in mutated and wild-type EGFR gene; $P<0.001$) and EGFR FISH (50 % vs. 14 % in EGFR FISH positive and negative; $P=0.016$) [103]. Those results were supported by Liu et al. that showed that both EGFR $(CA)_n$ and EGFR −216G>T germline polymorphisms were associated with outcome in 92 patients (vast majority Caucasians) with advanced NSCLC treated with gefitinib. Carrying a −216 T allele was associated with increase response rate ($P=0.03$) and improved PFS (4.1 vs. 2.1 months; $P=0.005$). For the EGFR $(CA)_n$ polymorphism, homozygous S allele, defined as ≤16 CA repeats, was associated with improved PFS (3.8 vs. 2.6 months; $P=0.03$) [104, 105].

Several studies evaluated East Asian patients treated with gefitinib. All reported an association for EGFR $(CA)_n$ polymorphism with outcome, and none showed a similar association with +497G>A polymorphism. Ma et al. reported in 84 Chinese patients with advanced NSCLC treated with second- or third-line gefitinib that germline EGFR $(CA)_n$ polymorphism was associated with response rate. Patients carrying at least one allele < 16 CA repeats had higher response rate (88.5 % vs. 48.3 %; $P<0.001$). However, no significant difference in PFS or OS was observed. They did not notice any association between EGFR +497 G>A polymorphism and outcome [106]. Nie et al. found Chinese patients ($n=70$) with advanced NSCLC given gefitinib with low number of germline EGFR CA repeats (any allele ≤16 CA) more likely to have a better response (57.8 vs. 28 %; $P=0.014$) and longer median survival (20 vs. 11 months; $P=0.039$) compared to patients with high number of EGFR CA repeats (both alleles >16 CA). No significant difference was found between EGFR +497G>A polymorphism and outcome [8]. Han et al. showed in 86 Korean patients with advanced NSCLC cancer treated with gefitinib that low EGFR CA repeats (sum of CA repeats ≤37) were associated with better response (HR = 7.1; 95 % CI 1.2–40.8; $P=0.029$) and time to progression (HR = 0.54;

95 % CI 0.34–0.88; $P=0.14$) [107]. Sasaki et al. did not find germline or tumor EGFR +497 G>A polymorphism to be associated with outcome in 46 Japanese patients with advanced NSCLC treated with gefitinib [108].

In summary, there is good evidence that low CA repeat number is predictive of better outcome in Asian and Caucasian patients with advanced NSCLC treated with gefitinib, despite using different cutoffs for the number of CA repeats defining low EGFR expression variants. As there seem not to be a correlation between EGFR polymorphisms and somatic EGFR mutations [8, 103, 107], which have been shown to predict outcome in patients with advanced NSCLC treated with gefitinib [109], EGFR $(CA)_n$ polymorphism may be an interesting predictive factor especially in Caucasian populations where EGFR mutations and amplification appear to affect a smaller proportion of patients. In that population the attributable role of EGFR polymorphisms may be greater. Germline genotyping is a convenient method, as DNA is extracted from blood. Indeed, it is not always possible to obtain good quality tumor tissue needed for somatic mutation analysis. The predictive value of the EGFR $(CA)_n$ polymorphism needs to be validated in a large study, and the cutoff for low and high expression variants should be standardized.

The EGFR +497G>A polymorphism seems not to be predictive of tyrosine kinase inhibitors efficacy in advanced NSCLC. The EGFR −216 G>T polymorphism may be associated with outcome as suggested by Liu et al. [104, 105]. Confirmation is needed in Caucasian populations, as its frequency in Asian populations is low.

2.2.4 Predictive Value for Gefitinib-Related Toxicity

Liu et al. also accessed if EGFR $(CA)_n$ and EGFR −216G>T germline polymorphisms were associated with toxicity in 92 Caucasian patients with advanced NSCLC treated with gefitinib. Carrying the variant allele was associated with increased risk of diarrhea (OR = 2.63; 95 % CI 1.12–6.17; $P=0.03$) or any grade of either rash or diarrhea (OR = 3.09; 95 % CI 1.42–6.71; $P=0.004$). The EGFR $(CA)_n$ polymorphism was not associated with toxicity [104, 105].

Three studies evaluated toxicity and EGFR polymorphisms in East Asian patients with advanced NSCLC treated with gefitinib and found divergent results. Nie et al. did not find any correlation with germline EGFR $(CA)_n$ or EGFR +497G>A polymorphisms and side effects in 70 Chinese patients [8]. Skin rash (any grade) was not associated with CA repeat status in 86 Korean patients [107]. However, Huang et al. reported that germline EGFR $(CA)_n$ polymorphism was associated with early (within 4 weeks) grade 2–3 skin toxicity in 52 Taiwanese patients with advanced NSCLC treated with first-line gefitinib. Patients with homozygous S allele, defined as 15–18 CA repeats, had higher rate of skin rash than S/L or L/L (71 % vs. 26.7 %; $P=0.031$). However, there was no significance difference in response rate between these three groups, possibly due to the small size of the study. The EGFR +497G>A polymorphism was not associated with skin rash [70, 71].

There seem not to be a reliable relation between toxicity and EGFR polymorphisms in patients treated with gefitinib. It may be related to the small studies' size or to toxicity assessment that varied between studies, with some authors looking at any grade of overall toxicity, any grade of specific toxicities, or ≥ grade 2 specific toxicities (which seem more appropriate). Recently, ≥ grade 2 skin toxicity was shown to correlate strongly with survival in two large phase III studies evaluating tyrosine kinase inhibitors (erlotinib) in advanced NSCLC [110]. Therefore, a proven association between EGFR polymorphisms and ≥ grade 2 skin toxicity would further support their predictive value for outcome.

2.2.5 Prognostic Value

There is no convincing data concerning an eventual prognostic value of EGFR polymorphisms in SCCHN. Etienne-Grimaldi et al. looked at the prognostic value of tumor and germline EGFR $(CA)_n$

repeat polymorphism in 112 patients with locally advanced SCCHN treated with various curative modalities. CA repeat polymorphism did not influence cancer-specific survival or OS, whatever cutoff was used [111]. Bandrès et al. evaluated if tumor EGFR +497 G>A, EGFR $(CA)_n$ repeat, and −216G>T polymorphisms predicted outcome in 78 patients with SCCHN that underwent surgery and/or chemoradiation. None showed a statistical association with disease-specific mortality. However, patients with both alleles containing ≥17 CA repeats tended toward lower disease-specific mortality compared with those with both alleles containing <17 CA repeats (RR = 0.36, 95 % CI 0.12–1.14) without reaching significance ($P = 0.07$) [112]. The small size and lack of significance of the latter study preclude from any conclusion. Further studies should access EGFR polymorphisms' prognostic value in homogenous patient population regarding treatment and stage.

Wang et al. evaluated the prognostic value of germline EGFR +497G>A polymorphism in 209 Chinese patients with colon or rectal cancer (40 % of patients). The high binding affinity EGFR +497 G/G genotype predicted a poor OS in patients with stage II or III disease ($n = 100$; $P = 0.003$) and in patients with metastatic disease ($n = 109$; $P < 0.01$), compared to A/G or A/A genotypes [94]. Those results suggest that EGFR +497G>A polymorphism's prognostic value may be similar in metastatic or non-metastatic setting. However, the inclusion in that study of a large proportion of patients with rectal cancer limits author's conclusions. Press et al. looked for the prognostic value of germline EGFR $(CA)_n$ repeat or EGFR +497G>A polymorphisms in 318 patients with metastatic colon cancer. When population was not separated by gender, both polymorphisms were not associated with OS. However, when the patient population was separated by gender, EGFR +497 G>A polymorphisms had opposite implications on OS based on gender (Pinteraction = 0.003). Male patients with the high binding affinity VEGF +497 G/G genotype ($n = 90$) had shorter OS (median OS = 10.3 months) than male patients with the A/G or the A/A variant ($n = 85$; median OS = 13.7 months). In female patients the opposite OS difference was found. The $(CA)_n$ repeat polymorphism also trended with a gender-related OS difference (Pinteraction = 0.11). The male patients with high expression homozygous <20 $(CA)_n$ repeat genotype ($n = 78$) had a shorter OS (median OS = 10.3 months) compared with males that had any ≥20 $(CA)_n$ repeat allele ($n = 96$; median OS = 13.1 months). In female patients the opposite OS difference was found. The combined analysis of those two polymorphisms showed again opposite implications for OS based on gender (Pinteraction = 0.002) [113]. The exact mechanistic interactions that contribute to those observations are unclear, as is the unexpected survival benefit seen in female patients with the high activity EGFR variants. It has been suggested that sex steroid hormones may play a role. Those data provide support for the predictive value of EGFR polymorphisms in patients treated with cetuximab-based treatments, as patients with high activity EGFR alleles have been suggested to have a better prognosis when treated with monoclonal antibodies targeting EGFR (as discussed previously). However, it needs to be validated in prospective biomarker-embedded trials.

Sasaki et al. looked in 206 Japanese patients with operated NSCLC for an association of tumor EGFR +497 G>A polymorphism and OS. In the entire cohort, EGFR +497 G>A genotype was not associated with outcome. However, in patients with node-positive disease, EGFR wild-type genotype (G/G) had significantly worse prognosis than the patients with G/A or A/A genotypes (RR = 2.4; 95 % CI 1.23–4.69; $P = 0.0072$). EGFR +497 G>A polymorphism did not predict outcome in patients with node-negative cancer [108]. Dubey et al. evaluated the prognostic value tumor EGFR $(CA)_n$ repeat polymorphism in 157 patients with NSCLC enrolled in the ECOG 3590 prospective trial that compared concurrent chemotherapy (cisplatin and etoposide) plus radiation to radiation alone in the adjuvant treatment. Patients with an allele CA sum of ≤35 had a median OS of 29.2 months whereas patients with an allele CA sum of >35 had a median OS of 41 months ($P = 0.03$). When controlling for treatment randomization, the difference in OS was still statistically significant. In that study, OS was identical between groups at different cutoffs for CA repeats (sum of allele ≤35 vs. >35; mean CA repeats ≤18 vs. >18) [114]. That study suggests that patients with low $(CA)_n$ repeats may live longer when treated with EGFR-TK inhibitors compared to patients with high $(CA)_n$ repeats. That hypothesis needs to be validated in prospective biomarker-embedded trials.

2.3 *Vascular Endothelial Growth Factor*

2.3.1 Background

Vascular endothelial growth factor (VEGF or VEGF-A) is the most potent angiogenic factor stimulating endothelial cell proliferation, survival, and vascular maturation. VEGF is highly implicated in the regulation of tumor angiogenesis through a complex interaction with multiple other angiogenic factors and proteases leading to newly formed vasculature with leaky and disorganized vessels. VEGF production can be induced by hypoxia, change in pH, and a variety of other mediators including cytokines. VEGF subsequently binds its receptor (VEGF receptors) and triggers multiple downstream pathways leading to endothelial cell survival, mitogenesis, migration, differentiation, vascular permeability, and mobilization of endothelial progenitor cells from the bone marrow into the peripheral circulation [115]. High circulating VEGF levels have been linked with poor prognosis in various solid tumors [116]. Since many solid tumors express VEGF receptors, VEGF may promote growth via an endothelial cell-independent pathway and may serve as an additional target for therapies [117–119].

Several anti-VEGF strategies have been developed, including antibodies neutralizing VEGF. In preclinical studies, anti-VEGF monoclonal antibodies have been shown to inhibit angiogenesis and growth of human tumor xenografts [120]. Bevacizumab, a humanized monoclonal antibody that binds VEGF, has been shown to be effective in various cancers including colorectal, renal, and lung cancers.

Several VEGF polymorphisms have been shown to be functional. Two polymorphisms situated in the VEGF gene promoter region, VEGF −1154G>A and −2578C>A, have been linked with VEGF production by blood mononuclear cells. Cells with VEGF −1154G/G genotype produced more VEGF than −1154A/A genotype. Similarly, cells with −2578C/C produced more VEGF than −2578A/A genotype [12]. Lambrechts et al. further confirmed VEGF −1154G>A polymorphism functionality. They found that the 1154A allele reduced VEGF transcription [11]. The third evaluated VEGF polymorphism, −634G>C, is situated in 5′ untranslated region. It has been shown to affect VEGF posttranscriptional level, with the −634G allele producing less VEGF compared to the −634C allele [11]. A fourth VEGF polymorphism situated in the 3′ untranslated region, VEGF +936C>T, was associated with VEGF plasma levels in 23 healthy male subjects. Subjects with a T containing VEGF 936C>T genotype had lower VEGF plasma than subjects with VEGF +936C/C genotype [15]. Krippl et al. confirmed those results in 21 nonsmoking postmenopausal women; carriers of a VEGF +936 T allele had significantly lower VEGF plasma levels than noncarriers [14]. A third study also showed in 88 patients with NSCLC that carrying homozygous VEGF +936 T allele predicted a lower serum VEGF level compared with patients homozygous for the +936C allele [13]. However, Awata et al. questioned VEGF 936C>T polymorphisms' functionality as they did not find an association between serum VEGF levels and this polymorphism in 64 healthy subjects [121]. The last VEGF polymorphism being evaluated is VEGF −1498G>A which has been suggested to be associated with increased breast cancer risk, the −1498 C/C genotypes being more common in cancer cases than controls [122, 123]. Its functionality has so far not been demonstrated.

2.3.2 Predictive Value

Schultheis et al. evaluated multiple germline polymorphisms in 53 patients with recurrent ovarian carcinoma treated in a phase II trial with oral cyclophosphamide and bevacizumab. They showed that VEGF +936 C>T polymorphism had a trend for an association with PFS. Patients with C/T genotype had a longer PFS (11.8 months; $P=0.061$) than patients with C/C genotype (5.5 months) or patients with T/T genotype (only 2 patients; 3.2 months). VEGF −634 G>C polymorphism was not associated with outcome.

Schneider et al. have looked in 363 patients with metastatic breast cancer that were enrolled in the phase III ECOG 2100 study evaluating palitaxel or paclitaxel with bevacizumab as initial chemotherapy, if tumor VEGF genotypes could predict outcome or toxicity. They evaluated 180 patients from the experimental arm and 183 patients from the control arm. The low-producing genotype of VEGF −1154G>A and −2578C>A polymorphisms was associated with outcome. Interestingly, those two polymorphisms did not predict response rate or PFS. Moreover, the haplotype analysis showed that patients homozygous for the VEGF −1154A/−2578A haplotype had an OS improvement in the experimental arm compared to all other genotypes ($P=0.041$). There was no statistically significant association between genotypes and VEGF expression in the tumor determined by IHC. However, the authors reported a trend for lower VEGF expression in patients with the −2578A/A genotype ($P=0.08$) or the VEGF −1154A/A genotype ($P=0.13$). In the toxicity analysis, they found that VEGF −634G>C and −1498G>A polymorphisms were associated with grade 3–4 hypertension. The high production VEGF −634C/C genotype ($P=0.005$) correlated with less hypertension when compared with all other genotypes. The −1498 T/T genotype was in same way associated with less hypertension ($P=0.022$) [122, 123].

In summary, low production VEGF −1154G>A and −2578C>A polymorphisms may predict a better outcome in patients with metastatic breast cancer treated with bevacizumab associated with paclitaxel. Those polymorphisms are not correlated with response rate and PFS, despite a doubling of response rate and PFS reported in the experimental arm in the original study. One explanation for this lack of concordance is that those polymorphisms may identify a subpopulation of patients that sustain benefit of short-term VEGF inhibition. Those provocative results need prospective validation. The association of the high production VEGF −634C/C genotype with hypertension is unexpected. Its biologic plausibility seems uncertain as the current explanation for hypertension induced by angiogenic factors involves decreased nitric oxide production by VEGF inhibition [124]. It seems counterintuitive that higher VEGF production is associated with better VEGF inhibition. Furthermore, hypertension was suggested to correlate with outcome in patients treated with anti-angiogenic treatments in various solid tumors [122, 123, 125–127].

2.3.3 Prognostic Value

The prognostic value of VEGF polymorphisms was assessed in different solid tumors, including ovarian and breast cancers. None of the previously discussed VEGF polymorphism alone was found to have a prognostic value in ovarian or in breast cancers [128, 129]. However, Hefler et al. found that simultaneous carriage of three high expression homozygous polymorphisms (VEGF −634C/C, VEGF −1154G/G, and VEGF −2578C/C) was independently associated with shortened OS (HR = 2.1; 95 % CI 1.1–3.9; $P=0.02$) in 563 Caucasian patients with ovarian cancer treated with surgery with the vast majority receiving also platinum-based adjuvant chemotherapy [130].

Most of the prognostic assessment for VEGF polymorphisms was done in gastrointestinal cancers. Four studies evaluated if VEGF +936C>T polymorphism was linked with survival in patients with gastric cancer with divergent results. Two relatively small studies that recruited 130 and 178 patients failed to correlate survival with the VEGF +936C>T polymorphism [131, 132]. In opposite to most publications, a larger study conducted in 503 Korean patients with gastric cancer, who have undergone gastrectomy, found that patients with the low expression +936 T/T genotype had a worse OS compared with the C/C genotype (HR = 3.23; 95 % CI, 1.13–9.25; $P=0.037$) [133]. Unfortunately, those studies did not access the −634G>C, −1154G>A, and −2578C>A polymorphisms. Tzanakis et al. also evaluated the prognostic value of VEGF +936C>T, however including the −634G>C, −1154G>A, and −2578C>A polymorphisms, in 100 Caucasian patients with gastric cancer (all stages). Only the high expression VEGF −634C/C genotype predicted a poor 10-year OS (46.7 %; $P=0.008$) compared to patients with G/C (59 %) or GG (65.5 %) genotypes [134].

View studies have evaluated the prognostic value of VEGF polymorphisms specifically in colon cancer, meaning excluding patients with rectal cancer. Lurje et al. evaluated the prognostic value of germline VEGF +936C>T and −634G>C polymorphisms in two different cohorts of patients, stage II or stage III colorectal cancer. Interestingly, they found a different genomic profile being at risk for stage II or stage III patients with colon cancer. Stage II patients with the high expression VEGF −634C/C homozygous genotype had a median TTR of 3.5 years, compared with 5.9 and 16.8 years in patients that were heterozygous and homozygous for the G allele, respectively ($P=0.028$) [135]. However, in stage III patients, the VEGF +936C>T polymorphisms was associated with outcome. Patients with the high expression VEGF +936 C/C homozygous genotype had a median TTR of 2.6 years compared with 11.1 years in patients heterozygous or homozygous for the T allele ($P=0.003$) [49, 50]. Those results suggest that the patients' angiogenic potential and associate risk for tumor recurrence may be different in lymph node-negative (stage II) and lymph node-positive (stage III) disease. Therefore, the assessment of the individuals risk should be accessed separately in those groups of patients.

In summary, there is good evidence that high expressing VEGF polymorphisms may predict poor outcome in patients with solid tumors. As there is increasing evidence that node-positive and node-negative colon cancer have distinct molecular pattern, prognostic markers should be assessed separately by stage. Current VEGF-targeted therapies seem to benefit more patients with low expressing variants; therefore, further drug development should aim primarily patients with high VEGF expressing variants, reflecting high circulating VEGF and poor prognosis.

2.4 HER2 Polymorphisms

2.4.1 Background

Human epidermal growth factor receptor (HER) 2 is a member of a family of transmembrane receptor tyrosine kinases that include also EGFR (HER1), HER3, and HER4. The HER family is involved in regulating a diverse repertoire of cellular processes that control cell growth, survival, differentiation, and migration. Unlike other HER family members, HER2 has no identified direct ligand and is constitutively active rendering it available for dimerization. It can occur between two different HER receptors (heterodimerization) or between two molecules of the same receptor (homodimerization). Homodimers weakly perpetuate signals compared with heterodimers [136]. The HER2 overexpression by IHC or FISH is found in patients with breast tumors and other tumors, such as gastric, prostate, ovarian, and NSCLC, and predicts poor prognosis [137–141]. HER2 targeting either by targeting the extracellular domain of the receptor with monoclonal antibodies (trastuzumab) or by inhibition of the HER2 tyrosine kinase activity with small molecules (lapatinib) has been shown to be effective in patients with breast cancer or gastric cancer when combined with chemotherapy and is currently used in clinical practice [98, 99, 142, 143].

The HER2 +655A>G polymorphism results in the substitution of isoleucine to valine in the transmembrane domain of the receptor, a region known to have an active role in the dimerization and activation of the HER2 protein. Fleishman et al. suggested, using a computational exploration of conformation space of the transmembrane segment of a HER2 homodimer, that the Ile variant (bulkier than the Val variant) destabilizes the formation of active HER2 dimers resulting in reduced protein kinase activity, even under conditions of HER2 overexpression [16]. Beauclair et al. confirmed its functionality in vitro and in vivo. The number of HER2/Val cells was greater than HER2/Ile cells when grown in absence of growth factors. They further showed that only HER2/Val-expressing cells develop tumors in nude mice. In their in vitro model, HER2/Val cells were more sensitive than HER2/Ile cells to trastuzumab [144]. Interestingly Papadopoulou et al. have also associated the Val allele

with HER2 overexpression [145] and with the truncated product of the receptor measured in the serum [146]. However, other authors have not confirmed a significant association of Val alleles with HER2 overexpression [147, 148].

2.4.2 Predictive Value

Beauclair et al. evaluated the predictive value of germline HER2 +655A>G polymorphism for outcome and toxicity in 61 patients with advanced breast cancer treated with first-line combination of trastuzumab with paclitaxel (87 %) or second-line or higher combination of trastuzumab with docetaxel (13 %). No significant link was shown between HER2 genotype and response- or disease-free survival. However, the HER2 +655A>G polymorphism was associated with cardiac toxicity. All cases were found in patients with Ile/Val (A/G) genotype (five patients had 20 % or more of left ventricular ejection fraction reduction), and none occurred in patients with Ile/Ile (A/A) genotype ($P=0058$) [144]. Those data suggest that cardiomyocytes containing the Val allele are particularly dependant on HER2 signaling and highly sensitive to trastuzumab. This observation warrants confirmation in a large prospective study.

2.4.3 Prognostic Value

Limited data are available for the prognostic value of HER2 polymorphisms in solid tumors. Papadopoulou et al. suggested in 56 Caucasian patients with breast cancer (all stages) that the presence of homozygous germline HER2 +655G allele was an independent poor prognostic factor of overall survival (HR=9.2; 95 % CI 1.1–80.1; $P=0.045$). Patients with the A/A genotype had a mean OS of 48 months compared to 34 months in patients with the G/G genotype ($P=0.011$) [146]. Their results suggest that the HER2 +655G allele predicts a more aggressive phenotype, consistent with preclinical studies. However, the small size of studied population, the inclusion of patients with metastatic disease, and the lack of treatment information limit the conclusions from that study. The prognostic value of HER2 +655A>G polymorphism should be confirmed in patients without HER2 overexpression or amplification, as those patients are currently not offered HER2-targeted treatments.

Pinto et al. evaluated the prognostic value of germline HER2 +655A>G polymorphism in 129 Caucasian patients with ovarian cancer (all stages) treated with paclitaxel and cisplatin. They reported that patients carrying the G/G genotype presented a lower mean survival (35.3 vs. 75.1 months; $P=0.002$ and PFS 14.2 vs. 75.3 months; $P=0.025$) than other patients. This study suggests that the G/G may predict resistance to cisplatin-based treatments in patients with ovarian cancer. Validation of those results is needed in a large clinical trial.

3 Polymorphisms in Genes of Drug Metabolism

3.1 MTHFR Polymorphisms

3.1.1 Background

The 5,10-methylenetetrahydrofolate reductase (MTHFR) gene located on chromosome 1 encodes for a key enzyme in folate metabolism that catalyzes the conversion of intracellular 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) to 5-methyltetrahydrofolate (5-methylTHF), the predominant circulatory form of folate. The substrate 5,10-methyleneTHF is required for DNA

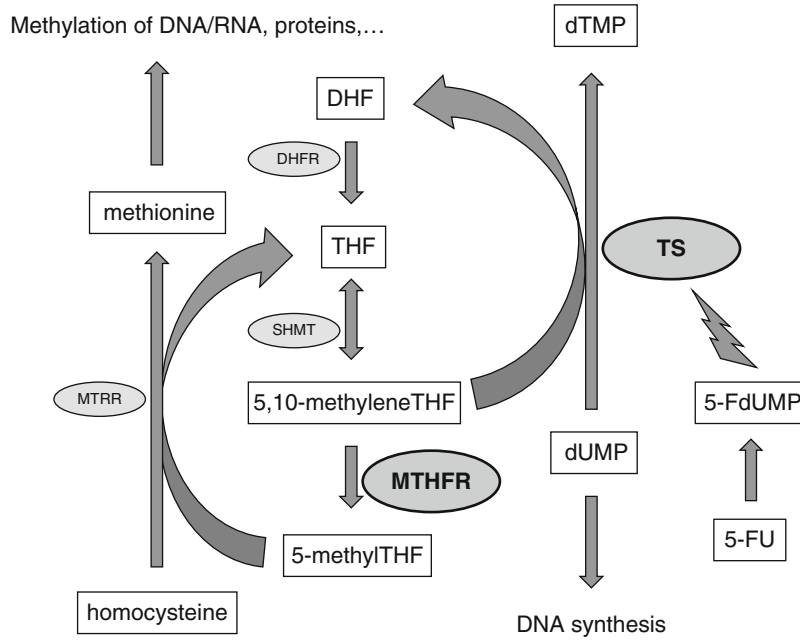

Fig. 2 Simplified view of the folate pathway. The 5,10-methylenetetrahydrofolate reductase (MTHFR) catalyzes the conversion of 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) to 5-methyltetrahydrofolate (5-methylTHF). MTHFR enzyme level or activity may influence cell chemosensitivity to 5-FU as the dissociation of 5-FdUMP, the active 5-FU metabolite, from the ternary complex with TS and 5,10-methyleneTHF is suppressed when levels of 5,10-methyleneTHF are increased. DHF, dihydrofolate; DHFR, dihydrofolate reductase; dTMP, deoxythymidine 5′-monophosphate; dUMP, deoxyuridine 5′-monophosphate; MTRR, methionine synthase reductase; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate; TS, thymidylate synthase; 5-FdUMP, 5-fluoro-2deoxyuridine-5′-monophosphate; 5-FU, 5-fluorouracil

synthesis and for maintaining the balance of the nucleotide pool, whereas 5-methylTHF is required for methylation reactions, including the methylation of homocysteine to methionine and the maintenance of DNA methylation patterns.

Directly linked to the 5-FU-mediated inhibition of TS is the presence of intracellular folate, because 5-FU activity is dependent on a competitive interaction with folate metabolism. It has been shown that the dissociation of 5-FdUMP, the active 5-FU metabolite, from the ternary complex with TS and 5,10-methyleneTHF is suppressed when levels of 5,10-methyleneTHF are increased [149]. As mentioned previously MTHFR catalyzes the conversion of 5,10-methyleneTHF to 5-methylTHF. Therefore, cellular MTHFR enzyme level or activity may influence the chemosensitivity of these cells to 5-FU (Fig. 2).

A common 677C>T transition in exon 4 (within the N-terminal catalytic domain) of the MTHFR gene results in alanine to valine substitution. The presence of the T allele has been shown in vitro to correlate with reduced enzymatic activity and thermolability [17]. Sohn et al. have shown that cells expressing the MTHFR 677 T allele had an accelerated growth compared to the cells with the wild allele. This observation is consistent with the accumulation of 5,10-methyleneTHF and a consequent increase in thymidylate and purine biosynthesis. The authors have further showed, in colon and breast cancer models, that mutant MTHFR 677 T allele increased the chemosensitivity to 5-FU [150]. The second polymorphism concerns a codon 1298 A>C transition in exon 7 (within the C-terminal regulatory domain) resulting in glutamic acid to alanine substitution. The variant is also associated with decreased enzymatic activity [18].

3.1.2 Colorectal Cancer

The predictive value for fluoropyrimidine-related toxicity was largely evaluated. Capitain et al. suggested that germline MTHFR 1298C/C genotype was associated with toxicity (definition not specified) compared to patients with A/A genotype (OR = 25.99; 95 % CI 1.76–384.32; $P = 0.018$) in 75 Caucasian patients with metastatic colorectal cancer treated with infusion 5-FU regimens. MTHFR 677C > T was not related to toxicity [33]. Gusella et al. showed that germline MTHFR 677 C/C genotype was associated with a reduced risk of grade 3–4 side effects versus the C/T genotype (OR = 3.10, 95 % CI 1.21–7.94, $P = 0.040$) in 130 Caucasian patients with metastatic colorectal cancer treated with adjuvant bolus 5-FU (Mayo schedule). MTHFR 1298A > C polymorphism was not related with toxicity [39]. In contrast to those results, Afzal et al. showed in a larger study that included 331 Caucasian patients treated with adjuvant bolus 5-FU (Mayo schedule) that the tumor MTHFR 677C/C genotype was related with an increased risk of grade 3–4 toxicity (OR = 1.83, 95 % CI 1.10–3.03, $P = 0.01$). A subgroup analysis further showed that this association was only significant with grade 3–4 diarrhea (OR = 1.99, 95 % CI 1.07–3.69, $P = 0.03$). The MTHFR 1298A > C polymorphism was not associated with any toxicity [151]. Tumor genotyping in this last study is unlikely to be responsible for those divergent results, as LOH has not been reported to impact MTHFR polymorphisms. Currently, mainly infusion 5-FU regimens are used in clinical practice limiting the value of those previous studies, as the toxicity incidence varies between bolus and infusion 5-FU schedules. In fact, predictive factors for bolus 5-FU regimen's toxicity are unlikely to be useful for 5-FU infusion regimens, as illustrated by Schwab et al. who did not find MTHFR polymorphisms to be associated with grade 3–4 toxicity in 683 patients treated with 5-FU (87 % of colorectal cancer; half of patients received infusion regimens) [32].

Only one small study evaluated patients treated with capecitabine. Sharma et al. looked in 54 patients with colorectal cancer treated with capecitabine monotherapy and reported that patients with the MTHFR 677 T/T genotype had a trend for lower incidence of grade 2/3 toxicity than patients with C/T and C/C genotypes (OR = 0.1; 95 % CI 0.01–1.0; $P < 0.05$). Patients with the MTHFR 1298A/A genotype suffered less grade 2–3 toxicity than patients with A/C and C/C genotypes (OR, 5.6; 95 % CI 1.73–17.98; $P < 0.01$). In addition, the haplotype analysis showed that the majority of the patients with one or two C–C haplotypes experienced grade 2/3 toxicity whereas considerably less patients with no C-C haplotypes experienced grade 2/3 toxicity (72 vs. 32 %; OR = 6.8; 95 % CI 2.05–22.27; $P < 0.01$) [152]. Those results need to be validated in a large clinical trial.

Ruzzo et al. looked in patients with colorectal cancer treated with 5-FU with oxaliplatin (166 patients treated with FOLFOX-4 regimen) [35] or with 5-FU and irinotecan (146 patients treated with FOLFIRI regimen) [36] and did not find any association of germline MTHFR polymorphisms with toxicity. In summary, there is no convincing data supporting MTHFR genotyping for drug dosage adaptation in patients treated with infusion 5-FU regimens with or without oxaliplatin or irinotecan.

Four studies looked if MTHFR polymorphisms could be associated with outcome in patients with stage II or III colon or rectal cancer treated with 5-FU-based chemotherapy. None have found MTHFR 677C > T or 1298A > C polymorphisms to be predictive of outcome [39, 49, 50, 153]. The largest study included 331 Caucasian patients with stage II or III colorectal cancer and 37 patients with a singular excised liver metastasis from colorectal cancer. All patients were treated with adjuvant bolus 5-FU (Mayo schedule). Tumor MTHFR polymorphisms were not associated with OS or relapse-free survival [151]. In consequence, there is currently no evidence that MTHFR polymorphisms may have a predictive value for stage II or III colorectal cancer patients treated with adjuvant 5-FU-based chemotherapy.

Several publications found that MTHFR polymorphisms could be associated with outcome in patients with metastatic colorectal cancer treated with fluoropyrimidines. Cohen et al. suggested in a

small study of 43 patients with metastatic colon or rectal cancer treated with various fluoropyrimidines that patients with germline MTHFR 677 T allele had better response rate (OR = 2.86; 95 % CI 1.06–7.73; $P = 0.035$) [154]. Jacobsen et al. also showed in 88 Caucasian patients with metastatic colorectal cancer treated with bolus 5-FU that the MTHFR 677 T/T genotype was linked with a higher response than the other genotypes combined (66 % vs. 27 %; $P = 0.04$). MTHFR 1298A > C polymorphism was not associated with response [56]. Etienne et al. also reported in 98 patients with metastatic colon or rectal cancer treated with infusion 5-FU regimens that patients with tumor MTHFR 677 T/T genotypes had higher response (56 %) compared to patients with 677C/C genotypes (40 %), with an odds ratio of 1.88 ($P = 0.04$). However, it did not influence cancer-specific survival. Interestingly, the authors suggested a prognostic value for the 1298A > C polymorphism. The MTHFR 1298C/C genotype was predictive of poor cancer-specific survival compared to the MTHFR 1298A/A genotype (RR = 2.05; 95 % CI 1.06–3.98; $P = 0.028$). MTHFR 1298A > C genotype was not associated with response rate [155]. Zhang et al. further reported in a larger study, including 318 patients with metastatic colon cancer treated with sequential 5-FU-based chemotherapies (large majority received oxaliplatin and irinotecan), that germline MTHFR 1298A > C and 677C > T polymorphisms were not associated with OS, when the population was not separated by sex. However, among females, patients with the MTHFR 1298C/C genotype (RR = 1.67; 95 % CI 0.78–3.6) and those with the A/C genotype (RR = 1.53; 95 % CI 1.01–2.3) had a greater risk of dying than noncarriers of the A allele [156].

Other relatively small studies did not find germline MTHFR polymorphisms to be associated with response or survival in colorectal cancer patients treated with 5-FU-based chemotherapies [33, 157, 158] or capecitabine alone [152]. Two relatively large studies published by the same group did also not find germline MTHFR polymorphisms to be associated with response or PFS in patients with colorectal cancer treated with 5-FU with oxaliplatin (166 patients treated with FOLFOX-4 regimen) [35] or with 5-FU and irinotecan (146 patients treated with FOLFIRI regimen) [36].

In summary, there are limited data suggesting that MTHFR 677C > T may predict response in patients with metastatic colorectal cancer treated with fluoropyrimidines alone, with a less clear repercussion on survival (PFS or OS). This association has not been reported in patients treated with combination regimen (oxaliplatin or irinotecan) suggesting a limited utility in clinical practice. However, early conclusions should not be made as most studies were small, included patients with metastatic colon or rectal cancer, and had given heterogeneous chemotherapy regimen with or without leucovorin modulation. MTHFR 1298A > C polymorphism seems to predict a gender-specific poor prognosis, most probably independently of 5-FU-based treatments, although definitive conclusion cannot be made without a controlled arm. Validation of the poor prognostic value of the MTHFR 1298C allele in females is needed in prospective clinical trials.

3.1.3 Rectal Cancer

A single study examined if MTHFR polymorphisms could affect outcome in patients treated with preoperative chemoradiation. Terrazzino et al. looked in125 patients with rectal cancer treated with preoperative 5-FU-based chemoradiation (35 % of patients received also platinum). MTHFR 677C/C homozygotes had higher response rate than carriers of the T allele. The percentage of responders was 57 and 34 %, respectively (OR = 0.32, 95 % CI 0.14–0.71, $P < 0.006$). MTHFR 1298A > C polymorphism was not associated with response. Haplotype analysis showed that patients with 677 T–1298A haplotype had a lower tumor regression rate compared with patients without this haplotype (OR = 3.85; 95 % CI 1.41–6.75, $P = 0.006$) [64]. It should be noticed that the authors have defined responders as no viable tumor cells (pCR) or presence of rare scattered residual cells, explaining the high response rate. Unfortunately, as tumor regression is not currently a validated surrogate for PFS or OS, it is difficult to predict any clinical usefulness for MTHFR 677C > T polymorphism.

3.1.4 Gastric Cancer

Huang et al. investigated the association of MTHFR 677C>T germline polymorphism with OS in 116 Chinese patients with gastric cancer treated with surgery and adjuvant 5-FU-based chemotherapy (88 % also were treated with concomitant oxaliplatin). They reported that patients with the 677C/C MTHFR genotype had a worse relapse-free survival (19.9 vs. 31.8 months; $P=0.043$) and OS (24.5 vs. 52.0 months; $P=0.040$) than patients with the T/T or C/T genotype. Those results remained significant in the multivariate analysis for relapse-free survival (HR = 1.72; 95 % CI 1.05–2.83; $P=0.031$) but not for OS (HR = 1.68; 95 % CI 0.99–2.86; $P=0.056$) [83]. However, in a preceding study, Ott et al. did not find any association of MTHFR 677C>T germline polymorphism with tumor-related survival in 135 Caucasian patients with locally advanced gastric cancer treated with preoperative 5-FU- and cisplatin-based chemotherapy [68] The different treatment settings (adjuvant vs. neoadjuvant), ethnicities (Asian vs. Caucasian), and concomitant drugs (cisplatin vs. oxaliplatin) make it difficult to compare those studies. The predictive value of 677C>T MTHFR should be further accessed in the perioperative setting in large clinical trials.

Interestingly, none of the two gastric cancer trials in the metastatic setting that evaluated an association between MTHFR 677C>T or MTHFR 1298A>C germline polymorphisms and outcome in Caucasian patients treated with 5-FU and cisplatin or oxaliplatin revealed an association with PFS, OS, or toxicity [73, 74].

3.1.5 Breast Cancer

There is currently very little data supporting a predictive value for of MTHFR polymorphisms in patients with breast cancer treated with fluoropyrimidines. Huang et al. investigated 192 Taiwanese patients with operated stage II or III breast cancer treated with a combination of adjuvant cyclophosphamide, epirubicin, and 5-FU (FEC). In univariate analysis, patients with the germline MTHFR 677C/C genotype had higher risk of early relapse, defined as recurrent or distant metastatic lesion within 2 years after receiving adjuvant chemotherapy, than patients with the C/T and T/T genotypes (27.7 vs. 10.4 and 15.4 %; $P=0.02$). Unfortunately, the multivariate analysis was not reported nor was the MTHFR 1298A>C polymorphism analyzed [83]. Largillier et al. evaluated prospectively the effect of germline MTHFR 677C>T and MTHFR 1298A>C polymorphisms on toxicity and efficacy in a phase II study with 105 advanced breast cancer Caucasian patients treated with capecitabine monotherapy. None of the MTHFR polymorphisms were related to efficacy or toxicity [40].

3.2 Dihydropyrimidine Dehydrogenase

3.2.1 Background

Dihydropyrimidine dehydrogenase (DPD) is the initial and rate-limiting enzyme in the catabolism of the pyrimidine bases uracil and thymine, but also of the pyrimidine analogue 5-FU. In fact, more than 80 % of the administered 5-FU is catabolized by DPD, mainly in the liver where DPD is abundantly expressed [159]. Monitoring DPD activity in peripheral blood mononuclear cells that correlate well with the DPD activity in the liver to predict 5-FU activity in tumor cells was inconclusive since modest correlation exists between DPD activity in normal and tumor cells [160]. In contrast, there is more evidence that low systemic DPD activity is associated with an increase risk of development of severe 5-FU-associated toxicities [161, 162]. However, not all publications found a significant association,

and 5-FU appears to be well supported in some patients with low DPD activity in mononuclear cells in peripheral blood [163].

Several DPD gene mutations or polymorphisms have been associated with 5-FU-related toxicity. The most frequent and well characterized is a G to A substitution within the 5′-splicing donor site of intron 14 (IVS14+1G>A). Processing of pre-mRNA bearing this substitution results in a loss of exon 14, immediately upstream of the polymorphic site. The resulting truncated protein product has a virtually absent catalytic activity [19]. As its allele frequency is just under 1 % in most Caucasian series, it is usually considered more as a mutation than as a polymorphism [164].

3.2.2 Predictive Value for 5-FU-Related Toxicity

Raida et al. looked in 25 patients that suffered grade 3–4 toxicity during bolus 5-FU treatment. One patient (4 %) was homozygous and 5 patients (20 %) were heterozygous for the IVS14+1G>A mutation [165]. Van Kuilenburg et al. evaluated 60 patients (mainly gastrointestinal cancers) who experienced grade 3–4 toxicities after the administration of 5-FU (regimen not specified). Sixteen patients (27 %) were heterozygous and one patient (2 %) was homozygous for the mutation ($P<0.001$ compared to allele frequency in the normal population) [166, 167]. Largillier et al. looked in 105 patients with advanced breast cancer treated with capecitabine and reported that the sole patient bearing the germline IVS14+1G>A mutation (heterozygous) deceased from hematologic toxicity [40]. Those preliminary data suggesting that carriers of the IVS14+1G>A mutation may be at risk of developing severe toxicity after the administration of 5-FU conducted to further investigations in larger trials.

Morel et al. conducted a prospective trial to determine the clinical relevance of 22 various DPD gene mutations. They included 487 patients with colorectal, head and neck, or breast cancer treated with 5 different types of infusion 5-FU-based regimens (59.2 % had also cisplatin, oxaliplatin, or irinotecan). They showed that 6 patients of the 10 (60 %) with germline IVS14+1G>A mutation had a grade 3–4 toxicity. They further reported that 6 patients of 10 (60 %) with a 2846A>T mutation experienced grade 3–4 toxicity. This mutation was previously shown to lead to an amino acid exchange (aspartate for a valine), interfering with cofactor binding or electron transport when analyzing the three-dimensional protein structure [166, 167]. There was a significant difference when patients with either of those two mutations (and a third rare 1679 T>G mutation) were compared to patients without a mutation (20 patients of 300 had grade 3–4 toxicity; $P=10^{-6}$). The 5-FU pharmacokinetics showed that these mutations were linked with significantly reduced median plasma clearance compared to patients without mutations (66.2 vs. 123.9 Lh/m^2; $P<0.001$) [168]. Schwab et al. also investigated prospectively the predictive value of germline DPD gene mutations for any grade toxicity in 683 patients treated with 5-FU monotherapy (87 % of patients had colorectal cancer; half of patients received infusion 5-FU). The IVS14+1A allele was associated with overall toxicity (OR=4.67; 95 % CI 1.54–14.2; $P=0.01$), with mucositis (OR=5.8; 95 % CI 1.71–19.4; $P=0.013$), and with leukopenia (OR=10.19; 95 % CI 3.0–35.1; $P=0.002$). Interestingly, they reported that the effect of IVS14+1A allele was strongly related with sex. It was associated with an independent increase in toxicity only in men (OR=41.8; 95 % CI 9.2–190; $P=0.0001$), but not in women (OR=1.33; 95 % CI 0.34–5.16; $P=0.68$). Recently, a third study published by Braun et al. did not find a significant association of germline (25 % of samples were extracted from the tumor) IVS14+1G>A mutation with delay and/or dose reduction within 12 weeks of starting treatment nor with any grade ≥3 toxicity event during the first 12 weeks of treatment. This study involved 1,188 patients with colorectal cancer treated with 4 different regimens: 5-FU monotherapy, 5-FU with irinotecan, 5-FU with oxaliplatin, or irinotecan monotherapy. No evidence for an association was found for any of the mentioned regimens [34]. Those negative results may partially be explained by the low observed genotype frequency (0.8 % of heterozygous, no homozygous) observed in the 629 successfully genotyped samples when compared to 1.8 and 1.9 % of heterozygous in the two previously discussed studies. Nevertheless, the two larger

positive studies showed that the sensitivity for the IVS14+1G>A mutation to detect grade 3–4 5-FU-related toxicity is low, between 5 % and 14 % with a positive predictive value between 50 and 60 %. In conclusion, the screening for DPD IVS14+1G>A mutation is currently not recommended because of its limited clinical impact. Further investigation is needed to confirm the IVS14+1G>A mutation/sex interaction.

3.3 Uridine Diphosphate Glucuronosyltransferase 1A1

3.3.1 Background

Irinotecan is metabolized by carboxylesterases to the active metabolite SN-38, which is 100–1,000-fold more potent than irinotecan as a topoisomerase I inhibitor. SN-38 is eliminated predominantly by the glucuronidation to SN-38G. This reaction is mediated primarily by Uridine Diphosphate Glucuronosyltransferase 1A1 (UGT1A1) polypeptide encoded by the UGT1A gene. This gene encodes several isoforms (9 identified isoforms: UGT1A1, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10) resulting from alternative splicing of unique alternate 13 first exons [169]. The most frequent investigated UGT1A1 polymorphism is a two-base pair insertion in the TATA box in the promoter region of the gene, resulting in a $(TA)_7TAA$ sequence. The variant allele is commonly designed as UGT1A1*28, with the wild-type $(TA)_6TAA$ allele being named UGT1A1*1 [170]. The variant allele has been consistently associated with reduced enzymatic activity, resulting in lower SN-38 glucuronidation [20, 21].

3.3.2 Predictive Value for Irinotecan-Related Toxicity

Tremendous work has been done to access the relationship between the incidence of irinotecan-induced grade III–IV hematologic toxicity in cancer patients and UGT1A1*28 polymorphism. Initial studies reported an increase risk of irinotecan-induced hematologic toxicities in patients who were homozygous for the UGT1A1*28 allele [171–174]. In 2005, this association led the US Food and Drug Administration (FDA) to advise the irinotecan manufacturer (Pfizer Pharmaceutical) to include this association in the product information and to recommend a lower starting dose for patients with homozygous UGT1A1*28 genotype. However, subsequent studies did not consistently report an association between toxicity and UGT1A1*28/*28 genotype. In fact, many patients with this genotype did not experience severe toxicity. One review [175] and a meta-analysis [176] tried to clarify the impact of UGT1A1*28 polymorphism in patients treated with irinotecan. Palomaki et al. concluded that the risk (RR) of hematologic toxicity, more specifically grade III–IV neutropenia, increased with the number of UGT1A1*28 allele, being 1.82 (95 % CI 1.16–2.85) in patients heterozygous for UGT1A1*28 allele and 3.51 (95 % CI 2.03–6.07) in patients homozygous for UGT1A1*28 allele [175]. Hoskins et al. investigated the relation between irinotecan dose and hematologic toxicity. They showed that when dose was used as a continuous variable, the risk of toxicity increased as irinotecan dose increased between patients with UGT1A1*28/*28 genotype and those with UGT1A1*1/*28 or UGT1A1*1/*1 genotypes ($P=0.028$). When irinotecan dose was used as a categorical variable, the risk was only higher in the medium (150–250 mg/m^2; OR = 3.22; 95 % CI 1.52–6.81; $P=0.008$) or high dose group (>250 mg/m^2; OR = 27.8; 95 % CI 4–195; $P=0.005$). In contrast, at low irinotecan doses (<150 mg/m^2), the risk was not statistically significant (OR = 1.8; 95 % CI 0.37–8.84; $P=0.41$) [176]. None of those studies reported a significant increase in gastrointestinal toxicity.

Since those two publications, several studies looking at the association between hematologic toxicity and UGT1A1*28 polymorphism were published (Table 5). Most of those studies confirmed an

Table 5 UGT1A1*28 polymorphism and hematologic toxicity

First author	Irinotecan dose (mg/m2)	Other drugs	Trial type	Cancer type	Toxicity (grade III–IV)	No. of patients	UGT1A1 *28/*28 patients (%)	Toxicity (%) in UGT1A1 patients				*P* value
								*28/*28	*1/*28	*1/*1	*28/*28 or *1/*28	
Kweekel et al. [177]	250 350	Capecitabine None	Phase III CAIRO1 trial, retrospective analysis	Metastatic colorectal	Febrile neutropenia	138 80	11 (8) 3 (3.8)	18.2 (2/11) 0	6.5 (4/62) 19.4 (6/30)	1.5 (1/65) 2.2 (1/46)		0.031 0.015
Braun et al. [34]	350 180	None 5-FU	Phase III FOCUS trial, retrospective analysis	Metastatic colorectal	Neutropenia	115 208	11 (9.6) 18 (8.6)	9 11		5 11		0.293 0.717
Ruzzo et al. [36]	180	5-FU	Prospective trial and analysis	Metastatic colorectal	Neutropenia	146	15 (10)	80 (12/15)		15.3 (9/59)		<0.05
Côté et al. [178]	180	5-FU	Phase III Accord02/ FFCD9802, retrospective analysis	Stage III colon	Hematologic Neutropenia	93	8 (9)	50 (4/8) 50 (4/8)		16.2 (6/37) 13.5 (5/37)		0.06 0.035
Liu et al. [104]	180	5-FU	Retrospective	Metastatic colorectal	Neutropenia	128	6 (4.7)			4.9 (5/102)	53.8 (14/26)	<0.01
Schulz et al. [179]	80	5-FU or oxaliplatin	Phase III FIRE-trial, retrospective analysis	Metastatic colorectal	Neutropenia	105	10 (9.5)			1 (0.9)	6 (3.1)	>0.05
Han et al. [180]	80 (76 %) or 65 (24 %)	Cisplatin	Phase II studies, retrospective analysis	Advanced NSCLC	Neutropenia	107	0		29 (4/14)	24 (22/93)		0.741

association between grade III–IV neutropenia and UGT1A1*28 polymorphism. However, Braun et al. did not find an association of UGT1A1*28 polymorphism with the incidence of toxicity-induced dose delay and/or reduction (primary endpoint) or with any grade ≥ III toxicity (secondary endpoint) within 12 weeks of starting irinotecan-based treatment in a planned analysis of 618 metastatic colorectal patients enrolled in a phase III trial (FOCUS). They further could not find an association with grade 3–4 neutropenia in an unplanned analysis of 323 patients [34]. Both studies that investigated the association of neutropenia with UGT1A1*28 polymorphism in patients treated with low-dose irinotecan (80 mg/m^2) were negative [179, 180]. However, one of them is difficult to interpret because it was conducted in East Asian patients, known to have a lower UGT1A1*28 allele incidence than Caucasian patients (0 patient with homozygous UGT1A1*28 genotype in that study). This study is also the sole recent trial that reported an association of UGT1A1*28 polymorphism with gastrointestinal toxicity (grade III diarrhea) in patients treated with irinotecan [180] (Table 6).

In summary, there is good evidence that the risk of grade III–IV neutropenia is increased in patients bearing a UGT1A1*28 allele, particularly in patients with the homozygous UGT1A1*28 genotype. There is most likely an irinotecan-dose relation, with higher risk of neutropenia in patients treated with doses ≥150 mg/m^2. However, its clinical relevance is still unclear, as very view studies have looked at the risk of febrile neutropenia (which is a more relevant clinical outcome), explaining why it is currently not a worldwide-recognized screening test. It has also been showed that the risk of neutropenia is associated with a number of clinical factors. Roth et al. presented at 2008 ASCO meeting a retrospective analysis of the phase III PETACC-3 trial looking at predictors of toxicity in patients treated with adjuvant 5-FU and irinotecan combination. They confirmed in the multivariate analysis that UGT1A1*28/*28 genotype was significantly associated with grade IV neutropenia (OR = 2.5; 95 % CI 1.3–4.9) as well as other previously documented risk factors: female sex (OR = 2.6; 95 % CI 1.4–4.6), performance status >1 (OR = 2; 95 % CI 1.1–3.7), and age < 60 years (OR = 0.6; 95 % CI 0.3–0.9) [181]. Therefore, UGT1A1*28 testing should be considered in patients with clinical risk factors before initiating irinotecan-based chemotherapies. A dose reduction (precise dose unknown) might then be offered to patients with UGT1A1*28/*28 genotype. There is little evidence that the UGT1A1*28 polymorphism predicts other toxicities, including gastrointestinal toxicities.

3.3.3 Predictive Value for Irinotecan-Related Efficacy

Palomaki et al. also evaluated in their review the association of UGT1A1 polymorphism with tumor response or with survival. They reported that patients with heterozygous UGT1A1*28 genotype had a similar response rate (RR = 1.09; 95 % CI 0.83–1.43) than patients with UGT1A1*1/*1 genotype. However, patients with homogeneous UGT1A1*28 genotype had a higher response rate (RR = 1.7; 95 % CI 1.24–2.33; $P < 0.001$) [175]. This analysis was based on only three studies totalizing 364 patients. In none of the studies did the increase in response rate translate in a significant increase in OS. It should be noted that in the largest study involving 250 patients with metastatic colorectal cancer, the increase in response rate in patients with UGT1A1*28/*28 genotype translated to a significantly longer TTP (HR = 0.52; 95 % CI 0.31–0.9) compared to patients with the homozygous wild-type genotype [182].

Since this review, several studies evaluated if UGT1A1 polymorphism could predict higher response rate, longer PFS (or TTP) and OS. None found a significant association with any of those outcomes [36, 104, 105, 177–179, 183]. Liu et al. further noted that the percentage of patients who required an irinotecan dose reduction was greater in patients who had a variant allele (42.3 vs. 12.7 %; $P < 0.01$), without affecting significantly response or DFS. Interestingly, all studies have not reported a lower irinotecan exposure in patients carrying the UGT1A1*28 allele [177, 179]. Nevertheless, the hypothesis that patient homozygous for the wild-type allele may in fact have insufficient irinotecan exposure has gained interest. Toffoli et al. recently reported in a genotype-driven phase I study that

Table 6 UGT1A1*28 polymorphism and gastrointestinal toxicity

First author	Irinotecan dose (mg/m2)	Other drugs	Trial type	Cancer type	Toxicity (grade III–IV)	No. of patients	UGT1A1 *28/*28 patients	Toxicity (%) in UGT1A1 patients				*P* value
								*28/*28	*1/*28	*1/*1	*28/*28 or *1/*28	
Kweekel et al. [177]	250	Capecitabine	Phase III CAIRO1 trial, retrospective analysis	Metastatic colorectal	diarrhea	138	11 (8)	36.4 (4/11)	22.6 (14/62)	21.5 (14/65)		0.43
	350	None				80	3 (3.8)	66.7 (2/3)	22.6 (7/31)	15.2 (7/46)		0.09
Braun et al. [34]	350	None	Phase III Focus trial, retrospective analysis	Metastatic colorectal	Diarrhea	115	11 (9.6)	9		8		0.763
	180	5-FU				208	18 (8.6)	11		4		0.311
Côté [178]	180	5-FU	Phase III Accord02/ FFCD9802, retrospective analysis	Stage III colon	Gastrointestinal	93	8 (9)	25 (2/8)		16.2 (6/37)		0.31
Liu et al. [104]	180	5-FU	Retrospective	Metastatic colorectal	Diarrhea	128	6 (4.7)			5.9 (6/102)	26.9 (7/26)	<0.01
Han et al. [180]	80 (76 %) or 65 (24 %)	Cisplatin	Phase II studies, retrospective analysis	Advanced NSCLC	Diarrhea (grade III)	107	0		7 (1/14)	11 (10/93)		1.0

patients with metastatic colorectal cancer bearing the UGT1A1*1/*1 or *1/*28 genotype could tolerate considerably higher irinotecan dose, respectively, 370 mg/m^2 and 310 mg/m^2, when combined with 5-FU (FOLFIRI regimen) than the current recommended irinotecan dose of 180 mg/m^2 [184]. Further studies are needed to evaluate if this dose increase can translate in a clinical benefit as well as to confirm its safety.

4 Conclusion

The ability of drug targets and metabolism polymorphisms to predict outcome may vary between tumors, stage, and ethnicity. In advanced gastric cancer, TS 3′-UTR polymorphism predicts outcome in East Asian patients, but in Caucasians, TSER polymorphisms seem to play a predominant role. When patients with rectum cancer are excluded from studies looking at TS polymorphisms' predictive value for 5-FU adjuvant treatment, TSER polymorphisms are significantly related with outcome. In stage II and III colon cancer, different VEGF polymorphisms are associated with outcome. Those observations highlight the necessity to study homogeneous populations, as they are emerging data suggesting that tumors without metastasis, with lymph node metastasis, or distant metastasis may not have the same molecular pattern and possibly not the same genetic background. In contrast, assessment of toxicity is less subject to variability. Overall, the data suggest that genotyping genes of drug targets and metabolism may improve tailored chemotherapies in the future. Limitation of genomic polymorphisms as molecular predictive or prognostic marker may arise from its static character. Expression profiles (gene and protein) may more accurately reflect the current situation in the tumor in second- or third-line chemotherapy. For example, upregulation of TS after first-line TS-inhibitor therapy (e.g., 5-FU) may occur differently in different patients.

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DNA Repair: ERCC1, Nucleotide Excision Repair, and Platinum Resistance

Eddie Reed, Teri L. Larkins, Cindy H. Chau, and William D. Figg

Abstract DNA excision repair plays a significant part in platinum-based chemotherapy by removing DNA lesions caused by platinum-containing drugs. The nucleotide excision repair (NER) pathway is the mammalian DNA repair mechanism that removes bulky DNA adducts induced by DNA damaging chemotherapeutic agents. Platinum compounds induce their cytotoxic effect by binding to a DNA molecule in the form of a platinum-DNA-adduct. The NER pathway is the main mechanism responsible for platinum resistance by increased platinum-DNA-adduct removal and the excision repair cross complementing-group 1 (ERCC1) gene plays a major role in the NER-pathway because of its damage recognition and excision ability. This chapter will review mechanisms of DNA repair and platinum resistance as it relates to the NER pathway and regulation of ERCC1. A brief discussion on the role of cancer stem cells in platinum resistance is also presented.

Keywords ERCC1 • Nucleotide excision repair • Hedgehog

1 Overview of Nucleotide Excision Repair

The repair of platinum-DNA damage is executed by the nucleotide excision repair, the DNA repair pathway. Understanding the molecular and pharmacologic control of nucleotide excision repair (NER) may allow for more refined development of new platinum anticancer agents. In addition, such information may contribute to the refined development of non-platinum agents that damage DNA and/or modulate DNA repair.

In nucleotide excision repair pathway, more than 30 genes are involved in the process, which goes from DNA damage recognition, through the helicase functions of XPB and XPD, through damage

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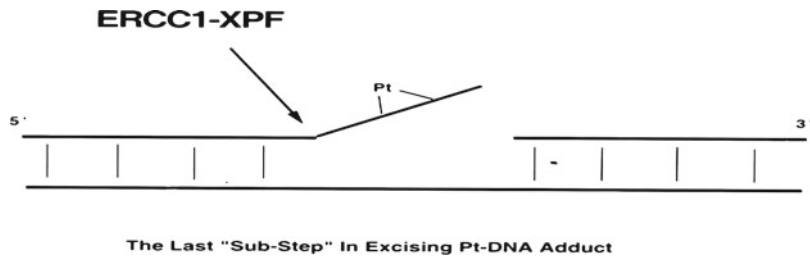

Fig. 1 Schematic of ERCC1 site of action in DNA damage excision

excision, through gap filling and ligation (reviewed in [1–4]). DNA damage excision is rate limiting to the process. In DNA damage excision, the last substep is the 5′ incision into the DNA strand, relative to the site of covalent damage. This 5′ incision occurs after the 3′ → 5′ and 5′ → 3′ helicase functions of the repairosome and after the 3′ incision. The 5′ incision is executed by the ERCC1-XPF heterodimer. This is graphically represented in Fig. 1.

ERCC1 is highly conserved in nature, with homologues in *Escherichia coli* [5, 6], in plants [7], and in every living organism yet examined. The *E. coli* homologue is UvrC, which performs the 5′ incision during the conduct of the NER process [5, 6]. More recent data suggests that ERCC1 and UvrC may differ, in that ERCC1 participates only in the 5′ incision in NER, whereas UvrC participates in the 5′ and 3′ incisions [5, 8, 9].

In one of our early reports, we studied paired Chinese hamster ovary (CHO) cells that did or did not have a functional ERCC1 [10]. Cisplatin-DNA adduct repair was assessed by atomic absorbance spectrometry (AAS) [10, 11]. Platinum-DNA adduct repair was assessed at the respective IC50 cisplatin dose, for each cell line. Part of this work is shown in Fig. 2. CHO cells lacking a functional ERCC1 (43:3B) were supersensitive to cisplatin and showed no detectable ability to repair cisplatin-DNA adduct. In the paired CHO cells that have a functional ERCC1 (83:J5), cisplatin-DNA adduct repair capability was intact, and there was an increased level of cellular resistance to cisplatin.

In this laboratory, we use AAS with Zeeman background correction as the tool for measurement of cisplatin-DNA damage [10–14]. This is for several reasons. AAS can be used to measure platinum-DNA damage after low, clinically relevant, levels of drug exposure. Measurements can be consistently obtained at the level of one platinum lesion per 100,000 bases of DNA. Further, AAS can be used to measure platinum in any subcellular compartment that can be faithfully isolated. This allows for detailed study of the subcellular pharmacology of platinum drugs.

ERCC1 is very difficult to study, because of the poor viability of ERCC1 defective cells [5, 6, 10]. Cells that are simply NER defective are more viable than cells that are specifically ERCC1 defective. ERCC1 has two known functions. In NER, the ERCC1-XPF heterodimer executes the 5′ incision into the DNA strand, freeing the DNA segment that has covalent bulky DNA damage [8]. The ERCC1-XPF heterodimer also is essential for drug-cross-link-induced double-strand break repair [15, 16] via an end-joining mechanism that is Ku86 independent [16].

Detailed structure–function analyses of both proteins show that XPF is a scaffold protein, upon which ERCC1 depends so that ERCC1 can perform the functions we know of at this time [17, 18]. It is possible, and likely, that ERCC1 has additional functions that are yet to be discovered. Prior to these reports, it was felt that BRCA1 and possibly BRCA2 were more important in these processes [19–21]. An association between ERCC1, BRCA1, and cisplatin resistance has been reported by several groups [19–22].

It has been long known that cisplatin induces double-strand breaks in the cellular DNA of treated cells [23, 24]. This is in addition to single-strand breaks, platinum-DNA adducts, and DNA-platinum-protein adducts [23, 24]. ERCC1 clearly plays a role in the excision of platinum-DNA damage from cellular DNA. Also, however, it now appears likely that ERCC1 may play a role in the repair of cisplatin-related double-strand break repair.

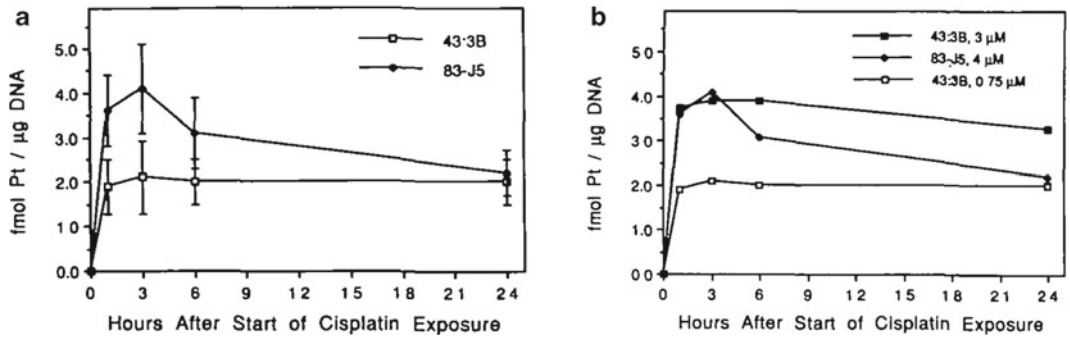

Fig. 2 (**a**) Pt-DNA adduct formation and removal profiles of paired CHO cells lines 43:3B (open squares; ICJQ = 0.75/µM) and 83-J5 (closed diamonds; ICjo = 4.0/µM) following treatment with their respective ICJO doses. (**b**) A third data set is added to the "means" of the two data sets presented in (**a**). The third data set is 43:3B cells treated at 3 µM (*closed squares*). For panels **a** and **b**, each data point is the mean and standard deviation of four or six separate determinations (Lee et al. Carcinogenesis 14:2177–2180, 1993. With kind permission from Oxford Journals)

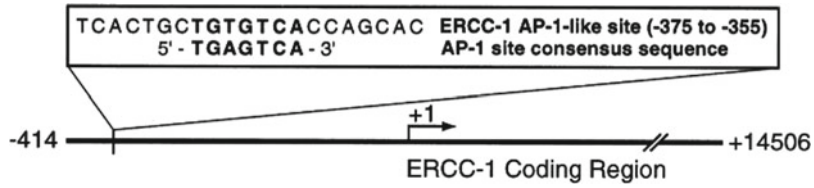

Fig. 3 Schematic of the 5′-flanking region of the ERCC-1 gene. The position of the AP-1-like site (*bold*) is pictured relative to the transcription start site (This figure was originally published in the Journal of Biological Chemistry. Li Q et al. J. Biol. Chem. 1998;273:23419–23425. © American Society for Biochemistry and Molecular Biology)

In our first examination of human ovarian cancer tissues, high levels of ERCC1 mRNA were demonstrated in tissues from patients that were clinically resistant to platinum therapy; and low levels of ERCC1 mRNA were demonstrated in tissues from patients that were clinically sensitive to platinum therapy [25]. This finding was in parallel with our in vitro CHO studies [10].

2 ERCC1, Activator Protein 1 (AP1), and Select NER Genes

After identifying ERCC1 as important in cellular resistance to cisplatin (18 and others), my group focused on the regulatory control of ERCC1 and of other genes involved in the excision of platinum-DNA damage. In human ovarian cancer cell lines, upregulation of ERCC1 is mediated by the jun-fos heterodimer, activator protein (AP1) [26–28]. The time course of the events of upregulation will be detailed below. The AP1-binding site in the 5′UTR of ERCC1 has been previously described by our group and is schematically shown in Fig. 3. Details of upregulation of ERCC1 are discussed below.

Laboratory studies, as well as clinical studies, show that ERCC1 is an excellent biomarker for the overall activity of NER in human cell lines and tissues (reviewed in [29–31]). ERCC1 is located on chromosome 19q13.2–13.4 and was the first gene cloned from the NER pathway [5, 6]. The coding region of the gene is 1.1 kb in length, with 10 exons. The non-functionality of ERCC1 results in the most severe DNA repair deficit phenotype yet described, in vitro or in vivo.

Exon VIII of ERCC1 has high homology with uvrC of *E. coli* [5, 8, 32]. In *E. coli*, the uvrABC protein complex executes NER types of DNA repair. Within the *E. coli* complex, uvrC executes the 5′ DNA strand-cutting step that excises platinum-DNA damage [8]. It is believed that in mammalian NER, exon VIII of ERCC1 may serve the same DNA strand-cutting function as uvrC in *E. coli* [5]. An alternatively spliced form of ERCC1 exists in human malignant and nonmalignant tissues, which lacks exon VIII. As discussed below, this alternatively spliced variant of ERCC1 may possibly have an inhibitory role for NER in human cells. These NER activities appear to be distinct from the ERCC1 role(s) in double-strand break repair [15, 16].

2.1 Upregulation of ERCC1

We were the first group to show that ERCC1 is inducible [26]. In human ovarian cancer cells, ERCC1 is upregulated after a 1 h treatment with cisplatin. However, a series of events precede the upregulation of ERCC1 [26–28]. After a 1 h cisplatin IC50 dose, A2780-CP70 human ovarian cancer cells upregulate the mRNA and protein of c-jun and c-fos. The peak in mRNA levels occurs at 1–2 h. C-Jun protein is then upregulated and peaks at 3–5 h after cisplatin. C-Jun protein has to be phosphorylated to be activated. C-Jun phosphorylation is greatly enhanced at 1 h after cisplatin treatment and peaks at 15-fold over baseline 3–5 h after cisplatin treatment. C-jun phosphorylation is necessary to activate AP1. AP1 activation leads to increased transcription of ERCC1.

ERCC1 mRNA is upregulated with peak levels at 3–4 h. This ERCC1 mRNA is degraded with a half-life of 24 h in cisplatin treated cells, as compared to a half-life of 14 h in cells not exposed to cisplatin [26], suggesting a cellular response to DNA damage that may prolong the period during which ERCC1 may be active. ERCC1 protein levels begin to rise within 1 h after cisplatin and peak at 24 h after cisplatin in these cells.

In separate sets of experiments, we showed that ERCC1 upregulation through AP1 may occur through at least two separate pathways; the JNK/SAPK pathway or the ERK pathway [27, 28]. The ERK pathway can be activated by cell exposure to phorbol ester.

AP1 was confirmed as the transcriptional regulator for ERCC1 by electrophoretic mobility shift assay (EMSA) and by supershift EMSA. We have performed dose-response experiments for ERCC1 and for AP1, in A2780-CP70 cells. For both mRNA and protein of these genes, peak levels are produced by an IC50 of cisplatin given as a 1 h dose, in monolayer culture (40 μm). When using phorbol ester, an IC50 dose is associated with maximal induction of ERCC1, as well. In Fig. 4, we show the AP1-binding site in ERCC1. We have identified AP1-binding sites for many of the genes known to exist in the NER repairosome. Figure 4 shows the AP1-binding sites for five of these genes within NER.

2.2 Factors That May Impact Upregulation of ERCC1

We reasoned that if ERCC1 is critical for NER activity, then we could alter platinum-DNA repair activity by altering ERCC1 upregulation. Further, such alteration of ERCC1 and of platinum-DNA adduct should alter cellular sensitivity to cisplatin. This work was done prior to the availability of siRNA approaches. One approach was to use a dominant negative to AP1 to disrupt the upregulation of ERCC1 [32]. This AdA-FOS construct was transfected into A2780-CP70 cells prior to cisplatin exposure. The dominant negative was designed to inhibit AP1 binding to its consensus binding sequence in the 5′UTR of the gene. In cells treated in this fashion, ERCC1 upregulation was severely

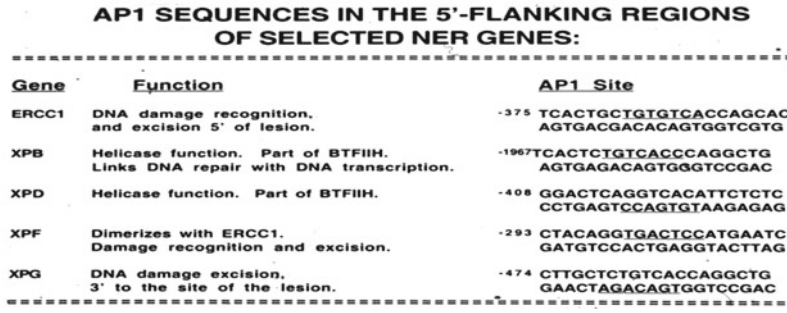

Fig. 4 AP1 binding sites for five NER genes

Table 1 Small molecules that modulate ERCC1 mRNA and protein

Drug	Known activity	ERCC1 effect
Pt, Cr, Cd, etc.	Damage DNA	Upregulates
Cycloheximide	Protein synthesis inhibitor	Downregulates
Alpha-amanitin	Global transcription inhibitor	Downregulates
Actinomycin D	Global transcription inhibitor	Downregulates
Interleukin-1 alpha	Biological therapeutic agent	Downregulates
TNF-alpha	Biological therapeutic agent	Downregulates
Phorbol ester	Protein kinase C modulator	Upregulates
Lactacystin	Proteasome inhibitor	Downregulates
N-ALLNL	Proteasome inhibitor	Downregulates
SU5416	VEGF inhibitor	Downregulates
Cyclosporin A	Immunosuppressant; blocks c-fos	Downregulates
Herbimycin A	PTK inhibitor, blocks c-jun	Downregulates

blunted after cisplatin exposures, platinum-DNA adduct repair was severely reduced, and cells were severalfold more sensitive to cisplatin treatment.

A series of pharmacologic agents were assessed for their ability to blunt ERCC1 upregulation, based on a range of known activities of the respective molecules [33–52]. All agents that blunted ERCC1 upregulation also inhibited platinum-DNA adduct repair and enhanced sensitivity to cisplatin. Molecules tested included the following (these all inhibited the process unless otherwise stated): a number of heavy metals such as platinum, chromium, and cadmium; cycloheximide (global protein synthesis inhibitor); alpha-amanitin (global transcription inhibitor); actinomycin D (global transcription inhibitor); interleukin-1 alpha (biological agent); TNF-alpha (no effect on ERCC1 upregulation); phorbol ester (upregulated ERCC1 through ERK pathway); lactacystin (proteasome inhibitor); N-acetyl-leucyl-leucyl-norleucinal (proteasome inhibitor); SU5416 (VEGF inhibitor); cyclosporin A (immunosuppressant, blocks c-fos); and herbimycin A (PTK inhibitor, blocks c-jun) (Table 1).

Although pharmacologic drugs may modulate ERCC1 expression, naturally occurring molecular factors also appear to impact ERCC1 and NER [53–59]. Molecular factors specific to ERCC1 that we have explored include the following. Alternative splicing of ERCC1 mRNA occurs in every human tissue and in every cell line that we have examined. One alternatively spliced form lacks exon

VIII (discussed above for its high homology to uvrC in *E. coli*). The percent occurrence of the form lacking exon VIII appears to correlate with a decrement in the ability of cells to repair platinum-DNA adduct (i.e., the higher the percent alternatively spliced ERCC1, the lower the DNA adduct repair capability). A second alternative spliced form of ERCC1 mRNA involves the 5′UTR and may involve transcriptional regulation by the gene RFX1 [57]. Additional polymorphic forms have been described by other groups.

My group was the first to describe the potential clinical relevance of a specific polymorphism of the NER gene ERCC1 in exon IV [58, 59]. This is a silent polymorphism at codon 118 that is associated with reduced mRNA expression of the gene, reduced protein expression, reduced platinum-DNA adduct repair, enhanced cellular sensitivity to cisplatin, and more favorable clinical outcomes from platinum-based chemotherapy. The association of the codon 118 polymorphism in tumor tissues, with positive response to platinum-based chemotherapy, has been observed in ovarian cancer, lung cancer, colorectal cancer, and other malignancies (reviewed in [3, 4, 29–31]).

Loss of heterozygosity may, or may not, occur in some ovarian cancer cells and tissues for the 19q region that contains ERCC1 [60, 61]. This has also been observed in malignant gliomas, with changes in gene copy number for ERCC1 and for ERCC2 (XPD) [62]. Whereas these changes occur with significant frequency, they do not correlate with alterations in mRNA or DNA expression of these genes, nor with observed clinical outcomes. For XPA, marked variations in mRNA expression of this gene occur in the absence of any evidence of mutations or changes in gene copy number [63].

2.3 MZF1 as a Transcriptional Repressor for ERCC1

Upon finding that AP1 is a transcriptional activator for ERCC1 and NER, we sought to identify a transcriptional repressor for ERCC1 and NER. Myeloid zinc finger 1 (MZF1) is a transcription factor that is important in hematopoietic transcriptional regulation [64–66]. It is located at the extreme end of 19q, less than 20 kb from the subtelomeric repeat region of 19q [66]. MZF 1 has a binding site in the 5′UTR of ERCC1, XPA, XPB, and XPD, among other NER genes, and is similar to AP1 in this respect [67]. AP1 is a positive transcriptional regulator for these NER genes and has a leucine-zipper-binding motif. MZF1, in contrast, has a zinc finger motif, which has the functions of a SCAN box that is leucine rich, and is predicted to form alpha-helices.

As a direct result of our analyses of the ERCC1 5′UTR [67], we explored the possibility that MZF1 may have a negative transcriptional regulatory role in NER [68]. The ERCC1 5′UTR has an MZF1-binding site, approximately 50 bases downstream from the AP1-binding site. The portion of the ERCC1 5′UTR that shows this is given in Fig. 6 (note arrows).

Electrophoretic mobility shift assay (EMSA) analyses show that in A2780-CP70 cells, the MZF1 ZN1-4 consensus region of the gene binds to the MZF1 site in the ERCC1 5′UTR [68]. At baseline, cells in log phase growth have high levels of MZF1. Concurrently, AP1 levels are low. Cellular treatment with cisplatin resulted in downregulation of the high baseline levels of MZF1 mRNA, and there was concurrent upregulation of AP1 (Fig. 5). This relationship reversed itself over the 48–72 time frame of the experiment. Stated simply, when AP1 is up, MZF1 is down. And, when AP1 is down, MZF1 is up.

Using a MZF1-luciferase construct, we showed that binding of MZF1 to the 5′UTR of ERCC1 directly resulted in suppressed expression of luciferase, further confirming the transcriptional repressive function of MZF1 [68]. We have also demonstrated that there are MZF1-binding sites in the 5′UTR of a number of genes in the NER repairosome, such as XPA, XPB, and XPD [67]. Thus,

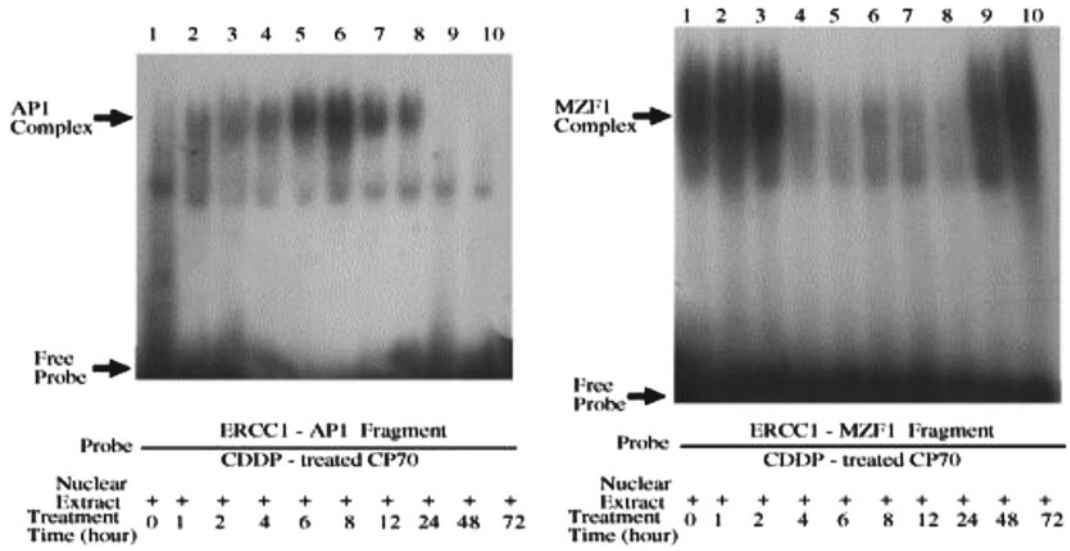

Fig. 5 Nucleotide sequence of the *ERCC1* promoter region. The binding consensus sites of transcription factors are labeled. The 5′-ends of various constructs for the CAT assay are indicated with *asterisks*. The *numbers* represent the nucleotide position relative to the transcription initiation site. The upstream region (UPSTR) and the downstream region (DNSTR) of the *ERCC1* promoter are depicted with *horizontal arrows*. *Bold letters* show the essential sequences of fundamental transcription of the *ERCC1* gene and regulatory elements within this region (Reprint from Yan et al. Biochem Pharmacol 2006;71(6):761–71 with permission from Elsevier)

we believe/hypothesize that MZF1 is an active transcriptional repressor for nucleotide excision repair.

2.4 *Coordinate Expression of NER Genes in Human Ovarian Cancer Tissues*

In clinical specimens from patients with human ovarian cancer, in nonmalignant bone marrow, and in human brain tissues, selected genes in the NER repairosome appear to be upregulated and downregulated together [69–75]. Such genes include ERCC1, XPA, XPB, and XPD, among others. Further, one can use mathematical linear regression approaches to assess the degree to which these genes are "coordinated." The more tightly coordinated the expression of these genes, the more the tissue appears to have effective NER activity and to be resistant to platinum-induced cell killing. Our first observation of coordinate expression was in nonmalignant human bone marrow specimens [69].

We followed the bone marrow studies with studies in human ovarian cancer [70–73]. In one study, we assessed NER genes ERCC1, XPA, XPB, and CSB, along with MDR1 and MT-II in the same ovarian cancer tissue specimens [72]. Figure 7 shows data taken from those studies. The NER genes we examined were upregulated in platinum-resistant tissues, together, in the absence of upregulation

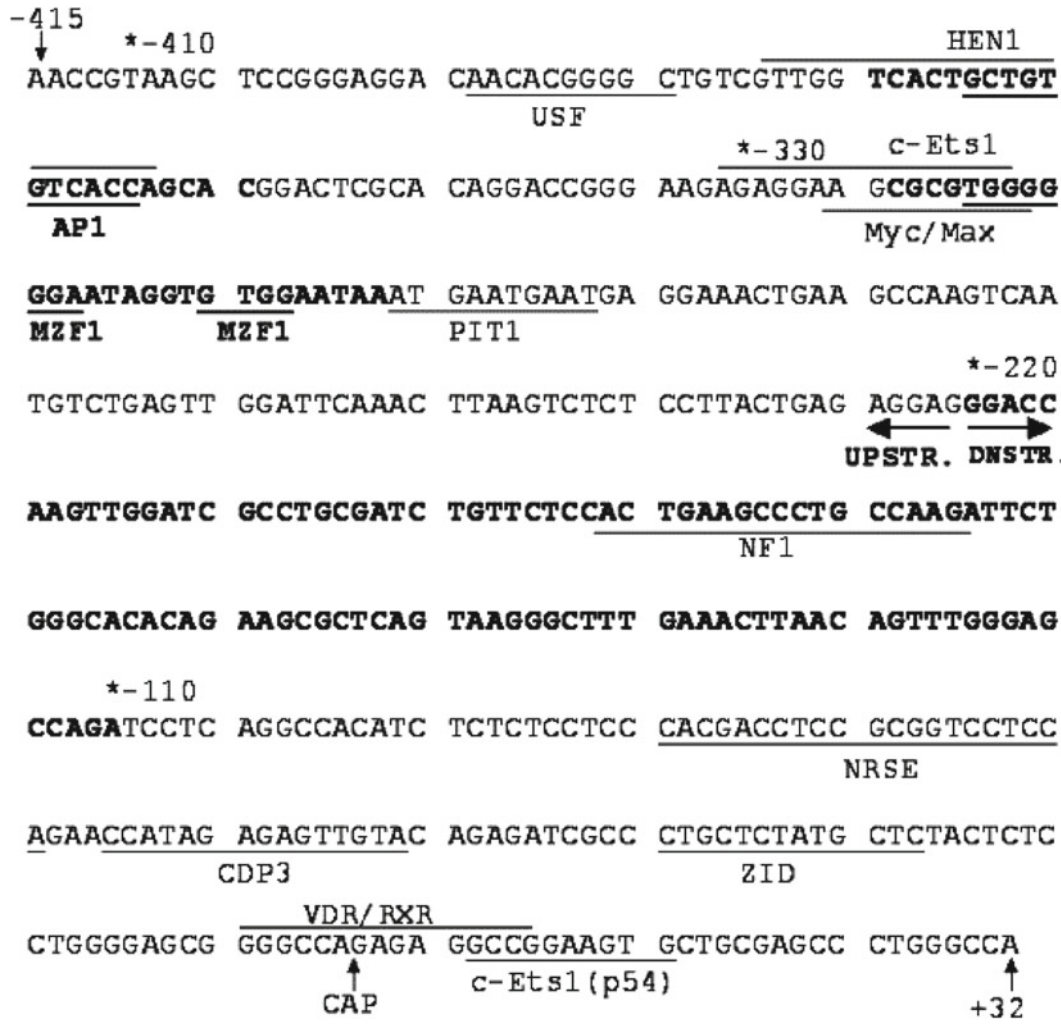

Fig. 6 Binding activities of AP1 element and MZF1 element within the ERCC1 promoter during time course after cisplatin treatment. A2780/CP70 cells were incubated for 1 h in the presence of medium containing 40 μmol/l cisplatin and continuously to be cultured in fresh medium (w/o drug) to the indicated timepoints. EMSAs were performed with nuclear extracts from above samples and [^{32}P]ATP-labeled oligonucleotides as described (Reprint from Yan et al. Biochem Pharmacol 2006 Mar 14;71(6):761–71 with permission from Elsevier)

of MDR1 and of MT-II. Tissues that proved to respond to chemotherapy (platinum-sensitive) consistently showed low levels of expression, together, of these same NER genes.

Another specific example of coordinate NER gene expression is clear cell tumors in human ovarian cancer [73]. More than 120 human ovarian cancer tissues were examined for coordinate mRNA expression of ERCC1, XPB, and XPD. Five different histologic types were investigated: clear cell, endometrioid, serous, mucinous, and undifferentiated. Clear cell tumors of the ovary are known for being particularly chemoresistant. In this study of >120 human tumor specimens, clear cell tumors had consistently higher mRNA levels of these NER genes, and the degree of coordinate expression was statistically significantly greater in clear cell tumors than in any of the other histologies. One set

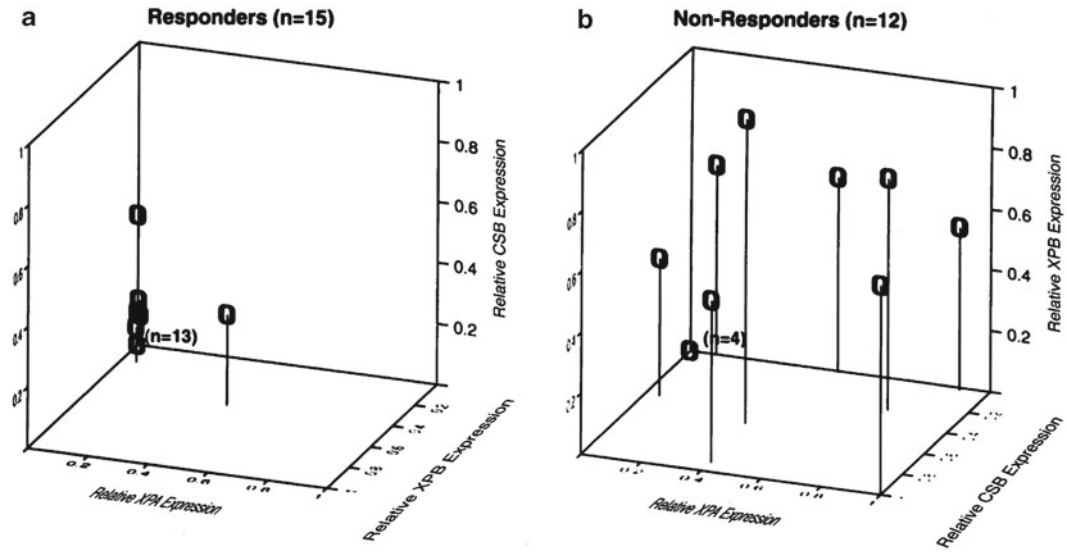

Fig. 7 Three-dimensional plots concurrently analyzing XPA, XPB/ERCC3, CSB/ERCC6 expression levels in ovarian tumor tissues from responders (panel A) and non-responders (panel B) (Reprint from Dabholkar et al. Biochem Pharmacol 2000;60(11):1611–1619 with permission from Elsevier)

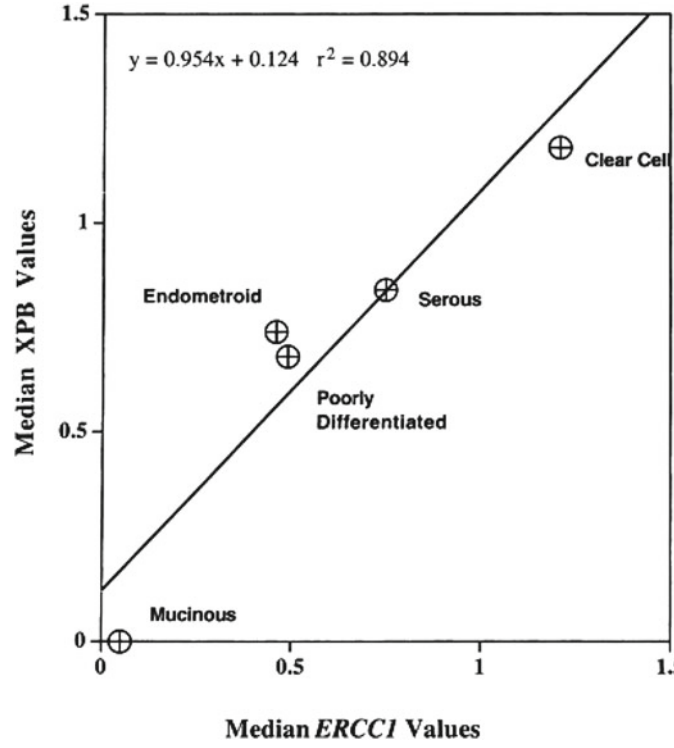

Fig. 8 The median mRNA values obtained by PCR for *ERCC1* and for *XPB* were plotted against one another for each of the five histological subtypes examined. The data were subjected to simple linear regression analysis, and the equation for the line is shown in the figure. On the basis of this analysis, the overall relationship approximates a slope of 1 (This figure was originally published in Clinical Cancer Research. Reed et al. Clin Can Res. 2003;9(14):5299–305. © American Association for Cancer Research Journals)

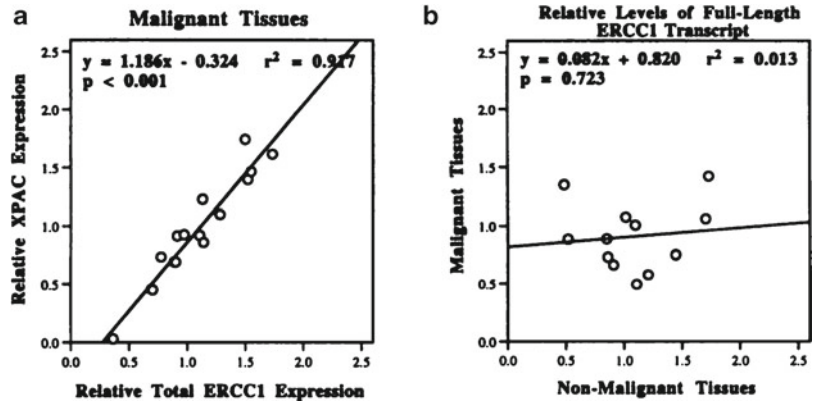

Fig. 9 Visual representation of the analyses for correlation of expression of NER genes in malignant and nonmalignant brain tissues. (**a**) Example of excellent concordance between ERCCl and XPAC in malignant tissues. (**b**) Example of poor concordance between malignant and nonmalignant tissues for ERCCl. Correlation coefficients obtained from linear curve fit analysis (CricketGraph) and Ps obtained from simple regression analysis (Statworks) are shown. (This figure was originally published in Cancer Research. Dabholkar et al. Can Res 1995;55(6):1261–6. © American Association for Cancer Research Journals)

Table 2 Chromosome location of selected NER genes

NER gene	Location	Gene size
ERCC1	19q13.2–3	1.1 kb; 10 exons
XPD	19q13.2–3	20 kb; 23 exons
XPB	2q21	45 kb; 14 exons
XPA	9q22.3	25 kb; 6 exons
XPF	16p13.3–11	28.2 kb; 11 exons
XPG	13q32.3–33.1	32 kb

of observations from that study is shown in Fig. 8. Here, ERCC1 values are plotted against XPB values for each of the five histologies that we studied. The higher mRNA levels observed in clear cell tumors, were statistically significant.

We have also studied in malignant, and adjacent nonmalignant, human brain tissues for evidence of coordinate expression of NER genes [74, 75]. Figure 9 shows data from one set of observations from these experiments. In high grade gliomas, there was excellent coordinate mRNA expression of ERCC1 and XPA, as assessed by linear regression analysis (Fig. 9, panel A). When malignant and nonmalignant glial tissues were assayed from the same patients, there was poor coordinate expression of ERCC1 mRNA (Fig. 9, panel B). This suggests that during the conversion of cells from the normal to the malignant state, ERCC1 is altered and possibly all of NER is altered. This type of circumstance has been confirmed using different DNA repair genes by another group, in their examination of direct reversal of DNA damage caused by methylating agents.

Overall, for human malignant tissues that may have some degree of clinical sensitivity to cisplatin and other platinum analogues, genes in the NER repairosome seem to display several common essential characteristics. First of all, higher levels of expression of mRNA and of protein are seen in platinum-resistant tissues, as compared to platinum-sensitive tissues. Secondly, the degree to which NER is tightly coordinated between the various genes involved in the process contributes to that tissue's

Table 3 Markers associated with spheroid-forming cells (cancer-initiating cells, or ovarian cancer stem cells) derived from monolayer forming cells

Markers of interest	Reference
Bmi-1, stem cell factor, Notch-1	Zhang et al. [71]
Nanog, nestin, ABCG1, Oct-4, CD117	Zhang et al. [71]
CD44+CD117+ (*profile of special interest*)	Zhang et al. [71]
CD44, EGFR, c-met, Vim, CK18	Bapat et al. [76]
Slug, C-kit, SCF, Snail, E cadherin	Bapat et al. [76]
AR, CD44, CD133, Nanog, Oct4	Gu et al. [77]
SOX2, Nestin, c-kit, p63, CK8/18	Gu et al. [77]

ability to repair platinum-DNA damage and resist platinum-based therapy. The extent to which the NER process is coordinated within a tissue can be assessed using mathematical linear regression analyses.

One of the things that make this observed coordinate expression of high interest is that these genes are located at disparate sites in the genome. Table 2 lists six of the NER genes that we've studied, which are located on five different human chromosomes. These facts raise the question, of what factors might play a role in establishing and maintaining coordinate expression of NER genes across chromosomes. If so, such factors may be operative in nonmalignant tissues, in human ovarian cancer tissues, in human brain cancer, and possibly in other malignancies.

3 Cancer Stem Cells and Drug Resistance

A number of laboratories have begun to investigate the concept of "cancer-initiating cells" in human ovarian cancer [78–81]. This concept is being developed in a number of malignancies, including prostate cancer, breast cancer, and lung cancer. One recent study by Zhang et al. demonstrates the power of this concept [78]. When human ovarian cancer cells are grown under conditions that support a subpopulation that grows in spheroids, this appears to select for cells that have a more potent ability to form new independent cancers.

In addition to becoming more potent in terms of forming new tumors, cells become much more drug resistant to a variety of agents, including platinum compounds [78–81]. It is not clear why the same cell line should become much more drug resistant, when the cells grow in spheroids as compared to growth in monolayer.

Spheroid-forming cells tend to show a set of molecular markers that differ from the same cell line grown in monolayer. Such markers are summarized in Table 3. In the Zhang report, as few as 100 spheroid-forming cells (cancer-initiating cells) could form new independent tumors when transferred to nude mice. In contrast, as many as 100,000 cells grown in monolayer were unable to form independent tumors. Another characteristic of these cancer-initiating cells is that they are particularly chemoresistant to cisplatin and paclitaxel. Another term that is commonly used for these spheroid-forming cells is "ovarian cancer stem cells."

If ovarian cancer stem cells are responsible for persistent low volume disease after induction of a clinical complete response, this means that eradication of ovarian cancer stem cells could result in cure of the disease. It is possible that the inability to eradicate such cells may be a function of cell dormancy and the relative inability of any chemotherapy to have a meaningful effect on cells in the dormant state. However, it is also possible that these cells may represent a state of extreme drug resistance on the molecular level.

Platinum-based therapy is the mainstay of systemic treatment for ovarian cancer. Nucleotide excision repair is one of the pathways of critical importance in the development of resistance to platinum agents. Therefore, the study of NER, and the regulatory control of NER, in ovarian cancer stem cells is of intense interest to our group.

3.1 "Stem Cells," Properties of Stem Cells, and Cisplatin Resistance

Stem cells have been defined in two ways. The phenotypic description is based, in part, on the ability of such cells to grow in non-adherent conditions and to exhibit enhanced resistance to anticancer agents. On a molecular level, stem cells are also defined by the specific molecular markers expressed by those cells (as listed above) and by activation of the Hedgehog pathway.

We have published extensively on the paired human ovarian cancer cell lines A2780 and A2780-CP70. These cells were developed by Hamilton, Ozols, and colleagues [82, 83] and have been studied extensively by our group [12, 26, 32]. The A2780 cell line is cisplatin sensitive, with an IC50 of ~3 μM. The A2780-CP70 cell line is cisplatin resistant and, in our hands, has an IC50 of ~40 μM.

Since stem cells are characterized by relative drug resistance, we chose to begin our investigations by asking the following question: are there molecular characteristics of "stem cells" that can be found at baseline, in either of these two well characterized human ovarian cancer cell lines? The molecular link(s) between the stem cell phenotype, and cellular resistance to platinum compounds, have yet to be determined.

One molecular pathway that is presumed to be critical to the stem cell phenotype is the Hedgehog pathway [84–86]. One critical gene in that pathway is Gli1, which was first described by Kinzler and Vogelstein [86]. Hedgehog ligand is thought to be produced in a range of cancers, by stromal cells, and not the cancer cells themselves [84, 87–89]. In ovarian cancer and in breast cancer, recent reports have shown that the hedgehog ligand is produced by the cancer cells themselves [88–90]. Proteins involved in the Hedgehog have been reviewed in detail elsewhere [84, 87–90]. Hedgehog ligand comes in three different forms: Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh). Hedgehog ligand binds to the Patched protein in the cell membrane. Patched, a 12 pass transmembrane protein, normally inhibits the 7 pass transmembrane protein Smoothened. Once hedgehog ligand binds to Patched, this disrupts the inhibition of Smoothened. Smoothened then is free to act on Gli1. Gli1 exists in the cytoplasm in cells that are not Hedgehog activated. Once Smoothened acts on Gli1, Gli1 can then translocate to the nucleus and act as a transcription factor for a range of genes that influence the biological state of the cell. The Hedgehog pathway is considered "activated" when Gli1 has translocated to the nucleus. Therefore, one characteristic of stem cells would be that Gli1 will have translocated to the nucleus. This finding would be consistent with the activation of the Hedgehog pathway (and activation of the Hedgehog pathway may be critical to the stem cell state).

We chose to ask whether there are molecular linkages between the Hedgehog pathway and Gli1 and/with the NER pathway and ERCC1. We began by addressing this question in the human ovarian cancer cell lines, A2780 cisplatin sensitive and A2780-CP70 cisplatin resistant. We studied the relationships between Gli1 expression, c-jun expression, ERCC1 expression, and cisplatin resistance in the human ovarian cancer cell lines A2780 and A2780-CP70. A2780-CP70 cells are 10–15-fold more resistant to cisplatin than A2780 cells [91, 92]. We used two different probes to modulate the Hedgehog pathway: (a) cyclopamine as a pharmacologic inhibitor of the Smoothened and (b) an anti-Gli1 shRNA to specifically inhibit Gli1. At baseline, cisplatin-resistant cells, expressed >10-fold more Gli1 protein than cisplatin-sensitive cells. In cisplatin-resistant cells, Gli1 protein was downregulated in response to cyclopamine and to anti-Gli1 shRNA. When A2780-CP70 cisplatin-resistant cells are pretreated with cyclopamine, or anti-Gli1 shRNA, common events occur. Also, disparate events occur when comparing the cellular response to cyclopamine versus the cellular response to anti-Gli1 shRNA. The common events are as follows. The c-jun Thr91/93 cascade is upregulated, which is associated with proapoptotic processes. The c-jun Ser63/73 cascade is suppressed. This cascade is associated with pro-growth processes. If cisplatin is given after cyclopamine or anti-Gli1 pretreatment, Gli1 protein is downregulated; the c-jun Thr91/93 cascade is upregulated; the c-jun Ser63/73 cascade is suppressed; and cells do not upregulate ERCC1, XPD, or XRCC1. As discussed above, the upregulation of ERCC1 and XPD is essential to the cellular response to cisplatin exposures by increasing the DNA repair capacity for cisplatin-DNA damage. The disparate events, when comparing cyclopamine versus anti-Gli1 shRNA, are more complex and are the subject of future publications.

However, it can be said that the normal cisplatin-induced upregulation of these three genes involved in NER (ERCC1, XPD) and in base excision repair (XRCC1) is suppressed by suppression of the Hedgehog pathway. We conclude that Gli1 and/or other genes involved in the Hedgehog pathway (which is critical for cancer stem cells) probably play key roles in the upregulation of DNA repair genes, specifically in response to DNA damaging agents such as cisplatin.

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Drug Interactions

Laurent P. Rivory

Abstract Drug–drug interactions in cancer patients are relatively common and can and do have significant impact on outcomes. Increasingly, it is being recognized that these arise through the complex interplay of drugs with both metabolic and transport processes. The complexity is increased through the time lags that may occur between the administration of interacting drugs, the fact that some drugs can both induce and inhibit metabolism, and the inhibition of multiple pathways by some. The potential for drug–drug interaction needs to be considered in the development of new drugs as well as in the routine use of existing agents.

Keywords Drug-drug interactions • Drug metabolism • Drug disposition • Transporters

1 Introduction

The setting of chemotherapy for cancer is rife with potential for significant drug interactions and this topic has been the subject of several excellent reviews [1–4]. Most patients receive multidrug combinations for their malignancy. Also, many of these patients are treated with intercurrent medication for comorbidity or for cancer-related disorders (coagulopathy, infection, pain, seizures, etc.). The clinical significance of these potential drug interactions is all the more relevant in cancer chemotherapy because many cytotoxic agents do not have clear therapeutic windows. That is, the doses selected produce toxicity in a significant proportion of patients without necessarily providing a demonstrable benefit. Drug–drug interactions can arise either at the sites of action (pharmacodynamic) or affect their disposition in the body (pharmacokinetic). The latter is the focus of this chapter.

Drug interactions causing an increased exposure of the patient to the cytotoxic agent may produce more severe side effects, whereas those causing a decreased exposure may jeopardize tumor control. Unfortunately, both the good and bad effects of chemotherapy are unpredictable, and the influence of drug interactions in either eventuality is almost impossible to detect in individual patients. These, however, may be borne out in large-scale studies or when combined with pharmacokinetic data (e.g., see [5, 6]). Therefore, most drug interactions in cancer chemotherapy may go undetected unless some a priori knowledge alerts the clinician or oncology pharmacist to their likelihood.

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The situation is further complicated by the fact that many patients will also take nutrient supplements and herbal medicines without necessarily informing oncology staff. Several of these, including St John's Wort, can lead to significant changes to the pharmacokinetics and activity of chemotherapy agents as exemplified with irinotecan [7].

When we think of drug–drug interactions, we usually think about classical interactions with the cytochrome P450 enzymes, as these have been well recognized and characterized over the last few decades. Certainly, this mechanism remains at the forefront of clinically significant drug–drug interactions. However, the pathways involved in the classical ADME of drug disposition (absorption, distribution, metabolism, and elimination) are all candidates for drug interactions. In particular, our understanding of transporters and their role in the systemic disposition of anticancer drugs has evolved exponentially over the last few years. They are now recognized as a major locus of drug–drug interaction [8] and increasingly of interest from that point of view for regulatory agencies [9].

The aim of this chapter is to review some of the potential mechanisms of drug–drug interactions and to illustrate these with published data. The various loci of drug interactions are considered in the setting of drug development of new molecular entities (NME) and their clinical scheduling rather than an exhaustive listing of all the known interactions. In addition, the possibility of exploiting drug–drug interactions to improve cancer chemotherapy is raised.

2 The Loci for Drug Interactions: Process by Process

As mentioned briefly in the previous section, any of the traditional processes implicated in drug pharmacokinetics (i.e., ADME) is a potential locus for drug–drug interactions.

2.1 Drug Absorption

For orally administered drugs, interactions leading to significant pharmacokinetic changes may arise as a result of changes in the gastric emptying time [10]. Food is the most widely accepted factor for increasing gastric transit time, but a number of drug-related factors may have similarly important roles. Drugs may affect directly the rate of gastric emptying with most slowing this process, although some, such as metoclopramide, actually speed it up [11]. Many cancer drugs produce transient nausea and vomiting. Nausea produces a slowing down of gastric emptying and may influence the rate and extent of absorption of oral chemotherapy. Many of the anticancer drugs given orally to date display wide variability in their absorption (e.g., mercaptopurine [12]), which would possibly mask these subtle effects. Small-intestinal transit time is also likely to be an important factor [13]. Certainly, the advent of rationally developed oral chemotherapy may, in the future, require specific consideration of these factors [14, 15].

Drug interactions during absorption may also follow from alterations in the gastrointestinal environment. For example, the camptothecins are unstable at physiological pH and drugs affecting intragastric pH could be of concern for the administration of these agents by the oral route. This was the basis for a study of oral topotecan with and without ranitidine [16]. Conversely, temozolomide is unstable at low pH and ranitidine was examined for an effect on drug absorption [17]. In neither case was there any significant effect. In some cases, the instability of drugs at acidic pH has led to the direct incorporation of inhibitors of gastric acid production into oral bioavailability studies [18]. Antacids may also be worthy of investigation from this point of view as a way of improving the bioavailability of labile drugs [19]. Mechanistic interpretation may, however, be difficult with some commonly used drugs. For example, members of the proton pump inhibitor family such as omeprazole and pantoprazole, which are used to reduce gastric acid production, are also inhibitors of drug transporters such as the half-transporter breast cancer-related protein (BCRP). Hence effects of omeprazole on the absorption of BCRP substrates such as topotecan could, if detected, be multifactorial.

Nonspecific interactions that may modulate drug absorption can arise from the coadministration of binding drugs such as cholestyramine. Some parenteral formulations have surfactants to solubilize hydrophobic drugs in aqueous solutions. Although it is attractive to use available intravenous formulations to investigate the oral route of administration of these drugs, the nonspecific effects of these surfactants may significantly modify the absorption of the compound of interest. In the case of paclitaxel, coadministration with its intravenous formulation surfactant, Cremophor EL, was shown to decrease greatly paclitaxel bioavailability, whereas polysorbate 80 (Tween 80) had the opposite effect [20]. Some of these surfactants may also affect transporters such as BCRP, but whether this is genuine inhibition or alteration of drug micellar distribution is unclear.

During their absorption from the gastrointestinal tract, drugs run the gauntlet of gut mucosal and hepatic drug metabolism, the so-called first-pass effect. As reviewed elsewhere in this book, a multitude of pathways are implicated in the metabolism of anticancer drugs. One of the first drug interactions observed in oncology was that which occurs between 6-mercaptopurine and allopurinol [3]. An important metabolic pathway for the catabolism of 6-mercaptopurine is mediated by xanthine oxidase, which is inhibited by allopurinol. In the study by Zimm et al., administration of allopurinol increased peak concentrations and the area under the concentration–time curve (AUC) of 6-mercaptopurine by fivefold, but only when 6-mercapoturine was administered orally [21]. Methotrexate is another, albeit weaker, known inhibitor of xanthine oxidase [22].

Inhibition of gut wall and hepatic metabolism may well be a requisite for appreciable absorption of some drugs from oral formulations. For example, the bioavailability of oral 5-fluorouracil (5-FU), which is of the order of 20–30 % [23], is limited by intestinal and hepatic dihydropyrimidine dehydrogenase (DPD), the major catabolic pathway for 5-FU [24]. Novel oral formulations of fluoropyrimidines often contain a DPD inhibitor to minimize this loss of drug to improve bioavailability. In the case of UFT, uracil is added in a 4:1 molar ratio as a competitive inhibitor of DPD with the 5-FU prodrug tegafur [25]. When combined with the DPD inhibitor ethyniluracil, the bioavailability of orally administered 5-FU approaches 100 % [26]. These are examples of how drug interactions can be exploited to improve the pharmacokinetic properties of important drugs. Another major class of drug-metabolizing enzymes present in the mucosa and liver is the cytochrome P450 (CYP450) superfamily. Again, these enzymes have been reviewed elsewhere in this book. In general, drug–drug interactions occurring at the CYP450 locus have been documented mostly in the context of parenterally administered cytotoxic drugs and these are discussed later.

As discussed elsewhere in this book, many of the ABC transporters including P-glycoprotein line the gastrointestinal lumen. By facilitating basal to apical fluxes, they move drug back into the intestinal lumen, thereby reducing absorption following oral administration [27]. This mechanism has now been extensively manipulated in the experimental setting to try and achieve oral chemotherapy of drugs previously considered too poorly absorbed [28]. The taxanes are avid substrates of P-glycoprotein and particularly suitable for testing the concept of modulation of this transporter on drug bioavailability [27]. Cyclosporin A and its non-immunosuppressive analog PSC833 were some of the first blockers of P-glycoprotein to be tested for this modulation, demonstrating impressive improvements in paclitaxel bioavailability in mice [29, 30]. Results in clinical trials of 60 mg/m^2 of oral paclitaxel combined with 15 mg/kg of oral cyclosporin also showed large increases in oral bioavailability of paclitaxel [31]. The bioavailability of the combination was approx. 30 %, which may, however, have been underestimated because of the nonlinearity of paclitaxel kinetics [31]. Nevertheless, targeting relevant concentrations (i.e., those achieved by intravenous administration) may prove difficult although possible [32]. Other P-glycoprotein modulators (e.g., GF120918) have been investigated in this setting [33, 34].

In the same vein, topotecan is a substrate of both P-glycoprotein and BCRP and both these transporters are expressed in the gut mucosa [35]. The P-glycoprotein modulator GF120918 is also a potent modulator of BCRP and caused a further increase in topotecan bioavailability in the mouse as a result of blocking this second transporter [35].

2.2 Drug Distribution

Aside from controlling the transfer of drugs across the gastrointestinal mucosa, the same transporters also control the distribution of drugs into other compartments (e.g., central nervous system, placenta). Inhibition or induction of transporters may therefore have an impact on the distribution of anticancer drugs. An effect of drug distribution would be detectable either from an effect on the volume of distribution of the drug or its pharmacokinetics in a specific compartment (e.g., central nervous system [CNS], cerebrospinal fluid [CSF], etc.). In a study of PSC833, Advani et al. examined the effects of this P-glycoprotein blocker (5 mg/kg po four times per day for 3 days) on a regimen of doxorubicin and paclitaxel [36]. Importantly, this was a crossover study and, although the sequences were not randomized, this design enabled the effect of PSC833 to be observed in each individual. The presence of PSC833 led to a doubling of the terminal half-lives of both doxorubicin and paclitaxel.

In the case of paclitaxel, the effect was primarily due to a trebling of the volume of distribution, indicating a substantial interaction with the distribution of this drug. In contrast, in the case of doxorubicin, the effect was attributable mostly to changes in total clearance. Specific compartments may well be targeted by such drug interactions. Indeed, there is the exciting possibility of improving drug distribution into the CNS by coadministration of blockers of P-glycoprotein and other transporters located at the blood–brain barrier [27, 37]. Importantly, however, an increase in CNS toxicity may be a downside to such strategies [38]. Also, issues in relation to the concentrations required need clarification [27]. There are conflicting data in animal models on the potential of cyclosporin A to modulate brain uptake of drugs [38–41], and this may reflect subtle differences in the probe drugs and their schedules of administration. Vinblastine does not readily penetrate into the CNS largely because of P-glycoprotein function, and mdr1a/1b knockout mice are at greater risk of neurotoxicity following administration of this agent [42]. The clinical interaction between itraconazole and vincristine [43, 44], therefore, may be due to modulation of CNS distribution of vincristine by P-glycoprotein.

In theory, the competition by two drugs for a plasma binding protein can lead to an increase in the free concentration of the displaced drug. This, however, depends largely on the physicochemical and pharmacokinetic properties of the drug in question. In most cases, the displaced drug distributes rapidly into tissue compartments and/or is eliminated more rapidly with no net effect on free plasma concentrations. If the tissue compartment contains the tumor or organs of toxicity, then there may be a clinical effect of this displacement. These are, however, relatively rare but may need to be considered for drugs with very high plasma binding and small volumes of distribution. Cyclosporin A has been reported as being able to cause an increase in the unbound fraction of teniposide and increase the myelosuppressant effect of the latter [45]. In such cases, however, it is difficult to discern whether the effect is exclusively pharmacokinetic and mediated through this mechanism. Other compounds implicated in affecting the protein binding of other drugs include the salicylates, other nonsteroidal anti-inflammatory drugs (NSAIDs), sulfonamides, phenytoin, tetracycline, chloramphenicol, and *p*-aminobenzoic acid.

2.3 Drug Metabolism

Arguably, the most clinically significant drug–drug interactions in medical oncology are caused by interference at this locus. Systemic drug clearance through metabolism occurs predominantly in the liver although other organs such as the kidneys, gut mucosa, and lungs may play a role. Several specific and nonspecific mechanisms are possible. Although it is beyond the scope of this chapter to explore all the possible enzymatic interactions, it is worth differentiating between some of the major mechanisms including those interactions that affect the activity of the metabolic enzymes from those that affect their transcription and/or translation. It is important to note that the time frames involved in

Table 1 Some types of interactions due to inhibition of metabolism and the approximate time-frame (relative to the affected drug) over which causative agent needs to be administered to produce significant interaction

Type of inhibition	Time frame of interaction	Causative agent	Drugs affected
Competitive	Immediate	Ketoconazole	CYP3A substrates, e.g., docetaxel, irinotecan, vinca alkaloids
Competitive + suicide	Immediate-days	Erythromycin, clarithromycin	CYP3A substrates, e.g., docetaxel, irinotecan, vinca alkaloids
	Days	Sorivudine[a]	DPD substrates, e.g., 5-FU
Enzyme production reduction	Days–weeks	5-FU, capecitabine	CYP2C9/2C19 substrates, e.g., warfarin, phenytoin

[a]Requires metabolism by intestinal bacteria to produce inhibitor

producing these various interactions vary significantly depending on the causative mechanism (Table 1). Also, some drugs are at the same time substrates, inhibitors, and inducers of the same metabolic pathway, which will result in complex interactions (e.g., Ritonavir).

2.3.1 Inhibition of Enzyme Activity

Inhibition of metabolism by coadministered drugs can occur by a range of mechanisms including those that are reversible (competitive, noncompetitive) and the irreversible "suicide inhibition." Competitive inhibition is the dominant mechanism when two drugs compete for the same metabolic enzyme and a reduction of the metabolism of one occurs due to displacement from the substrate binding site by the other. Erythromycin and cyclosporin A, for example, are competitive inhibitors of cytochrome P450 3A [46]. However, in many cases, the inhibiting drug is transformed into a highly reactive species by the action of the enzyme, and the two react to form a covalent complex. This type of inhibition, referred to as "mechanism based" or "suicide inhibition" is potentially more significant than competitive inhibition because the extent and duration of enzyme inactivation can both be extensive. Many inhibitors of CYP, which behave as competitive inhibitors in short-term incubations, lead to the formation of nitrosoalkane intermediates. The latter form tight complexes with the CYP heme which, although reversible in theory, are almost impossible to dissociate under physiological conditions, leading to a progressive loss of enzyme activity. This is true for many of the compounds that undergo N-dealkylation reactions such as the macrolide antibiotics (erythromycin, troleandomycin, clarithromycin), some local anesthetics (e.g., lidocaine), diltiazem, fluoxetine, and tamoxifen [47] as well as HIV protease inhibitors [48]. This leads to auto-inactivation of metabolism and may account for nonlinear kinetics of such drugs [49]. Because the complex typically requires significant preincubation with NADPH and enzyme, such interactions may not be detected on a casual screen of concurrent incubation. As a result, the screen may detect only the immediate competitive component (if present) and greatly underestimate the possible interaction which may operate in vivo. Indeed, this form of complex may explain why some drug interactions with drugs such as macrolide antibiotics are much more extensive (see Fig. 1) than predicted from inhibition constants estimated from competitive inhibition experiments [50]. Long-term treatment with such drugs depletes the affected CYP until equilibrium of CYP synthesis and deactivation is established [51]. There is growing realization of the importance of this and other forms of time-dependent inhibition, and hence a greater emphasis by the FDA in recommending such studies be performed during the evaluation of NMEs [52]. The majority of drug companies have in place high-throughput screens for the detection of drugs likely to lead to interactions of this type and standard protocols have been published [53].

Suicide inhibition is not limited to CYP-mediated reactions. For example, an interaction between an antiviral nucleoside, sorivudine, and 5-FU is suspected of resulting in 18 deaths in Japan [54].

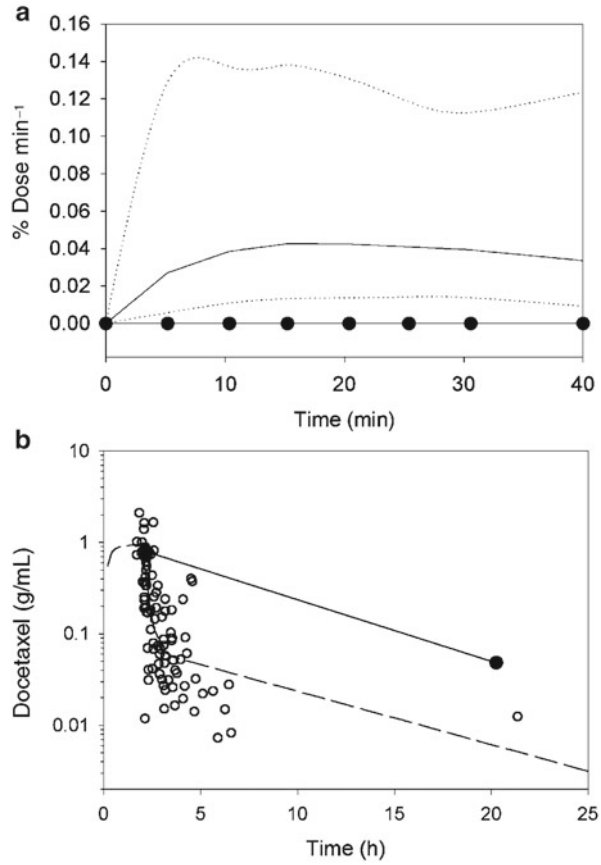

Fig. 1 (**a**) The results of the [^{14}C]erythromycin breath test performed on 54 cancer patients prior to treatment with single-agent docetaxel. The *solid* and *dotted lines* represent the mean and 95 % confidence intervals, respectively, for the evolution of breath radioactivity as part of this test. In general, subjects with early appearing radioactive have greater cytochrome P450 3A activity [169].The results from a subject who was receiving intercurrent clarithromycin for a chest infection at the time of treatment are shown as a *solid line* and *symbols*. The lack of measurable radioactivity in this individual suggests substantial impairment of drug metabolism as a result of a drug–drug interaction. (**b**) Sparse docetaxel plasma concentration data for the same individuals as in (**a**). The patient with impaired CYP3A drug metabolism is identified again with *solid symbols* and *line*. Identification of outliers in such data "clouds" may lead to the detection of unsuspected drug–drug interactions in patient population pharmacokinetic studies

An investigation into the metabolism of sorivudine found that the drug was hydrolyzed into 5-(2-bromovinyl)uracil by intestinal bacteria [55]. The latter is a potent suicide inhibitor of DPD, the major catabolic pathway of 5-FU. Blockage of DPD effectively transforms these patients to a DPD-deficient phenotype [56] with possible lethal consequences. Ironically, 5-(2-bromovinyl)uracil was a well-recognized inhibitor of DPD and had previously been shown to be of benefit when combined with reduced doses of 5-FU in animal models [57]. Indeed, tumoral overexpression of DPD is a recognized mechanism of resistance to fluoropyrimidines, and other suicide inhibitors of DPD have been developed clinically to reduce both the systemic and intratumoral metabolism of 5-FU [58].

Although suicide inhibition is an attractive mechanism to exploit in cancer chemotherapy (e.g., exemestane and aromatase [59]), the situation with sorivudine exemplifies that drugs that produce suicide inhibition of key catabolic enzymes can have serious consequences unless they are administered for the specific purpose. Preclinical screening of compounds for their potential to inhibit

DPD has become commonplace [60, 61]. Several 17α-ethinyl-substituted steroids such as gestodene and ethinylestradiol are suicide inhibitors of CYP3A, and this would suggest potentially multiple drug–drug interactions. However, clinical studies looking for such effects with, for example, ethinylestradiol have not been in support of a major effect under therapeutically relevant conditions [62].

Some of the most potent inhibitors of CYP3A activity are found among the azole antifungals and the HIV protease inhibitors (ritonavir, indinavir, etc.). The latter may be problematic in the setting of HIV-related (e.g., Kaposi's sarcoma) and coincident malignancy, although there is very little prospectively collected data to determine the impact of drug–drug interactions in this setting [63]. Interactions with azole antifungals are likely to occur in the routine oncology setting where they are used in antifungal treatment and prophylaxis. Such combinations may lead to severe, occasionally fatal interactions [64]. The clearance of cyclophosphamide in children receiving fluconazole is reduced by almost 50 % relative to controls, and in vitro experiments were in support of a role of decreased CYP metabolism in this interaction [65]. With the majority of drugs being substrates for CYP3A, there is a growing list of drug–drug interactions involving the azoles [66, 67]. Ketoconazole has long been used as an inhibitor of steroidogenesis in the setting of hormone-dependent prostatic carcinoma [68]. More recently, this property has been identified to be due to inhibition of a different cytochrome P450 species CYP17, and, as reviewed in Reid et al. [69], androgen signaling remains important in many "castration-resistant" prostate cancers, thus motivating the development of new and more selective CYP17 inhibitors. Nevertheless, ketoconazole still features in experimental studies of prostate chemotherapy, sometimes as part of multidrug regimens containing known CYP3A substrates [70].

Other anticancer drugs metabolized by CYP3A include topotecan, irinotecan [71, 72], paclitaxel [73], and docetaxel [74]. With irinotecan, the importance of the CYP3A pathway was recently made clear in a study of ketoconazole pretreatment. Administration of 200 mg of ketoconazole 1 h prior to and 23 h after the infusion of irinotecan did not impact on the clearance of irinotecan. However, it significantly shifted the metabolic profile away from CYP3A-mediated deactivation toward carboxylesterase-mediated activation [75]. The apparent metabolic ratio for CYP3A was decreased approx. 10-fold. Therefore, this interaction could provide a significant safety risk to patients receiving this combination.

The azole antifungals are also inhibitors of CYP2C8, albeit at higher concentrations [76]. Paclitaxel is metabolized more avidly by this isoform [73] and an interaction with high-dose ketoconazole was thought possible on the basis of in vitro studies [77]. However, acute administration of ketoconazole after paclitaxel or 200 mg orally 3 h prior to paclitaxel had no pharmacokinetic consequences [78]. Whether this implies safety of paclitaxel with steady state ketoconazole administration cannot necessarily be implied. Ketoconazole and the other azole antifungals are also inhibitors of P-glycoprotein [79] (but possibly not substrates [80]), and Kehrer et al. considered this as a potential factor in the drug interaction with irinotecan. Drug–drug interactions with azole antifungals may therefore be mediated at least partially through modulation of P-glycoprotein transport. Conversely, many of the investigated inhibitors of P-glycoprotein are inhibitors of CYP3A4, and this may contribute to drug–drug interactions [30]. This includes some of the later generation inhibitors such as PSC833 [81].

One of the issues with investigating drug–drug interactions with several drugs including tamoxifen and the oxazaphosphorines is the fact that their metabolism is extremely complex, with some pathways responsible for both activation and deactivation reactions. The end result is often unpredictable although the multiplicity of pathways usually leads to the lack of a clear effect. For drugs with activation and deactivation reactions catalyzed with different classes of enzymes, inhibition of deactivation can lead to increased activation and toxicity without necessarily impacting on the overall clearance of the parent drug. The previously discussed increased activation of irinotecan to SN-38 which results when ketoconazole inhibits the CYP3A-mediated metabolism of the parent drug [75] is a case in point.

The metabolism of thiotepa, which is at least partially mediated by CYP enzymes [82], has been shown to interfere with that of cyclophosphamide. Significant reductions in the Cmax and AUC of the active 4-hydroxycyclophosphamide metabolite were observed when thiotepa was administered 1 h

prior to cyclophosphamide [83]. Recently, it has been demonstrated that thiotepa is a potent, selective inhibitor of CYP 2B6, one of the key pathways in oxazaphosphorine metabolism [84]. Carmustine, which inhibits human aldehyde dehydrogenase 1, causes a reduction in the deactivation of the aldocyclophosphamide intermediate [85].

2.3.2 Reduced Expression of Metabolic Enzyme

Many significant drug interactions involving fluoropyrimidines (e.g., 5-FU, capecitabine and tegafur) have been reported, particularly in relation to the CYP2C9 substrates phenytoin and (S)warfarin [86–89]. Recently, the clearance of the cytotoxic drug indisulam, which is metabolized by CYP2C9 and CYP2C19 [90], was found to be significantly inhibited by capecitabine in the second cycle, leading to significantly more severe myelosuppression [91]. The mechanism for these interactions remains unclear but is unlikely to be mediated through inhibition of enzyme activity per se as 5-FU does not inhibit the activity of these enzymes [92]. This is supported also by the time lag in the effect of the interaction. For example, in the three cases of phenytoin toxicity described by Brickell [93], patients became symptomatic only several weeks into their fluoropyrimidine regimen. Instead, it is more likely that fluoropyrimidines lead to a change in the expression of metabolic enzymes through their effects on transcription or translation. In a rat model, Yoshisue et al. demonstrated that oral administration of 5-FU reduced the activity of several drug-metabolizing enzymes (phases I and II). The loss of activity was apparently mediated by a reduction of these proteins within the cells and displayed little specificity [94] and occurred over the time frame of 4–7 days. The effect was more marked in enterocytes than hepatocytes, suggesting that interactions might be more pronounced for orally administered drugs. The addition of oxonate appeared to have a protective effect [94], but recently, a report of phenytoin toxicity in a patient receiving S-1 (tegafur, 5-chloro-2,4-dihydroxypryridine and potassium oxonate) [95] suggests that these protective effects are not necessarily transferable to the clinical setting. With the acceptance of oral fluoropyrimidines into the therapy of many common solid cancers, there is an urgent need to better understand and characterize the mechanism for this clinically significant phenomenon. There has also been a case report of a gemcitabine–warfarin interaction [96], but an analysis of the Eli Lily safety data records of Gemcitabine available in 2000 indicates that the risk of interaction is low [97].

Cytokines are able to downregulate the expression of many cytochrome P450 enzymes, and this may explain the apparent link between inflammatory diseases and decreased metabolic drug clearance observed in several disease states [98] including cancer [99]. Exogenous cytokines can have the same effect and the administration of interferon-α to patients was associated with a significant 37 % decrease in cyclophosphamide clearance [39]. This was consistent with a reduction in metabolic activation, because the AUC of the activated metabolite 4-hydroxycyclophosphamide was correspondingly reduced by 45 %. Similar data demonstrating a reduction in 5-FU clearance when administered with interferon-α have been reported [100], but this effect has varied between studies, possibly reflecting differences in schedules and doses [101–103].

As pointed out previously, some of the clinically significant interactions seen in cancer patients arise when the anticancer drug inhibits the metabolism of drugs used for comorbid conditions or in supportive care. Drugs such as some of the anticoagulants (e.g., warfarin) and the anticonvulsant phenytoin are frequently affected. In many cases, a drug–drug interaction is suspected of being mediated through inhibition of drug metabolism although the exact mechanism is not demonstrated. Many of these are detected on the basis of toxicity (e.g., CNS symptoms with phenytoin) or altered blood coagulation parameters (warfarin). Some examples include interactions of erlotinib [104], with warfarin and/or phenytoin. Specific interactions are then examined with either in vitro or in vivo studies of drug metabolism and disposition [105].

2.3.3 Induction of Enzyme Expression

Cytochrome P450s in general are inducible enzymes. In the case of CYP3A4, it is well recognized that glucocorticoids (dexamethasone), barbiturates, rifampicin, and several anticonvulsant agents (phenytoin, carbamazepine) are able to upregulate enzyme activity at the transcriptional level [106] through activation of a number of nuclear receptors including the pregnane X receptor [107]. Drug interactions resulting from increased CYP3A-mediated metabolism following the administration of anticonvulsants and steroids are among the most commonly encountered and problematic drug–drug interactions in medical oncology [5].

Among the CYP3A substrates that have been demonstrated as being affected are the Vinca alkaloids [108], cyclophosphamide [6], irinotecan [109], etoposide, and teniposide [110]. In the study of Baker et al. [110], the range of teniposide clearance in six children on anticonvulsants was 21–54 mL/min/m^2 (22 courses) as compared to 7–17 mL/min/m^2 in a control group matched for age and sex. The clearance of topotecan has also been shown to increase by approx. 50 % when phenytoin is coadministered with concomitant increases in the AUC of the CYP3A metabolite *N*-desmethyltopotecan [111]. Steroids have been reported to induce the metabolism and/or clearance of other drugs, including paclitaxel [112, 113]. Many of these inducers also induce other CYP450 isoforms, including members of the CYP2C superfamily [114]. The oxazaphosphorines ifosfamide and cyclophosphamide display autoinduction of metabolism with unregulated expression of several enzymes in vitro [115]. However, this is a complex effect and its role in drug interactions is not well established. It is complicated by the fact that cyclophosphamide metabolites can also inactivate CYPs, as discussed previously. A confounding factor for the interpretation of drug–drug interactions with drugs such as the anticonvulsants, steroids, and other CYP-inducing drugs (e.g., rifampicin and phenobarbital) is that these compounds can coordinately upregulate P-glycoprotein and other transporters. Hence, even drugs that are not extensively metabolized by the cytochrome P450 system may have altered pharmacokinetics, presumably through an effect on transporter-mediated excretion [116].

2.4 Excretion

Drug interactions leading to modified drug excretion generally relate to interference with transporter function. Specifically, drug transporters in the proximal tubule of the kidney and the bile canaliculi are most likely involved. A major problem is identifying the tissue and transporter most at play in any observed drug–drug interaction. For example, cyclosporin A is an inhibitor of not only CYP3A but also of the transporters MRP-2 and P-glycoprotein. In addition to the effects on drug distribution discussed previously, cyclosporin also modulates both the renal and nonrenal clearance of several drugs including etoposide [117].

2.4.1 Renal Excretion

Many drugs undergo active tubular secretion in the kidney, and there is the potential for drug interactions at that locus. The interaction between methotrexate and probenecid is one of the best known examples [118]. Several transporters of the organic anion transporter (OAT) family are present in the tubular epithelium of the kidney and their inhibition by probenecid, various antibiotics, and NSAIDs is the likely mechanism [119–122]. This type of interaction may also occur with other antifolate analogs, but the clinical significance is not clear. For example, ibuprofen was found to reduce the clearance of pemetrexed by 16 %, whereas aspirin was without effect [123]. Alterations in tubular secretion may also be modified through less direct effects. For example, Beorlegui et al. reported an apparent interaction

between omeprazole and methotrexate [124]. They argued that inhibition of the tubular proton pump by omeprazole caused a reduction in methotrexate secretion because of the requirement for protons by the latter. A recent study has examined the ability of proton pump inhibitors to inhibit BCRP-mediated excretion of methotrexate. Although relatively weak inhibitors of BCRP, concomitant administration of proton pump inhibitors was associated with delayed elimination of methotrexate in a study of 74 patients receiving high-dose chemotherapy [125].

2.4.2 Biliary Excretion

Biliary excretion is a major route of excretion of many anticancer drugs [126, 127]. As mentioned previously, several transporters are present at the canalicular membrane including P-glycoprotein, MRP1, MRP-2 (cMOAT), and MRP-3. Evaluation of drug interactions specifically involving biliary excretion is problematic because measurement of biliary excretion is not routinely possible. Nevertheless, some of the pharmacokinetic interactions that have been reported might be attributable to this locus [128], but confirmatory evidence is not available. Preclinical experiments using perfused rat liver preparations have shown that inhibitors of P-glycoprotein may reduce biliary excretion of compounds known to be excreted such as doxorubicin [129, 130]. Using a similar model, Smit et al. demonstrated the potential for interactions between Vinca alkaloids and doxorubicin [130].

Transporters, as with metabolic enzymes, can be involved in drug–drug interactions as a result of induction of activity. It is increasingly recognized that the induction of transporters such as P-glycoprotein can be caused by agents that overlap substantially with those capable of inducing CYP3A [131]. As a result, some interactions with anticonvulsants may actually be the results of upregulation of important transporters. This is a possible mechanism for the interaction between methotrexate and anticonvulsants [116]. BCRP induction and increased biliary clearance of the active metabolite of irinotecan, SN-38, has been proposed [132] to explain the reported reduction in irinotecan in a patient receiving irinotecan and the fluoropyrimidine-based S-1 [133].

3 When the Problem Is Not the Drug But Its Vehicle

In some instances, apparently classical drug–drug interactions have subsequently been shown to have little to do with the drugs involved. Consideration of these alternate mechanisms should be part of the investigation of drug–drug interactions.

Many drugs are not sufficiently soluble to be administered in purely aqueous solvents. Instead, a formulation component is frequently a surfactant (e.g., Cremophor EL, sorbitol, and Tween). These compounds should not be dismissed as inactive and inconsequential. For example, Cremophor EL has some membrane effects and has been investigated for its ability to reverse P-glycoprotein-mediated multidrug resistance [134]. It also modulates differentially the toxicity of cisplatin to marrow and tumor cells [135, 136]. Cremophor EL has been shown to modulate the distribution and elimination of doxorubicin in both preclinical and clinical studies [134, 137, 138]. In the study of Millward et al., 11 patients were randomized to receive either 50 mg/m^2 of doxorubicin alone or in combination with 30 mL/m^2 of Cremophor EL. They were then crossed over to the alternative regimen. Cremophor significantly reduced the clearance of doxorubicin by approx. 20 %. The metabolite doxorubicinol was present in higher concentrations after Cremophor EL administration, resulting in an almost doubling of its AUC. Cremophor EL is also the likely major component of the interaction between Taxol® and anthracyclines. When Taxol® (paclitaxel formulated in Cremophor EL) is administered immediately prior to a doxorubicin infusion or as an infusion prior to bolus doxorubicin, the clearance of doxorubicin is reduced by 20–30 % and concentrations of doxorubicinol are again greatly increased

[128, 139, 140]. The latter suggests a possible reduction in the biliary clearance of the metabolite or a redistribution phenomenon secondary to the membrane effects of the Cremophor EL. Indeed, even when paclitaxel is administered 24 h after doxorubicin, sudden rebound profiles of doxorubicinol are observed [140]. The effects of Cremophor EL on the hepatic disposition of paclitaxel itself also appear to be due to nonspecific effects on distribution rather than direct effects on biliary excretion [141]. Polysorbate 80 (also known as Tween 80) is used in the current formulation of docetaxel and etoposide. The coadministration of polysorbate 80 by itself or in the etoposide formulation had up to a twofold effect on doxorubicin AUC, mainly through increased early tissue distribution [142]. The effect of etoposide on methotrexate pharmacokinetics reported by Paal et al. may also be due to the nonspecific effects of the Tween 80 present in etoposide [143]. Indeed, the rebound profile of plasma methotrexate that occurred a few hours following etoposide is qualitatively similar to the rebound of doxorubicinol concentrations when paclitaxel is administered in Cremophor EL [140].

4 Predicting Interactions

The prediction and evaluation of significant drug–drug interactions has in many instances been a rather piecemeal process. However, significant inroads in our understanding of the loci of these interactions have been made in vitro through to in vivo [50], and there have been instances of systematic screening for these interactions during drug development [144]. Significant potential for drug–drug interactions with an NME is usually considered a block to further development. Because of the mounting costs of drug development, particularly during late-stage clinical trials, early detection of such properties is vital. Withdrawal of FDA approval of registered drugs, such as was the case for terfenadine, has led to much closer scrutiny of potential interaction by a variety of means.

4.1 Historical Data

In assessing likely interactions for a new drug, it is enormously useful if the compound has metabolism and excretion properties similar to those of previously characterized analogs. In the absence of such information, the usual sequential processes of in vitro and preclinical studies need to be carried out. One of the major problems is the manner in which these experiments have been performed often varies significantly from study to study, and the data are reported in a range of formats. There is therefore a real need for consistent experimental design and reporting in such studies [145]. In the absence of such a platform, however, other groups have assembled electronic databases, which at least should enable some qualitative predictions of potential drug–drug interactions [146]. A drug–drug interaction locus identified in vitro will be relevant only if this pathway represents a major route of elimination for the candidate drug [147]. This is one of the major issues that databases such as the one proposed by Bonnabry et al. are trying to address [146].

4.2 Predicted from In Vitro Experiments

The advantage of being able readily to obtain drug metabolism enzymes from recombinant sources (insect cells, human lymphoblastoid cells, yeast, bacteria) has greatly facilitated the task of establishing the important routes of metabolism likely to be encountered when administered to patients. Similarly, these systems can also be used to search for potential drug interactions. Rapid throughput

systems have been developed that use specific fluorogenic substrates to facilitate the large-scale and thorough screening required [148, 149]. The much larger potential problem is that relating to drug transporters. Here, the best systems are likely to be panels of stably transfected cells expressing each known transporter, starting probably with P-glycoprotein, BCRP, and MRP-1 and MRP-2. Alternatively, cells mimicking the relevant in vivo system may be used to test simultaneously the effects of expression of transporters and metabolic enzymes [150]. A specific problem with the in vitro testing of drug interactions with alkylating anticancer drugs relates to the fact that these have complex pharmacology featuring multiple, often unstable species. Comprehensive studies of metabolic interactions with such drugs are difficult to perform and to interpret [65, 83–85, 115, 151–153].

4.3 In Silico

Ultimately, the structure–activity relationships for each enzyme/transporter may become sufficiently documented by the use of large databases to enable direct in silico predictions of the metabolic and transporter properties of new drugs. These in turn would enable extrapolation of likely in vivo disposition, pharmacogenetics, and possible drug interactions. The use of such predictive models and their validation has been reviewed [154]. As mentioned previously, predictions are complicated by the overlap between transporters and metabolism. The interaction of etoposide with cyclosporin reported by Bisogno et al. [155] could, on the basis of other observations discussed above, be the result of interaction with metabolism, biliary and intestinal secretion, and protein binding. Likewise cimetidine, which is often used as a relatively nonselective CYP450 inhibitor, is also an inhibitor of OAT in the kidney. Hence the mechanism for the drug interaction reported between cimetidine and epirubicin is also difficult to identify [156]. Ultimately, therefore, classical in vitro and in vivo approaches for the investigation of drug interactions are required.

4.4 Preclinical Studies

Animal models may enable certain drug–drug interactions to be examined in a more physiologically meaningful fashion [157]. However, some major interspecies differences exist with respect to the metabolism and excretion of drugs. For example, rats are relatively deficient in the aldo-ketoreductases that metabolize anthracyclines to their corresponding C-13 alcohols [158]. The cytochrome P450 enzymes also often differ markedly between species, not only in terms of their substrate affinities and reaction products [159, 160] but also in their susceptibility to inducers and inhibitors [161]. The regulation of CYP450 is also highly species dependent. For example, induction of CYP3A by rifampicin is pronounced in humans and rabbits but not in rats [162]. Hence, investigations of drug interactions require the use of the most representative animal or in vitro system to ensure relevance.

4.5 Clinical Studies

Many of the clinical drug interactions reported have been detected on the basis on comparisons of relatively small patient groups. In most cases, data are compared to historical or case-matched groups. Even less reliable are isolated case reports that report suspected drug interactions on the basis of abnormal drug disposition. Because of the large interindividual differences in metabolism and

disposition, many of these studies are underpowered and biased. Ideally, clinical studies should be performed using a randomized crossover design as is normally used in trials to support of registration of compounds. FDA guidelines are available for the design of such trials (http://www.fda.gov/cder/guidance/). However, in medical oncology, many of the drugs are inherently unsafe. Usually drug–drug interactions are investigated because they pose a threat either to the safety or the efficacy of a compound, and it becomes ethically difficult to propose trials likely to produce these outcomes in patients who cannot really afford either. One alternative is to incorporate extensive population pharmacokinetics as part of Phase II and III trials of anticancer drugs. This then enables the identification of patients with unusual pharmacokinetic data and the identification of possible interactions [163, 164]. One of the down sides of this approach is that studies may require a very large number of subjects (several hundred) unless the interacting drug is very commonly used in the intended setting (e.g., anticonvulsants in CNS malignancy). The drug candidates most worthy of further study for possible interactions are obviously those that are likely to be coadministered and for which there is some support from in silico, in vitro, preclinical, or clinical data. Other factors to be considered include the doses of the agents, their dosing regimen (single or multiple doses), and the timing of the administration (A before B, A after B, or A + B). The latter is particularly important when the drug interaction is modulated through alteration of gene expression. For example, a study on the effects of rifampicin on drug clearance would require several days of rifampicin treatment prior to administration of the test drug to ensure maximal induction of the relevant enzyme system. Drug scheduling is also a vital consideration given that drugs that may lead to minor interactions when given concurrently can be problematic over longer time-frames of administration through delayed suicide inhibition (Table 1).

4.6 Clinical Setting

A number of tools and databases have been developed to assist clinicians and oncology pharmacists to consider the likelihood of patients experiencing drug–drug interactions during chemotherapy. Using one such software-based system, Riechelmann et al. found that 109 of 405 patients who self-reported their medication status could have been the subject of 276 drug interactions, the majority of which were based on noncancer drugs. Not surprisingly, the risk of these was increased in patients taking larger numbers of drugs and those with comorbid conditions or brain tumors [165]. Increasingly sophisticated recording of electronic patient cases and interrogation with software-based filters may provide improved safety of patients through detection of possible drug–drug interactions. These should not substitute for the vigilance of oncology staff however.

5 Exploiting Drug Interactions

5.1 Oral Delivery of Drugs

Drug interactions, as shown in the relevant sections above, can have both detrimental and beneficial effects in chemotherapy. In particular, the inhibition of gastrointestinal transporters and enzymes greatly improves the bioavailability of oral drugs and enables oral delivery of some drugs that could previously not be administered orally. Poor bioavailability is usually associated with highly variable systemic concentrations and an additional possible benefit could be a reduction in intra- and interpatient variability in pharmacokinetics, although this is unlikely to be reduced to less than that encountered

with parenteral administration. An example of successful exploitation of this strategy is the development of orally administered fluoropyrimidines that incorporate an inhibitor of DPD [25]. Certainly in the therapy of HIV, some of the newer protease inhibitors are best administered with low-dose ritonavir (an earlier protease inhibitor) purely to enhance their pharmacokinetic profiles through inhibition of CYP3A (reviewed in [166]).

5.2 Systemic Administration

As previously discussed for the DPD inhibitors, administration of an inhibitor may greatly reduce intratumoral metabolism that in some cases may act as a significant mechanism of resistance in vivo. The second possible advantage of the suicide inhibitors of DPD (e.g., ethinyl uracil) is that when coadministered, the elimination of 5-FU is no longer mostly via DPD catabolism but by renal excretion. The latter pathway is inherently less variable and more predictable than DPD, thereby potentially facilitating the dose individualization of treatment [26], although this has not been exploited in Phase III trials of the combination [167].

Other DPD inhibitors have been developed in combination with oral prodrugs of 5-FU. These combination products include S-1 and UFT. S-1 also contains potassium oxonate as an inhibitor of thymidine kinase, and this assists in reducing activation of the 5-FU in the gastrointestinal mucosa.

The reduction in fluoropyrimidine dose that is made possible with DPD inhibitors underscores another way in which drug interactions can be exploited. In the early years of cyclosporin A use, the interaction between diltiazem was characterized and exploited to enable substantial cost savings. However, interactions based on reversible mechanisms are very difficult to predict because of constantly varying profiles on the target and inhibitor drug. In oncology, because of the limited safety of many of these agents, introduction of an additional variable into the chemotherapy regimen may become counterproductive, and such strategies are used only in the experimental setting. Such interactions also need to be proven from the aspect of activity, and the substantial costs of the additional Phase III trials might outweigh the savings. Ironically, once the cost of cyclosporin was reduced, it was subsequently proposed as a modulator of several agents to improve their pharmacokinetic and pharmacodynamic properties.

6 Future Directions

From a drug development aspect, the most exciting proposition is that accumulated knowledge about current drugs and their metabolism will enable some in silico prediction of likely mechanism of clearance and the potential drug interactions that might arise. This could greatly assist the rational refinement of drug leads and reduce the expense of development. The possibility of using well-characterized drug–drug interactions to enable oral therapy may prove obvious potential advantages, but this has not yet been developed into the routine clinical setting. Finally, genomic advances are likely to advance greatly our understanding of drug–drug interactions, particularly as they relate to instances in which drug metabolism is induced. For example, cytochrome P450 3A expression is extremely sensitive to control from interactions of nuclear receptors with the RXR. Some of the nuclear factors involved (CAR, PXR, and PPAR) have been shown to be subject to functional polymorphic variation [168]. The elucidation of genotype/phenotype associations may help ultimately enable prediction of drug–drug interactions in individual subjects.

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ABC Transporters: Involvement in Multidrug Resistance and Drug Disposition

Paul R. Massey, Tito Fojo, and Susan E. Bates

Abstract ATP-binding cassette (ABC) transporters, among the largest of the transporter superfamilies, are found in normal tissues and transport a wide range of substrates important in normal physiology. One normal tissue function of these transporters is protection from toxic compounds including xenobiotics. By limiting tissue exposure to substrates, ABC transporters may contribute to the blood–brain barrier, the maternal–fetal barrier, and the mucosal barrier that limits oral absorption of compounds. Because a range of chemotherapeutic drugs have been found to be substrates for several of these transporters, many have inferred that expression of transporters in tumor cells has the potential to confer drug resistance; indeed, several transporters are referred to as multidrug transporters. This chapter will review the role of these multidrug transporters in oncology and in normal tissue.

Keywords P-glycoprotein • ABCG2 • Multidrug transporters • ABC transporters

1 Mechanisms of Drug Resistance

1.1 Cellular Mechanisms of Drug Resistance

Multiple cellular mechanisms of drug resistance have been described, and a schematic for these is shown in Fig. 1. These can be classified as those that (1) affect drug accumulation through reduced uptake or enhanced efflux, (2) impact drug metabolism leading to decreased activation or increased inactivation, (3) alter the molecular target either by reducing expression or by altering affinity, (4) increase repair of drug-induced damage, and (5) support cell survival following drug exposure, allowing time for repair and/or adjustments to be made. Anticancer agents may be subject to one or all of these mechanisms of resistance. Doxorubicin is an illustrative example: (a) drug accumulation may be reduced by the MDR1/Pgp multidrug transporter; (b) glucuronidation may inactivate the drug once inside the cell; (c) its molecular target, topoisomerase IIa, may be reduced to levels that impair drug

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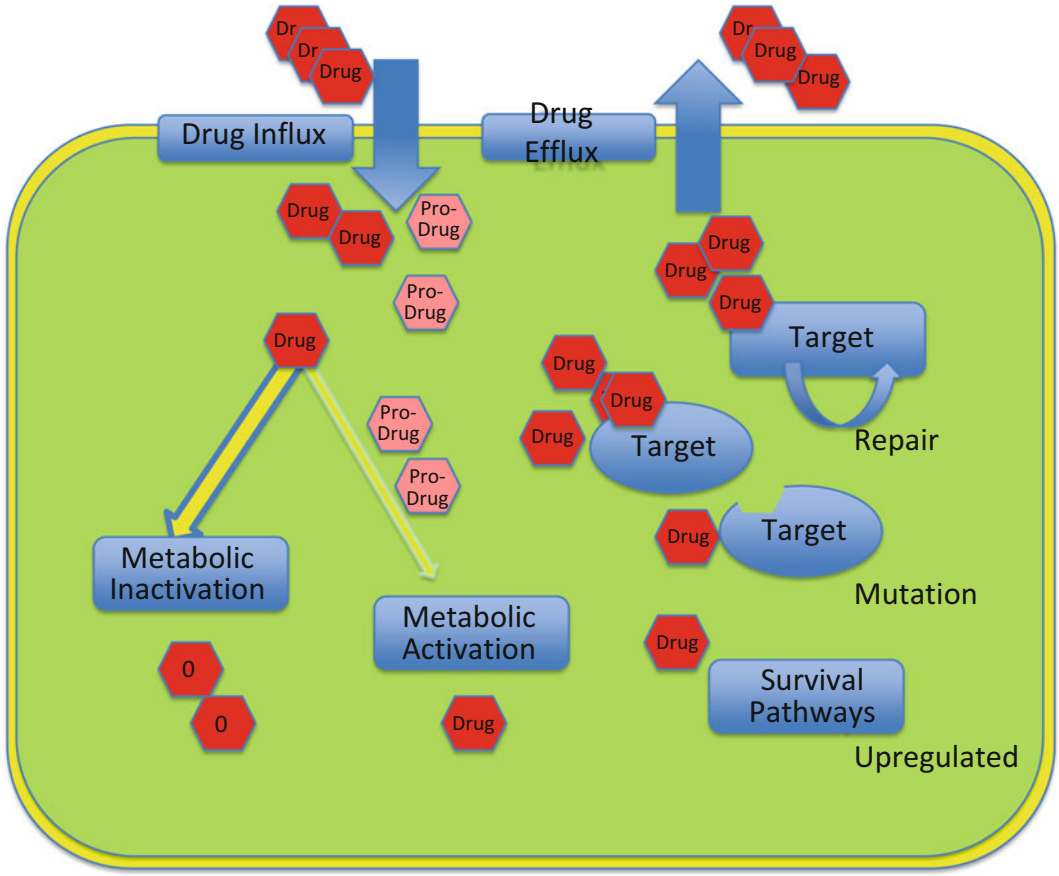

Fig. 1 Drug resistance mechanisms. Schematic showing levels of cellular mechanisms of drug resistance: reduced accumulation through reduced drug influx or increased drug efflux, altered drug metabolism including both decreased activation and increased inactivation, altered drug target through mutation or changed expression level, and altered repair through enhanced damage repair pathways or enhanced survival pathways. Most cellular mechanisms of drug resistance can be classified according to this schema

activity or may be rendered insensitive by an acquired mutation; and (d) p53-mediated G1 arrest or Bcl-2 overexpression may promote cell survival by inhibiting apoptosis. Imatinib provides another example: (a) increased expression of P-glycoprotein (Pgp) and ABCG2 confers resistance in in vitro models, as does decreased expression of the organic cation uptake transporter gene (OCT1) [48, 190]; (b) increased levels of a_1-acid glycoprotein (AGP) can alter its disposition [56]; (c) mutation of the target, Bcr-abl, has been linked to resistance through reduced affinity; and (d) cellular quiescence is thought to render a fraction of cells tolerant [17]. The purpose of this chapter is to evaluate the contribution of ABC transporters to drug resistance and to the pharmacology of anticancer drugs as one of the contributors to the problem of drug resistance. However, as the examples cited indicate, a narrow focus on one resistance mechanism underestimates the complexity of the problem.

The number of possible mediators of drug resistance has expanded beyond what single gene analysis can accomplish. It is thus important that clinical samples be assayed for multiple mechanisms of drug resistance. Assays should include uptake and efflux transporters, detoxification and conjugating enzymes, and survival and repair mechanisms. New technologies that allow unbiased simultaneous screening of drug resistance mechanisms should be widely employed in the effort to improve the clinical outcome of cancer therapy.

1.2 ABC Transporters

The functional ATP-binding cassette (ABC) transporter requires at a minimum two transmembrane domains (TMDs) and two ATP-binding domains [42]. The latter bind and hydrolyze ATP, and the derived energy is used for transport of the substrate by the TMDs across the cell membrane [42]. The ATP-binding domains are highly conserved across the ABC superfamily, and characteristic sequence motifs known as the Walker A, Walker B, and Walker C signature regions are critical for ATP binding and hydrolysis. Conservation of these regions is such that ABC transporters can be identified by homology searches in genetic databases. Human genome sequencing identified 48 ABC transporters that group into seven subfamilies based on ATP-binding domain homology and designated ABCA through ABCG. Table 1 lists the seven ABC subfamilies and known members of each, with currently understood functions and known syndromes associated with mutations or deletion of each transporter.

1.3 Multidrug Transporters

Although 48 ABC transporters have been definitively identified, a more limited number are thought to transport anticancer compounds and to have a potential role in multidrug resistance, itemized in Table 2. These include P-glycoprotein, MRP1–5, BSEP (sPgp), ABCA2, and ABCG2. While it has been difficult to establish the contribution of these transporters as mediators of drug resistance in the clinic, steadily expanding data from pharmacology suggests a major role in determining drug absorption, distribution, and entry into sanctuary sites. Thus, "pharmacologic" resistance could result from higher levels of expression of the transporters resulting in decreased drug absorption and decreased entry into sanctuary sites. Resistance of this nature could augment cellular mechanisms of resistance, including but not limited to transporter-mediated drug efflux.

2 P-Glycoprotein

2.1 Background

The most intensively studied multidrug transporter is P-glycoprotein, the product of the MDR1 gene. Pgp has the characteristic "full transporter" structure with two ATP-binding domains and two transmembrane domains each comprising six transmembrane segments that evidence indicates confer substrate specificity. Pgp is known to transport commonly used cancer chemotherapeutics including anthracyclines such as daunorubicin and doxorubicin, taxanes, vinca alkaloids, and podophyllotoxins (Table 2). However, work with the National Cancer Institute (NCI) drug screen suggested there were hundreds, if not thousands, of Pgp substrates [8, 108]. This is not surprising given the presumed role of Pgp in normal physiology: protection of the organism from exposure to xenobiotics.

2.2 Physiologic Role of Pgp

A protective role for Pgp in normal physiology has received support from several sources. First, the substrate profile encompasses a wide array of structurally unrelated natural products or xenobiotics

Table 1 Human ABC transporters

HUGO name[a]	Common name	Disease associated with absence or mutation	Function
ABCA1	ABC1	Tangier disease	Cholesterol efflux onto HDL
ABCA2	ABC2	Alzheimer's disease	Lipid transport
ABCA3	ABC3, ABCC	Fatal surfactant deficiency, interstitial lung disease	Pulmonary surfactant secretion
ABCA4	ABCR	Stargardt disease, retinitis pigmentosum, cone-rod dystrophy	*N*-retinylidiene-PE efflux
ABCA5			Lysosomal trafficking
ABCA6			Lipid homeostasis
ABCA7			Lipid transport
ABCA8			Lipid metabolism
ABCA9			Macrophage lipid homeostasis
ABCA10			Lipid transport
ABCA12		Harlequin ichthyosis, lamellar ichthyosis type 2	Lipid trafficking
ABCA13			
ABCB1	MDR1, P-glycoprotein		Xenobiotic protection
ABCB2	TAP1	Immune deficiency	Peptide transport
ABCB3	TAP2	Immune deficiency	Peptide transport
ABCB4	MDR2, MDR3	Progressive familial intrahepatic cholestasis type 3	Phosphatidylcholine transport
ABCB5			
ABCB6	MTABC3		
ABCB7	ABC7	X-linked sideroblastic anemia and ataxia	
ABCB8	MABC1		
ABCB9			
ABCB10	MTABC2		
ABCB11	SPgp, BSEP	Progressive familial intrahepatic cholestasis type 2	Bile salts
ABCC1	MRP1		Xenobiotic protection
ABCC2	MRP2, CMOAT	Dubin–Johnson syndrome	Bilirubin glucuronide
ABCC3	MRP3		
ABCC4	MRP4		
ABCC5	MRP5		Urinary excretion of bile salts
ABCC6	MRP6	Pseudoxanthoma elasticum	
CFTR	CFTR	Cystic fibrosis	Chloride ion channel
ABCC8	SUR	Familial hyperinsulinemic hypoglycemia of infancy	Regulation of insulin secretion
ABCC9	SUR2		
ABCC10	MRP7		
ABCC11	MRP8		Bile acids and conjugated steroids
ABCC12	MRP9		
ABCD1	ALD, ALDP	X-linked adrenoleukodystrophy	Regulation of VLCFA metabolism and transport
ABCD2	ALDL1, ALDR, ALDRP		
ABCD3	PXMP1, PMP70		
ABCD4	PMP69, P70R		
ABCE1	OABP, RNS4I		Initiation of translation of proteins
ABCF1	ABC50		Initiation of translation of proteins
ABCF2			

(continued)

Table 1 (continued)

HUGO name[a]	Common name	Disease associated with absence or mutation	Function
ABCF3	GCN20		Initiation of translation of proteins
ABCG1	ABC8, White		
ABCG2	MXR, ABCP, BCRP	Gout, hyperuricemia	Xenobiotic protection, uric acid modulation
ABCG4	White2		
ABCG5	White3	Sitosterolemia	Plant sterols
ABCG8		Sitosterolemia	Plant sterols

Abbreviation: *VLCFA* very long chain fatty acids
[a]Adapted from [42, 43, 62, 90]

expected for a protein tasked with a protective function. Second, the distribution of Pgp includes high levels on the apical surface of cells in the gastrointestinal tract and the kidney and on the canalicular surface of hepatocytes in the liver—all organs involved in the elimination of toxins. High levels are also found in the adrenal cortex, where Pgp is thought to protect the adrenal cell membrane from the toxic effects of glucocorticoids [13] and in the capillary endothelium of the brain and the testis where Pgp is thought to have a role in the blood–testis and the blood–brain barrier [14, 158, 174]. The latter is complemented by the high Pgp levels on the apical surface of the epithelial cells of the choroid plexus, which may prevent movement of compounds out of the CSF into the brain parenchyma [147]. Third, knockout studies in mice support a protective role for Pgp. Although mice lacking the orthologues for Pgp, *mdr1a* and *mdr1b*, developed and reproduced normally, the mice died unexpectedly when exposed to the neurotoxic effects of the prophylactic pesticide ivermectin [159]. Pharmacokinetic studies demonstrated an 87-fold increase in ivermectin levels in the brain with a 3.3-fold increase in plasma levels in the knockout mouse. As shown in Table 3, further studies in knockout mice have shown altered blood and brain accumulation of numerous compounds, including cyclosporine A [161], digoxin [161], vinblastine [178], loperamide [160], verapamil [67], quinidine [53], nelfinavir [33], and corticosterone [81]. Drug accumulation in the brain of *mdr*-deficient mice is 35-, 29-, 22-, and 17-fold higher for digoxin, quinidine, vinblastine, and cyclosporin A, respectively. These studies in mice lacking the MDR1 orthologues have provided pharmacokinetic information not otherwise attainable in humans, although the extent to which the findings in this model can be extrapolated to humans is unknown. The development of radiotracers from some of these substrates may offer the possibility of clinical study of this question [95].

Consistent with a protective role for Pgp, several studies have shown that Pgp limits the absorption of its substrates from the gastrointestinal tract, presumably by pumping them back into the intestinal lumen. Studies with Pgp inhibitors administered either orally or intravenously (discussed in more detail below) have shown increased bioavailability of orally administered Pgp substrates by coadministration with a Pgp inhibitor. For example, oral absorption of paclitaxel was increased eightfold when administered concurrently with cyclosporin A [125]. Similarly, oral bioavailability of paclitaxel calculated at 30 % ± 15 % when the drug was administered alone was increased to 50 % in patients when administered in combination with elacridar [121]. In animal models, similar increases for paclitaxel have also been reported in combination with valspodar, OC144-093, and MS-209 [64, 88, 179]. Thus, the ability to modulate oral bioavailability could improve the efficacy of anticancer agents, a clinically valuable approach given that anticancer agents available as oral formulations are a preferable alternative to parenteral administration for patients.

Table 2 Anticancer agent specificities of ABC transporters

HUGO name[a]	ABCA2	ABCB1	ABCB4	ABCB1	ABCC1	ABCC2	ABCC3	ABCC4	ABCC5	ABCC6	ABCC10	ABCC11	ABCC12	ABCG2
Common name	ABC2	MDR1	MDR2 MDR3	SPgp	MRP1	MRP2	MRP3	MRP4	MRP5	MRP6	MRP7	MRP8	MRP9	BCRP
Adriamycin		+			+	+								
Paclitaxel		+	+	+		+					+			
Vinblastine		+			+	+					+			
Vincristine		+			+	+					+			
Etoposide		+			+	+	+			+				+
Cisplatin					+	+			+	+				
Organic anions					+	+	+	+	+	+		+		
Methotrexate		+			+	+	+	+	+	+				+
17β-E2G					+	+	+	+		+	+	+		
6-Mercaptopurine								+	+					
6-Thioguanine								+	+					
Topotecan		+				+		+						+
SN-38		+			+	+		+						+
Mitoxantrone	+	+			+	+								+
Estramustine	+													
Gefitinib		+												+
Erlotinib		+												+
Imatinib		+			+									+
Dasatinib		+			+									+
Nilotinib		+			+									+
Lapatinib		+												+
Sorafenib		+												+
Sunitinib		+												+

Abbreviations: *SN38* active metabolite of irinotecan, *17β-E2G* 17β-estradiol glucuronide

[a]Compiled from [20, 41, 97, 98, 102, 103, 144, 171, 173]

Table 3 Increased drug levels in MDR-deficient (*mdr1a*$^{-/-}$ or *1a/1b*$^{-/-}$) mice

Drug	*T* (h)	Deleted	Blood	Liver	Kidney	Lung	Testis	Brain	Reference
Ivermectin	4.0	*Mdr1a*$^{-/-}$	3.3	3.8	3.0	4.0	n.a.	87.0	[159]
Cyclosporin A	4.0	*Mdr1a*$^{-/-}$	1.4	1.2	1.0	1.2	2.6	17.0	[161]
Digoxin	4.0	*Mdr1a*$^{-/-}$	1.9	2.0	1.9	2.2	2.8	35.3	[161]
Morphine	4.0	*Mdr1a*$^{-/-}$	1.1	1.1	0.8	1.1	0.7	1.7	[161]
	4.0	*Mdr1a*$^{-/-}$	1.2	n.a.	n.a.	n.a.	n.a.	2.6	[195]
Dexamethasone	4.0	*Mdr1a*$^{-/-}$	1.0	1.1	1.2	0.8	1.2	2.5	[161]
	1.0	*Mdr1a*$^{-/-}$	1.2	1.1	n.a.	n.a.	n.a.	4.2	[126]
Vinblastine	4.0	*Mdr1a*$^{-/-}$	1.7	2.4	2.3	2.1	2.5	22.4	[178]
Loperamide	4.0	*Mdr1a*$^{-/-}$	2.0	3.1	1.5	1.7	3.8	13.5	[160]
Ondansetron	0.5	*Mdr1a*$^{-/-}$	1.0	0.9	0.9	1.1	1.9	4.0	[160]
Verapamil	1.0	*Mdr1a*$^{-/-}$	1.2	1.1	1.2	0.9	3.4	9.5	[67]
Doxorubicin	4.0	*Mdr1a*$^{-/-}$	0.9	4.5	1.0	1.0	n.a.	2.8	[180]
Quinidine	4.0	*Mdr1a*$^{-/-}$	3.7	4.3	2.5	2.7	n.a.	29.2	[53]
Nelfinavir	2.0	*Mdr1a*$^{-/-}$	1.1	n.a.	n.a.	n.a.	4.1	27.9	[33]
Corticosterone	1.0	*Mdr1a*$^{-/-}$	0.9	1.0	n.a.	n.a.	1.1	1.3	[81]
Cortisol	1.0	*Mdr1a*$^{-/-}$	1.0	1.0	n.a.	n.a.	1.1	3.9	[81]
Sparfloxacin	4.0	*Mdr1a*$^{-/-}$	0.97	n.a.	n.a.	n.a.	n.a.	3.2	[40]
Grepafloxacin	1.0	*Mdr1a/1b*$^{-/-}$	1.1	1.0	1.4	1.0	0.62	2.8	[196]

2.3 Pgp in Multidrug Resistance

The role of Pgp in conferring multidrug resistance has been addressed in numerous preclinical and clinical studies. Two approaches have been used to assess its importance. The first approach determined the expression of Pgp in tumor tissues and sought to correlate expression levels with outcome. Unfortunately, a validated clinical test with a specific and sensitive antibody has never been developed. Most studies assessing expression have been small and anecdotal. The second approach looked for clinical benefit following the addition of a Pgp inhibitor to a chemotherapy regimen. A third approach, which has never been fully developed, employs radionuclide imaging to evaluate tumor uptake of a chemotherapy agent or surrogate.

2.3.1 Pgp Expression as a Marker of Drug Resistance

Attempts to correlate Pgp expression with outcome led to studies that found Pgp expression in numerous tumor types. In the majority of studies, Pgp expression has been detected using antibodies; in patients with acute myelogenous leukemia (AML), functional studies examining daunorubicin accumulation, for example, confirm expression. In some studies, its encoding mRNA, MDR-1, has been analyzed. High levels of expression are found in renal, gastrointestinal, adrenocortical, hepatocellular, and pancreatic cancers and AML [49]. Lower levels that increase with treatment have been observed in breast cancer, ovarian cancer, leukemia, lymphoma, lung cancer, and sarcoma [15]. The most consistent expression data have been reported in AML, where expression is observed in 30–50 % of newly diagnosed cases. The lack of a validated assay for Pgp has meant that large variations in expression levels have been reported. For example, a meta-analysis concluded that 40 % of breast cancers express Pgp, but the studies included in the analysis reported expression levels ranging from 0 to 80 % [176]. Some of this variation is due to different patient populations—Pgp expression was reported in 11 % of pre-therapy and 30 % of posttreatment samples [124]—but the extreme variability from 0 to 80 % highlights the need for a validated assay.

Table 4 Pgp substrates and inhibitors in clinical use in oncology

	Pgp inhibitors		
Pgp substrates	First-generation agents	Second-generation agents	Third-generation agents
Doxorubicin	Verapamil	R-Verapamil	Elacridar (GF120918)
Daunorubicin	Quinidine	Valspodar (PSC833)	Laniquidar (R101933)
Epirubicin	Quinine	Dexniguldipine	Tariquidar (XR9576)
Paclitaxel	Amiodarone	Biricodar (VX710)	Zosuquidar (LY335979)
Taxotere	Nifedipine		Dofequidar (MS-209)
Vincristine	Cyclosporine A		OC144-093
Vinblastine			CBT-1
VP-16			
Mitoxantrone			
Bisantrene			
Homoharringtonine			
Actinomycin D			

2.4 Inhibition of Pgp in the Clinic

The second approach used to assess the importance of Pgp has been to look for benefit in clinical trials using inhibitors of Pgp. With the description by Tsuruo in 1981 of the ability of verapamil to inhibit Pgp-mediated drug efflux, the reversal of drug resistance became a tantalizing possibility [177]; indeed, verapamil would be the first in a series of inhibitors to be tested in patients. These inhibitors can be roughly grouped based on chronology and on clinical characteristics (Table 4).

2.4.1 First-Generation Pgp Inhibitors

The identification of verapamil as a Pgp inhibitor was all the more provocative because it was a compound already in clinical use. Soon thereafter, additional agents were identified as inhibitors of Pgp-mediated efflux and, like verapamil, these were already available for use in clinical trials. These first-generation agents were rapidly tested as inhibitors of Pgp in numerous clinical trials, but the outcome was inconclusive and, side effects prevented advancement of drug doses [137].

Several studies suggest that many of these first-generation inhibitors were not sufficiently potent to inhibit Pgp in vivo. Abraham et al. reported results of a trial in adrenocortical cancer using mitotane [2]. Rhodamine efflux from Pgp-expressing circulating $CD56^{+}$ cells, an established surrogate, failed to demonstrate inhibition of Pgp in patients despite steady-state mitotane levels exceeding 10 mg/ml, a level that could readily block Pgp in vitro. Cisternino could not demonstrate increased brain uptake of vinblastine in mice following brain perfusion with 1–2 mM verapamil, although vinblastine accumulation was increased 2.7-fold in *mdr1a*$^{-/-}$ mice and 3-fold in valspodar or elacridar-treated mice [35].

Together, data suggest the failure of the first-generation trials was due to a lack of potency in Pgp inhibition. A single trial alone suggested an advantage when cyclosporine A was added to a standard daunorubicin and Ara-C regimen in AML [113]. Although there was no difference in complete response rates (33 % vs. 39 % for the combination), a statistically significant difference in resistant disease (47 % vs. 31 %), 2-year relapse-free survival (9 % vs. 34 %), and overall survival improvement (12 % vs. 22 %) was observed.

2.4.2 Second-Generation Pgp Inhibitors

A group of second-generation antagonists were then developed with the hope of achieving increased potency to inhibit Pgp. One of these, valspodar (PSC833), an analog of cyclosporine D, was widely

tested in phase I clinical trials, but its use usually required dose reduction in the anticancer agent to avoid excessive toxicity. A reduction of as much as 66 % in dosage was needed to avoid toxicity when valspodar was coadministered with some anticancer agents. Dose reductions required in a series of clinical trials with valspodar are summarized in Table 5. Biricodar (VX710), another second-generation agent, was also found to reduce the clearance of anticancer agents, requiring a dose reduction of 54 % for paclitaxel [163, 175].

Table 5 Completed clinical trials with second- and third-generation Pgp inhibitors: required or planned dose reductions

Trail	*n*	Drug regimen	Cancer type	% dose reduction required for Pgp substrate drugs[a]	Ref.
PSC 833					
Phase II	10	Mitoxantrone (2.5 mg/m^2)/etoposide (170 mg/m^2)	AML	66/66	[94]
	37	Mitoxantrone (4 mg/m^2)/etoposide (40 mg/m^2)/Ara-C	AML	44/58/0	[4]
	66	Daunorubicin (40 mg/m^2)/etoposide (60 mg/m^2)/Ara-C	AML	33/45/0	[107]
	30	Mitoxantrone (6 mg/m^2)/etoposide (60 mg/m^2)	AML	40/40	[27]
	43	Daunorubicin/Ara-C	AML	None	[45]
	23	Mitoxantrone/VP-16/Ara-C	AML	25/62.5/0	[184]
	58	Paclitaxel, 3 h[b] (70 mg/m^2)	Ovarian	60	[51]
	33	Doxorubicin (35 mg/m^2)/cisplatin	Ovarian	30/0	[9]
	39	Vinblastine (2.1–6.3 mg/m^2)	Renal cell	25	[16]
Phase III	120	Daunorubicin/VP-16/Ara-C	AML	33/40	[10]
	129	Mitoxantrone (4 mg/m^2)/etoposide (40 mg/m^2)/Ara-C	AML/high-risk MDS	50/60/0	[63]
	410	Daunorubicin (40–90 mg/m^2)/etoposide (40 mg/m^2)	AML	55/60	[92]
	419	Daunorubicin (45 mg/m^2)/Ara-C	AML	22/0	[182]
	94	Vincristine (0.4 mg/m^2)/doxorubicin (9 mg/m^2)	Multiple myeloma	50/30	[52]
	762	Paclitaxel (80 mg/m^2)/carboplatin	Ovarian	54	[110]
VX-710					
Phase II	37	Paclitaxel, 3 h (80 mg/m^2)[b]	Breast	54	[175]
	29	Doxorubicin (60 mg/m^2)	Sarcoma	None	[21]
	50	Paclitaxel, 3 h (80 mg/m^2)[b]	Ovarian	54	[163]
	40	Mitoxantrone (12 mg/m^2)/prednisone 5 mg	Prostate	None	[146]
	36	Doxorubicin (45 mg/m^2)/vincristine (0.7–1.4 mg/m^2)	Small cell lung	None	[57]
Zosuquidar LY335979					
Phase I	16	Daunorubicin (50 mg/m^2)/cytarabine (200 mg/m^2)	AML	None	[59]
	40	Doxorubicin (45–75 mg/m^2)	Advanced malignancy	None	[156]
	19	Vinorelbine (22.5–30 mg/m^2)	Advanced solid tumor	25	[117]
	15	Vincristine (1.4 mg/m^2)/doxorubicin (50 mg/m^2)/cyclophosphamide (750 mg/m^2)/prednisone 100 mg/day	Non-Hodgkin's lymphoma	None	[130]
	16	Daunorubicin (45 mg/m^2)/cytarabine (100 mg/m^2)	AML	None	[106]
Tariquidar XR9576					
Phase I or II	26	Vinorelbine 20 mg/m^2 weekly	Advanced malignancy	30	[3]
	17	Paclitaxel (80–175 mg/m^2)/doxorubicin (50 mg/m^2) or docetaxel (30–100 mg/m^2)	Breast	None	[145]

(continued)

Table 5 (continued)

Trail	*n*	Drug regimen	Cancer type	% dose reduction required for Pgp substrate drugs[a]	Ref.
Laniquidar R101933					
Phase I	17	Docetaxel (60–100 mg/m^2)	Advanced malignancy	None	[183]
CBT-1					
Phase I	18	Paclitaxel (135 mg/m^2)	Advanced malignancy	25	[134]
Dofequidar MS-209					
Phase III	227	Doxorubicin (25 mg/m^2)/fluorouracil (500 mg/m^2)/ cyclophosphamide (100 mg)	Breast	16.6	[155]
Elacridar GF120918					
Phase I	46	Doxorubicin (50–75 mg/m^2)	Advanced malignancy	None	[168]
	46	Doxorubicin (50 mg/m^2)	Advanced malignancy	None	[143]
	39	Topotecan (2.5 mg)	Advanced malignancy	20	[100]

Drugs separated by the slash mark, /, correspond to dose reductions separated by slash mark; doses for Pgp substrate drugs indicated

[a]Dose reduction at the MTD in the presence of the Pgp inhibitor, compared to MTD in the absence of the Pgp inhibitor, or planned dose reduction in the case of a randomized trial

[b]Dose reductions relative to doxorubicin, based on 50 mg/m^2 q 3 weeks as standard dose, and for paclitaxel, 175 mg/m^2 as standard dose

Because Pgp knockout alone does not markedly increase plasma concentrations of chemotherapeutics (Table 3), other explanations were considered. Inhibition of cytochrome P450 (CYP450) emerged as the primary mechanism for the reduction in drug clearance with bile flow attenuation as a contributing factor. In vitro studies demonstrated that both valspodar and biricodar inhibit CYP450 [154, 188]. Further, drug clearance is determined in part by biliary flow, which in turn is influenced by the transport of bile salts through the ABC transporter BSEP (bile salt exporter protein) [99, 151]. BSEP was initially labeled "sister of Pgp" due to its high homology with Pgp [58]. The transport of taurocholate and other bile salts is inhibited by cyclosporine A and valspodar, and in animal models valspodar reduced bile flow, which in patients could impair drug clearance and explain the elevated bilirubin levels observed in some clinical trials [18, 166].

While reductions in the doses of chemotherapeutic agents are not desirable in patients with cancer, it was hoped that the reductions would result in comparable areas under the concentration curves (AUCs) at the maximum tolerated doses (MTD) for the anticancer agent under study. However, significant inter-patient variation was observed, most likely due to differences in CYP450 activity which have been reported to vary by as much as tenfold among patients, when evaluated by midazolam or erythromycin disposition [39, 61]. In our study combining valspodar with paclitaxel, the calculated mean AUCs for paclitaxel were comparable alone and with valspodar, despite a 50 % paclitaxel dose reduction [30]. However, individual AUCs for paclitaxel with valspodar revealed one-third of patients underdosed, and a comparable number of patients overdosed. This finding echoed that of Dorr et al., who reported on inter-patient variation in a trial combining valspodar with daunorubicin and Ara-C in patients with AML [45].

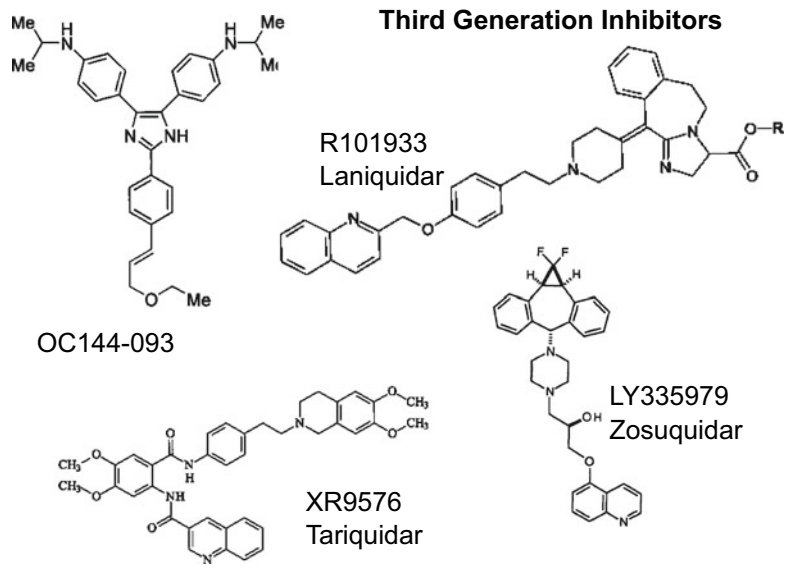

Fig. 2 Structures of Pgp inhibitors

Concurrent with—and most likely related to—the problematic dose reductions summarized in Table 5 were poor response rates in trials with valspodar. While high response rates were not anticipated in early trials, it was hoped that some evidence of drug resistance reversal would be observed. If first-generation agents failed because toxicity prevented the administration of doses sufficient to overcome drug resistance, this was not the central problem for valspodar or biricodar. Both compounds were shown to overcome Pgp in patients using surrogate studies that were developed to reflect Pgp inhibition [16, 57, 141].

Virtually all randomized phase III trials with valspodar were disappointing. In ovarian cancer, valspodar plus paclitaxel and carboplatin with paclitaxel dosage reduction from 175 to 80 mg/m^2 (54 %) showed no improvement over paclitaxel and carboplatin alone [110]. The valspodar arm of a 1998 phase III study of patients with previously untreated AML was suspended in 1999 due to excess mortality [10]. Friedenberg et al. of the Eastern Cooperative Oncology Group later demonstrated worsened median progression-free survival with the addition of valspodar to the VAD regimen in patients with refractory multiple myeloma [52]. Greenberg et al. found no improvement in patients with refractory AML and high-risk myelodysplastic syndrome when treated with mitoxantrone, etoposide, and Ara-C [63].

2.4.3 Third-Generation Pgp Inhibitors

Third-generation agents thus entered clinical trials without successful precedent. These agents included elacridar (GF120918), laniquidar (R101933), tariquidar (XR9576), zosuquidar (LY335979), OC144-093, and CBT-1 [50, 100, 106, 133, 135, 183]—structures are drawn in Fig. 2. Third-generation agents are characterized by little impact on drug clearance, minimal or no requirement for reduction in the dose of the anticancer agent since they have no significant CYP450 interactions, and no significant toxicity in combination with a chemotherapeutic agent [133].

Indeed, laniquidar (R101933) was shown to have no effect on docetaxel plasma levels in patients in a phase I study [183]. Zosuquidar (LY335979) was studied in several phase I trials [59, 106, 130, 156].

No dose reductions were required across three of these; in two trials, reductions of just 12 % and 25 % were needed when used in combination with a vincristine/doxorubicin regimen and vinorelbine, respectively. Similarly, dose reductions were not required for tariquidar (XR9576) in combination with doxorubicin, paclitaxel, or vinorelbine, although admittedly conservative doses were selected for the trials: 50 mg/m^2 doxorubicin, 135 mg/m^2 paclitaxel, and 20–22.5 mg/m^2 vinorelbine [50]. Both CBT-1 and elacridar have been in extended clinical trials, without evident need for significant dose reduction [100, 135, 143, 168].

Tariquidar was shown to be effective in preventing the efflux of ^{99m}Tc-sestamibi from Pgp-bearing tumors and tissues. A single 150 mg dose inhibited Pgp-mediated rhodamine efflux from CD56$^+$ circulating cells for 48–72 h without re-administration [3]. Nonetheless, Pusztai et al. found tariquidar to have limited clinical activity when added to paclitaxel (80–175 mg/m^2) or doxorubicin (50 mg/m^2) in women whose breast cancer had progressed on a prior chemotherapy regimen [145]. Two large randomized phase III trials of tariquidar in lung cancer closed early due to toxicity in the experimental arm [50].

One third-generation Pgp inhibitor, dofequidar fumarate (MS-209), was found to be well tolerated in a randomized, double-blind, placebo-controlled phase III trial and demonstrated significant improvement in progression-free survival in advanced breast cancer patients who were premenopausal and were therapy-naive [155]. Saeki et al. posit that such data suggest Pgp inhibition can be used to reverse MDR in carefully selected and narrowly defined patient cohorts, as in the advanced disease, premenopausal patient group that benefited from dofequidar [155].

2.5 Surrogate Endpoints

Two surrogate assays have been developed to confirm inhibition of Pgp in patients—the CD56$^+$ rhodamine assay and the ^{99m}Tc-sestamibi scan. The rhodamine efflux assay takes advantage of the ability of naturally high levels of Pgp in circulating CD56$^+$ mononuclear cells to rapidly efflux rhodamine, a fluorescent Pgp substrate. When administered to patients, both second-generation valspodar and third-generation tariquidar revealed excellent inhibition of efflux from CD56$^+$ cells [3, 148, 193]. The second strategy to confirm Pgp inhibition is ^{99m}Tc-sestamibi imaging. Shown by Pinwica-Worms et al. to be a Pgp substrate, ^{99m}Tc-sestamibi has long been used in cardiac imaging [142] and was thus easily adapted to imaging tumors and tissues for Pgp inhibition. As shown in Fig. 3, the liver and kidneys of a patient with adrenocortical cancer are not well visualized in scans obtained prior to the administration of the Pgp inhibitor [5]. However, both the liver and kidneys retain ^{99m}Tc-sestamibi following administration of tariquidar, confirming Pgp inhibition in normal tissue. A marked increase in uptake in retroperitoneal and lung disease confirms the ability of Pgp inhibition to increase accumulation of the radionuclide in tumors (Fig. 3).

Uptake of sestamibi in lung cancer has been correlated with response to therapy—and absence of uptake with nonresponse [128]. We recently reported marked heterogeneity of uptake in sestamibi in lung cancer, with minimal change following tariquidar [85]. Traditional sestamibi imaging is relatively insensitive and work has been underway to identify other radionuclide imaging agents that offer the option to perform PET imaging studies. These have included [11C]-gefitinib [84], 4-[18F] paclitaxel [101], and a metabolite of loperamide [95], as well as PET-sestamibi [26]. These imaging agents offer the possibility to test the Pgp hypothesis (that drug accumulation can be increased by Pgp inhibitors) as well as to systematically approach the question of drug uptake in cancer therapeutics. When it has been studied in the past, tumor drug uptake has been far more variable than assumed, offering compelling rationale for the development of sophisticated imaging technologies to determine whether cancer chemotherapeutics are being adequately distributed throughout tumors.

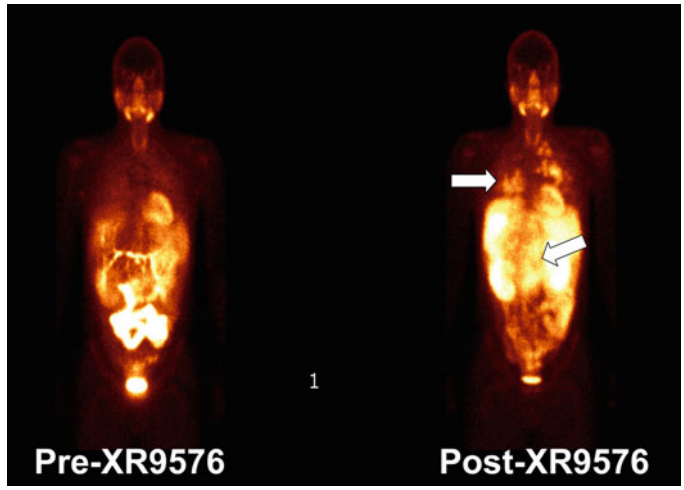

Fig. 3 ^{99m}Tc-sestamibi imaging of Pgp inhibition. The cardiac imaging agent sestamibi has been shown to be a Pgp substrate and has been used to indicate Pgp inhibition. In this figure, images were obtained before (*left*) and after (*right*) administration of the Pgp inhibitor tariquidar (XR9576) to a patient with adrenocortical cancer enrolled on a clinical trial combining tariquidar with vinorelbine. In the image on the *left*, sestamibi accumulation is observed in the gastrointestinal tract and in the bladder following excretion from the liver and kidneys. In the image on the *right*, sestamibi accumulation is observed in pulmonary metastases (*arrow*), in metastases in the retroperitoneum (*arrow*), in the liver, and in the kidneys. The retention in normal tissues indicates that sestamibi can be used as a marker of Pgp inhibition, while retention in tumor tissue indicates that sestamibi can be used as a diagnostic assay for Pgp function. Reprinted from Gottesman et al., Nature Reviews in Cancer, vol 2 p 48–56, 2002

3 The MRP Family of Drug Transporters

3.1 Background

Subsequent to the discovery of ABCB1, a number of other ABC transporters were identified that could also efflux chemotherapy drugs; among these, the largest is the MRP or ABCC transporter subfamily. Cloned by Cole et al., MRP1 was found to have an additional five transmembrane segments in a single domain at the amino terminus (TMD_0) [36]. Since the original description of MRP1 in 1992, 12 members of the ABCC subfamily have been described, of which nine have been labeled as MRPs due to homology with MRP1. All characterized MRP transporters appear to be organic anion transporters of varying specificity (Table 2). MRPs 1, 2, 3, 6, and 7 contain TMD_0, while MRPs 4, 5, 8, and 9 do not, suggesting TMD_0 is not critical for transport [11]. However, the adjacent linker region is essential and is present in all MRPs [12, 172]. MRPs 1–3 have been shown to transport glutathione and glucuronide conjugates, sulfates, and other organic anions. MRP2 transports bilirubin glucuronide from the liver. MRP4 and MRP5 have been shown to transport cyclic nucleotides including cAMP and cGMP, as well as nucleoside analogues [28, 78, 162, 192]. Interestingly, glutathione depletion was recently reported to reduce the export of cAMP in MRP4-expressing cells [104]. MRP7 has been confirmed as both a glutathione conjugate transporter and a broad-spectrum chemotherapy drug transporter [89].

3.2 MRP Family: Subcellular Localization and Substrate Specificity

The substrate specificity as well as localization within the body varies for the different members of the MRP family. MRP1 is ubiquitously expressed at low levels in most normal tissues. It routes to the basolateral surface of epithelial cells, including the choroid plexus epithelium, where it may be involved in the transport of substrates from the CSF into the bloodstream [147]. Murine data suggest a degree of synergy between MRP1 and ABCB1; *mrp1*$^{-/-}$ knockout fails to significantly alter plasma efflux of methotrexate in normal cells compared to wild type [82]. However, when etoposide was instilled into the CSF of a *mdr1a/1b*$^{-/-}$*/mrp1*$^{-/-}$ (triple-knockout) mouse, a tenfold increase in accumulation occurred, relative to that observed in *mdr1a/1b*$^{-/-}$ mice [191]. In the liver, MRP2 is found on the canalicular surface of the hepatocyte, transporting substrates into the bile, while MRP3 is found on the basolateral surface, presumably with the functional assignment of pumping substrate back into the bloodstream should biliary flow be interrupted [93]. Like MRP1 and MRP3, MRP6 demonstrates basolateral localization [157], while MRP4 demonstrates apical localization [181].

Most MRPs transport chemotherapeutic drugs, although the MRP substrate profile with respect to anticancer agents is less straightforward than that for Pgp. Currently understood substrate profiles for MRP family members are shown in Table 2. It has been shown that in addition to the transport of glutathione conjugates, MRP1 is able to cotransport anthracyclines, vinca alkaloids, or etoposide together with free glutathione without conjugation [98]. Methotrexate, a substrate for MRP1, is also transported by MRPs 2–5 [29, 89, 98]. Because methotrexate is an organic anion, transport does not require conjugation or cotransport with GSH. In contrast, the polyglutamylated forms of methotrexate are not transported by the MRPs [199]. MRP3 has been shown to confer resistance primarily to etoposide and to favor glucuronides over glutathione conjugates [198]. MRP4 and MRP5 have been shown to transport the nucleoside analogues 6-mercaptopurine and 6-thioguanine [28, 192]. PMEA, an antiretroviral nucleoside agent, is an MRP4 substrate [162]. MRP4 overexpression reduces both the cytotoxic and antiviral activity of PMEA. Recently, MRP7 overexpression has been correlated with resistance to docetaxel, paclitaxel, and vincristine in vivo [71] and in vitro [132].

3.3 Physiologic Role of MRP Family Members

Like Pgp, MRP1 is thought to protect the organism from xenobiotics. This idea is reinforced by in vitro studies using cells from knockout mice. When fibroblasts from mice lacking the orthologues for both Pgp and MRP were studied, increased in vitro sensitivity was observed compared to the Pgp knockout alone: for paclitaxel (22 vs. 16-fold), vincristine (28 vs. 2.4-fold), doxorubicin (7.1 vs. 4.7-fold), and etoposide (7.0 vs. 1.0-fold) [6]. These results suggest that there is redundancy in the protection of normal tissue from xenobiotics and that the drug transporters contribute substantively to drug resistance. Knockout mice in which MRP alone had been deleted exhibited increased sensitivity to etoposide in the oropharynx [116], but little difference in most drug pharmacokinetic profiles. MRP2 is responsible for the efflux of bilirubin glucuronide from the liver. Mutations that result in a nonfunctional MRP2 are the basis of the Dubin–Johnson disorder, characterized by failure to transport bilirubin into the bile. Mutations in MRP6 in patients with pseudoxanthoma elasticum impair transport of the glutathione conjugates leukotriene and NEM-GS [73].

4 ABCG2: Breast Cancer Resistance Protein

Cloned by three independent labs, ABCG2 was originally named BCRP for breast cancer resistance protein, ABCP for ABC transporter in placenta, and MXR for mitoxantrone resistance gene [7, 46, 127]. ABCG2 is a "half transporter," comprising six transmembrane segments in one TM domain and a

single ATP-binding site at the amino terminus that dimerizes to generate a fully functional ABC transporter. Nonreducing conditions and studies using cross-linking agents show high molecular weight forms, suggesting that higher order multimers may also be involved [80, 115]. Immunofluorescence studies show ABCG2 at the plasma membrane of drug-resistant selected cell lines [114, 150].

ABCG2 is expressed in the placenta, the small and large intestine, the liver, and the endothelium of CNS tissues. As is the primary function of ABCB1 and MRPs, the major role of ABCG2 in normal human physiology is protection of the organism from exposure to environmental xenobiotics. In the placenta, ABCG2 is found at high levels in the syncytiotrophoblast, suggesting a distinct role in fetal protection and in the excretion of toxins from the fetal circulation [119]. Indeed, increased fetal penetration of topotecan was demonstrated following treatment with an inhibitor of ABCG2 efflux [79]. Evidence for a protective role in the gastrointestinal tract and an impact on oral drug absorption includes studies showing that in mice lacking the Pgp orthologues, oral administration of elacridar, both a Pgp and ABCG2 inhibitor, increased the absorption of orally administered topotecan, an ABCG2 substrate [79]. If also true in humans, inhibition of ABCG2, as theorized for Pgp, may be exploitable in the development of oral agents for anticancer therapy.

Finally, significant energy has gone toward the study of the "side population" of stem cells found in bone marrow, skeletal muscle, and neural cells, identified by ABCG2-mediated Hoechst 33342 dye efflux [202]. Mice lacking the *Abcg2* gene reproduce and mature normally, despite absence of this Hoechst 33342-defined side population of stem cells [201]. These mice have normal steady-state hematopoiesis, but marrow cells are more sensitive to the toxic effects of mitoxantrone.

Overexpression of ABCG2 leads to reduced drug accumulation and thereby resistance to mitoxantrone, topotecan, and irinotecan [22, 83, 114, 120, 152]. Only with a gain of function mutation replacing the arginine with threonine or glycine at position 482 does ABCG2 confer resistance to the anthracyclines doxorubicin, daunorubicin, and epirubicin and to rhodamine and VP-16 [69]. ABCG2 has also been shown to transport methotrexate, its polyglutamates [185], glucuronide-conjugated SN-38 [131], and sulfates [74, 186]. This organic anion specificity differentiates the transporter from Pgp.

A number of nontraditional anticancer agents have also been reported to be substrates for ABCG2, as shown in Table 2. Imatinib, nilotinib, and dasatinib, drugs that target Bcr-abl, are substrates for both ABCG2 and Pgp—suggestive of a possible inhibitor function instead [65, 72, 118, 138]. Indeed, our data, and those of others, suggest that tyrosine kinase inhibitors are substrates only over a narrow concentration range and act as inhibitors at higher concentrations [60, 68, 164]. Among TKIs, dasatinib appears to be an exception—more a substrate of Pgp and ABCG2 than a modulator [44, 60, 68]. Similar findings by Elkind et al. for gefitinib led to suggestion of a decreased off-rate with increasing drug concentration [47]. While in vitro data seem clear at this point, even more striking is evidence in murine knockout studies that TKIs are substrates for Pgp and ABCG2, with a notable increase in brain uptake when both transporters are deleted [41, 102, 103, 144] (Fig. 4). Data derived from the studies represented in Fig. 4 suggest that inhibitors of ABCG2 and Pgp may be useful to increase the CNS uptake of the tyrosine kinase inhibitors. An increase in the incidence of CNS metastases following long-term control of systemic disease by lapatinib, erlotinib, sorafenib, and sunitinib in breast, lung, and renal cancer, respectively, makes the goal of increasing CNS uptake of these agents very compelling.

5 Other ABC Transporters

Other ABC transporters with distinct roles in normal physiology have been shown to transport anticancer agents. These transporters provide an interesting proof of principle in cell physiology—transporters may be subverted to protect the cell following a challenge by a cytotoxic agent. This flexibility in enlistment of transporters offers a keen potential explanation for the failure of Pgp inhibitors in the clinic—co-expression of other transporters may confound any benefit achieved by Pgp inhibition.

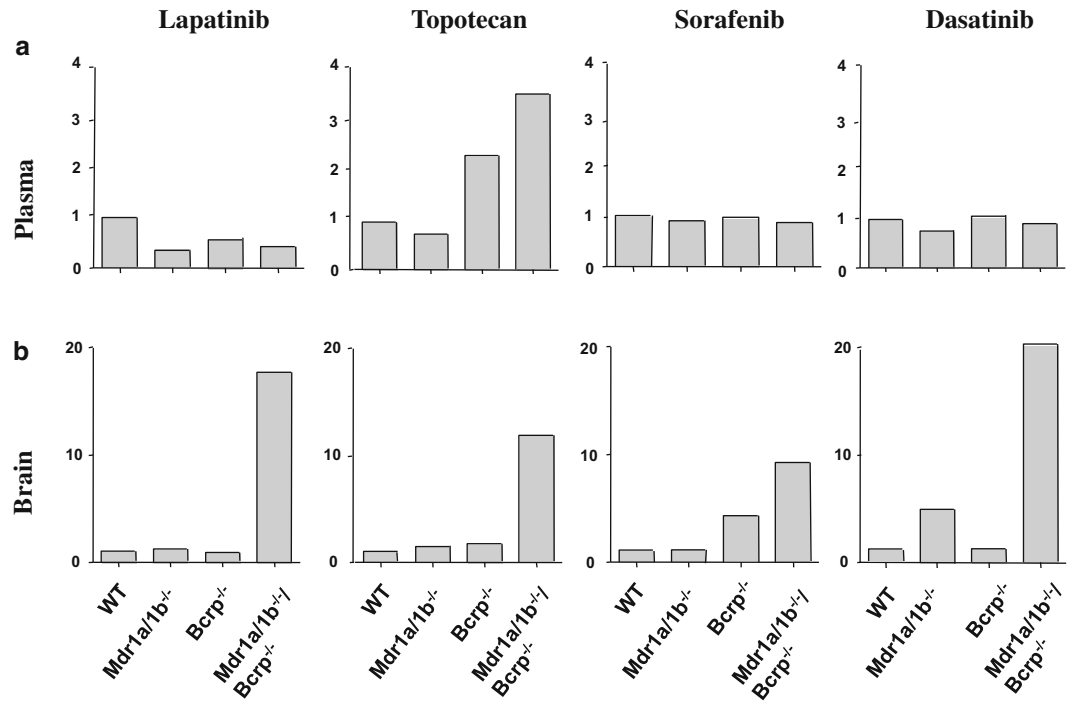

Fig. 4 Dual effect of Pgp and ABCG2 knockout on CNS penetration. Relative plasma and CNS uptake in 4 murine knockout studies in which the gene *Mdr1a/1b*$^{-/-}$ *or Abcg2*$^{-/-}$ (*Bcrp*$^{-/-}$), or both, has been deleted. In 3, knockout was followed by concomitant intravenous anticancer agent administration. Values for sorafenib follow oral drug administration. For comparison, wild-type control samples from all studies were assigned a value of 1. Panel **a** comprises ratios of plasma area under the concentration curve values ($AUC_{0\text{-}6h}$), usually in *μg/ml*, for knockout (KO) relative to wild type (WT). Panel **b** comprises CNS uptake data as measured by a C_{brain} drug ratio, usually calculated in *μg/g*, between KO and WT mice. Lapatinib values in Panels **a** and **b** are represented in C_{ss}, or steady-state plasma concentration. Graphs depict data in [41, 102, 103, 144]

ABCB11 was originally termed "sister of Pgp (sPgp)" based on its homology with Pgp [31]. ABCB11 mutations in liver tissue obtained from patients with Type II progressive familial intrahepatic cholestasis led to the recognition that sPgp encoded the ATP-dependent bile salt export protein (BSEP) [170]. Absence of ABCB11 leads to impaired bile salt transport and progressive liver damage. In vitro studies have demonstrated the ability of BSEP/sPgp to confer resistance to paclitaxel, which can be reversed by CsA, PSC 833, or verapamil [31]. ABCA2 is expressed at high levels in the brain and is involved in cholesterol transport [42, 189]. ABCA2 (originally termed ABC2 when cloned from cells with high levels of estramustine resistance) confers resistance to estramustine and mitoxantrone in experimental systems [42, 105, 187]. ABCA3 mutation has been identified as a cause of surfactant deficiency leading to respiratory distress syndrome in infants [165]. It has also been linked to drug resistance and was noted to be overexpressed in leukemic samples from patients with childhood AML [19, 169].

6 ABC Transporters: An Expanding List of Substrates

The breadth of Pgp substrates is unsurprising given its vital role in protecting the organism. Substrate profiles for the MRPs and for ABCG2 generally appear more restricted. Nonetheless, recent reports have shown that MRP4 and MRP5 confer resistance to the nucleosides 6-MP and 6-TG, previously not considered substrates of ABC transporters, thus raising the possibility that transporters for other

nucleoside analogues such as cytosine arabinoside may be identified. Wild-type ABCG2 has also been shown to confer resistance to flavopiridol, in addition to mitoxantrone, topotecan, and irinotecan [149]. Numerous tyrosine kinase inhibitors have been shown to be substrates for Pgp and ABCG2 [23, 41, 44, 66, 91, 102, 103, 122, 136, 144]. The PARP inhibitor olaparib is a substrate for Pgp [197]. This line of thought seems to suggest a drug transporter may exist for every anticancer agent developed. It is beyond the scope of this chapter to discuss the numerous non-oncologic substrates that have been identified as interacting with ABC transporters—and possible roles in medicine in psychiatry, epilepsy, arthritis, and endocrine diseases [32, 112].

7 ABC Transporters: Single Nucleotide Polymorphisms

Regardless of whether or not inhibition of MDR transporters can be utilized to mitigate cellular drug resistance (through increasing drug accumulation), evidence has mounted suggesting that the transporters are important in pharmacology. ABC transporters affect drug absorption through the GI tract, excretion through the kidneys and liver, and drug distribution. As noted in Table 3 and Fig. 4, the impact of *Abcb1* and *Abcg2* knockout in the mouse blood–brain barrier exceeds the impact on plasma pharmacokinetics. Recognizing that inter-patient variability in drug efflux may be explained by transporter variants, there has been intense investigation into the impact of single-nucleotide polymorphisms (SNPs) on pharmacokinetics and drug distribution.

A diversity of coding variants in ABCB1, ABCG2, and ABCC1 has been described, generating a conflicting literature. Table 3 hints at the cause of difficulties in assessing impact on pharmacokinetics; even complete knockout of mouse Abcb1 does not always result in significant plasma accumulation of known substrate drugs. Progress in the field has also been made difficult by frequent assay of SNPs of only single transporter genes, failing to consider the multiplicity of genes that play a role in drug metabolism.

A number of polymorphic variants have been implicated in impaired expression or function of ABC transporters. Studies of common coding variants are most extensive for Pgp. SNPs are the most widely studied, with 32 novel variants currently characterized [34]. Three SNPs in particular have been discovered to have strong linkage, thus a haplotype comprising *1235C>T*, *2677G>T/A*, and *3435C>T* [54].

Variations across the *1236C>T-2677G>T/A-3435C>T* haplotype are preferentially expressed by ethnicity: the reference allele *(ABCB1*1) 1236C-2677G-3435C*, for example, commonly occurs among Africans, while the variant allele *(ABCB1*2 or 1*13) 1236T-2677T-3435T* is more common among Asians [25]. The *ABCB1*1* allele was found at a frequency of 43.3 % in European Americans ($n=37$) and 69.5 % in African Americans ($n=23$) [86]. The ABCB1*13 allele occurs at a frequency of 32 % in Caucasians, 5 % in African Americans, 27 % in Asians, 35 % in Mexican Americans, and 33.3 % in Pacific Islanders [96]. Variance along this haplotype has been posited to associate with MDR1 expression; some studies suggest the TTT haplotype confers lower levels of Pgp expression [77]. Jamroziak et al. hypothesize that lower levels of Pgp expression in the duodenum may result in increased exposure to carcinogenic factors in the environment [77]. Researchers have struggled to correlate *1236C>T-2677G>T/A-3435C>T* to clinical outcomes, with limited success.

Kimchi-Sarfaty et al. hypothesized that the functional change in MDR1 caused by the *3435T>C* SNP is rooted in changes in protein folding. Specifically it is posited that the *3435T>C* SNP results in a codon that uses a rare tRNA. A pause results during transcription of the variant allele [87]. This pause results in a differential protein conformation, which may reduce ATP-binding affinity, disrupt disulfide bond formation, or lead to a loss of ATP hydrolysis [54].

Taken together, the association between the ABCB1 haplotype and Pgp expression and function suggests the possibility of significant pharmacological implications. However, the literature continues to be conflicting on this point; for example, one widely studied marker for Pgp function, digoxin, has evaded consensus on the impact of allelic variation on pharmacokinetics [1].

Non-synonymous SNPs have been identified across the coding region of MRP1 (reviewed in [37]). In vitro, several polymorphisms showed impaired transport of different organic anions, such as LTC4, E217BG, and MTX, but no single SNP was consistent across all three [109]. In patients, clinical associations with these variants have been limited. As noted in Table 1, mutations in at least 4 of 13 genes in the ABCC subfamily cause genetic disorders, including mild conjugated hyperbilirubinemia, cystic fibrosis and persistent hyperinsulinemic hypoglycemia of infancy, and pseudoxanthoma elasticum. This would suggest that SNP variance should have some impact on pharmacology, but clear documentation of this has been limited to date. There is no clear association of ABCC1 and ABCC2 variance with methotrexate toxicity, for example. For irinotecan, SNPs in a multiplicity of genes have been cited as related to toxicity and treatment response: UGT1A, ABCC1, ABCB1, SLCO1B1, and ABCG2 [76].

At least 14 non-synonymous SNPs have been identified in ABCG2. The most studied of these is *C421A (Q141K)*. This polymorphism is found in the ATP-binding region between the Walker A and Walker B motifs [70]. Functional studies have linked *Q141K* to both impaired function and reduced cell surface expression of ABCG2 [55, 75, 129].

Important associations of this variant with gout in GWAS studies led to the identification of a normal physiologic role of this transporter in uric acid excretion [123, 194]. In oncology, this variant has been linked to increased toxicity in gefitinib and impaired pharmacokinetics of diflomotecan, irinotecan, topotecan, and gefitinib [24, 38, 111, 167, 200].

8 Conclusions

The role of ABC transporters in pharmacology, oncology, and medicine at large is still under investigation. The earliest studies with inhibitors of Pgp focused on reversal of multidrug resistance in oncology. These trials were limited by inferior antagonist potency and the need to reduce the dose of the anticancer agent. Newer inhibitors of Pgp have reduced toxicity, greater potency, and minimal pharmacokinetic interaction, and surrogate studies have confirmed that these inhibitors are able to inhibit Pgp in vivo. However, interest in the oncologic community in the study of transport inhibitors as resistance modulators has waned due to negative results from early trials, coupled with the conviction that the Pgp hypothesis applies to classical cytotoxic therapies. To date, drugs remaining under limited development as Pgp inhibitors include only CBT-1, tariquidar, and zosuquidar.

Disappointing clinical results have led to myriad explanations for the failure of Pgp inhibitors. Among these is the view held by Patel and colleagues that Pgp inhibitors cause disproportionate drug uptake in tumor cells proximal to capillaries and blood vessels, while accomplishing the purported goal of Pgp blockade, inhibition comes as a detriment to intermediate and distal tumor cell drug exposure [140]. Furthermore, we do not know whether the inhibitors were able to accumulate in tumors in the clinical trials. Some of the same factors limiting drug penetration into tumors may have also limited penetration of the inhibitors.

Without the development of new or unexpected data, renewed interest in the field of Pgp modulation in its current form is unlikely. One potential direction from which such data may emerge lies in efforts to alter CNS uptake of anticancer agents. As observed in Fig. 4, the effect of combined knockout of *Bcrp* (ABCG2) and *mdr-1a/1b* on the CNS retention of several TKIs is dramatic. As patients experience increasing degrees of systemic tumor control with targeted therapies, an increased incidence of intracranial metastases is being observed. The use of TKIs in combination with an inhibitor of ABC transporter-mediated efflux could potentially increase CNS uptake and enable the eradication of micrometastases. This potential change in uptake was recently demonstrated in a murine PET imaging model using Pgp inhibitors in combination with radiolabeled 14C-gefitinib [84].

New data supporting continued investigation into the role of ABC transporters in cancer has been provided by Sven Rottenberg and colleagues [197]. In a genetically engineered murine *brca*$^{-/-}$;

p53$^{-/-}$ orthotopic transplantation model, Pajic et al. generated spontaneous mammary tumors, which then became refractory post-doxorubicin therapy [139]. Although the study was conceived as an unbiased search for drug resistance mechanisms, the resistant phenotype was associated with concomitant increases in *mdr1a/1b* expression levels. The same results were observed when resistance to the PARP inhibitor olaparib emerged [153]. In that model, addition of tariquidar delayed the onset of resistance. In yet a third model, administration of topotecan induced heightened expression of ABCG2 in drug-resistant tumors, and the same experiment performed in mice in which ABCG2 had been deleted doubled the time it took for resistance to emerge [197]. These in vivo data implicate ABC transporters in the development of multidrug resistance in cancer and argue for some role for ABC inhibition in the clinic.

Taken together, the data presented in this chapter suggest that there may be important avenues to therapeutically exploit ABC transporters. Validation that preclinical models apply in humans is a critical step. Several important strategies can be considered: to overcome sanctuary site protection for the tyrosine kinase inhibitors, to increase the oral absorption of traditional anticancer agents that could be administered without the discomfort and expense of infusional therapy, to increase or equalize absorption of oral agents currently given at fixed doses, or to increase cellular accumulation of an anticancer agent. Whatever the strategy, studies should be systematic and conducted with the considerable range of confounding transporters, both efflux and influx, well in mind.

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Solute Carriers

Richard H. Ho and Richard B. Kim

Abstract Membrane transporters are important for regulating cellular and physiologic solute and fluid balance and can generally be grouped into two major classes—uptake and efflux. These proteins are critical to human physiology in the maintenance of normal homeostasis via transport of endogenous substrates, but are also important to the disposition of xenobiotics such as environmental toxins, dietary constituents, drugs, and their metabolites, thereby modulating or altering drug response. In particular, over the past decade, members of the solute carrier (SLC) superfamily, including the organic anion-transporting polypeptide (*SLCO*; OATP) family, the organic cation/anion/zwitterion transporter (*SLC22*; OCT and OAT) family, the concentrative nucleoside transporter (*SLC28*; CNT) family, and the equilibrative nucleoside transporter (*SLC29*; ENT) family, have been shown to have important physiologic, pathologic, and therapeutic implications. Although generally recognized as drug transporters, these proteins have only recently been determined to be important factors in the disposition of commonly used antineoplastic chemotherapeutic agents and thereby serve as key determinants of pharmacokinetics, pharmacodynamics, and pharmacogenetics. In this chapter, we focus on the field of solute carriers and provide a comprehensive overview of the significant and emerging roles these proteins play in anticancer drug disposition and response.

Keywords Transporter • Uptake • Drug disposition • Organic anion • Organic cation • Nucleoside • Pharmacogenetics • Pharmacokinetics • Pharmacodynamics • Drug toxicity • Genotype • Phenotype • Chemotherapy • Polymorphism

1 Introduction

Membrane transporters have long been recognized to be an important class of proteins for regulating cellular and physiologic solute and fluid balance. Thus, it is not surprising to note that in the human genome nearly 1,000 genes encode transport proteins [1, 2]. Transporter proteins can be generally

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categorized into two major classes—uptake and efflux. Some facilitate the cellular entry of solutes or substrates while others prevent their entry through extrusion or efflux processes. Not only are transporter proteins critical to human physiology in the maintenance of normal homeostasis via transport of endogenous substrates but in many cases are important to the disposition of numerous xenobiotic compounds such as environmental toxins, dietary constituents, drugs, and their metabolites. In particular, the study of drug transport across biological membranes has garnered considerable attention over the last two decades as a result of rapid advances in genetic and molecular biological tools which delineated the emerging functions of the responsible transporter proteins expressed in organs such as the liver and intestine, as well as in healthy and cancerous cells.

The interplay between substrate metabolism and transport that occurs locally within tissues determines its overall absorption, distribution, and elimination. For many compounds, particularly those capable of significant diffusion across membranes, the major impact of metabolism overshadows the influence of facilitated membrane transport on disposition. The dynamic, reversible nature of transport has made it challenging for investigators to understand its role in normal human physiology and/or pharmacokinetics. The importance of uptake transporters to endobiotic/exobiotic disposition is most apparent for compounds that are metabolically inert or whose rates of metabolism are significantly affected by transporter-mediated delivery to eliminating enzymes. While efflux transporters, primarily composed of members of the ATP-binding cassette (ABC) superfamily of transporters, have been extensively characterized in the oncology field due to their important roles in chemotherapy disposition and the so-called multidrug resistance phenotype that mediates acquired resistance to chemotherapeutic regimens, significantly less is known with regard to solute carriers and their roles in anticancer drug disposition and response.

The goal of this chapter is to summarize the current state of knowledge in the area of uptake transporters, comprised primarily of the so-called solute carriers, and their relevant roles in anticancer drug disposition. As the name suggests, uptake transporters facilitate the movement of drugs into cells. The Human Genome Organization (HUGO) Nomenclature Committee Database provides a comprehensive and growing list of solute carrier (SLC) gene superfamily members comprising passive transporters, ion-coupled symporters, and antiporters in the plasma membrane and other cellular membrane compartments (http://www.genenames.org). We will focus on four main families of transporters: the solute carrier organic anion transporter family (*SLCO*), which includes members of the organic anion-transporting polypeptides (OATP); the solute carrier family 22 (*SLC22*), which includes members of the organic cation transporters (OCT) and organic anion transporters (OAT); the solute carrier family 28 (*SLC28*), which includes members of the concentrative nucleoside transporters (CNT); and the solute carrier family 29 (*SLC29*), which includes members of the equilibrative nucleoside transporters (ENT). We will begin with a review of nomenclature followed by sections covering relevant polymorphic variants, protein structure, transport mechanisms, and substrate specificity.

2 Nomenclature

Currently, the list of transporter families of the SLC gene superfamily includes 43 families and 298 transporter genes [3]. In general, the genes are named using the root symbol SLC, followed by a numeral, the letter A which acts as a divider between the numerals, and finally by the number of the individual transporter.

2.1 SLCO Nomenclature

The rules of SLC gene nomenclature have been modified and extended in further detail for the SLCO family (formerly SLC21) which encodes the organic anion-transporting family (OATP). After the discovery of the first Oatp [4] over 15 years ago, the rapid identification of new members of this transporter family in multiple species along with the various naming systems proved to be a source of confusion particularly for those not closely following the field. However, in 2004, a new classification and nomenclature system was developed by Hagenbuch and Meier and subsequently approved by the HUGO Nomenclature Committee [5]. In this system, Oatps are classified according to conventions originally established for the cytochrome P450 (CYP) enzyme superfamily that are based on amino acid sequence identities [6]. For the Oatps, the italicized gene symbol begins with *Slco* while the encoded proteins are named with the root Oatp. Oatps within the same family share ≥40 % amino acid sequence identity and have root names followed by Arabic numbers, of which 6 families in humans are known. Subfamilies share ≥60 % amino acid sequence identity and are indicated by letters following the family designation. For example, OATP family 1 (OATP1) has three subfamilies (A, B, and C). Specific transporter proteins are then assigned numerical designation after the subfamily heading. Therefore, OATP1B1 is the first named member belonging to family 1, subfamily B. The final numeral is named chronologically and allows for unambiguous identification of Oatp transporters between species. Ultimately, OATP family members mediate the sodium-independent transport of a variety of structurally divergent, mainly amphipathic, organic compounds, such as bile salts, hormones and their conjugates, toxins, and various drugs [7].

2.2 SLC22 Nomenclature

SLC22 is comprised of the organic cation/anion/zwitterions transporter family. Nomenclature is derived according to the conventional naming of SLC family members as described above. Members tend to be grouped by the types of substrate they transport. For instance, *SLC22A1–3* contains the three subtypes of organic cation transporters (OCT1–3), while *SLC22A6–8* and *11* include the organic anion transporters (OAT1–4) [8, 9]. While these transporters demonstrate generalized selectivity in the types of substrates they transport, they share a common characteristic of being polyspecific in their capacity to accept compounds with different sizes and molecular structures.

2.3 SLC28 Nomenclature

SLC28 contains members of the concentrative nucleoside transporter (CNT) family. This family includes three members, CNT1, CNT2, and CNT3, encoded by *SLC28A1–3*, respectively [10]. In a sodium-dependent manner, CNT1 primarily transports pyrimidine nucleosides, while CNT2 prefers to transport purine nucleosides. CNT3 demonstrates broad substrate selectivity and the ability to transport nucleosides in both sodium- and proton-coupled manners. All three CNT family members play important roles in nucleoside balance, synthesis, and salvage but also, to varying degrees, transport nucleoside-derived antiviral and anticancer drugs and hence may have important roles in drug disposition.

2.4 *SLC29 Nomenclature*

SLC29 includes members of the equilibrative nucleoside transporter (ENT) family. Four ENTs, ENT1–4, encoded by *SLC29A1–4*, have been identified to date and mediate low-affinity, equilibrative nucleoside transport processes in mammalian cells [11]. ENT1 and ENT2 have similar, broad selectivity for transporting purine and pyrimidine nucleosides, but ENT2 also transports nucleobases [12]. ENT3 also transports nucleosides and nucleobases but, interestingly, appears to function in intracellular membranes, including lysosomes. ENT4 is able to transport a variety of organic cations but also transports substrates such as adenosine and serotonin. ENTs function in nucleoside salvage pathways as part of normal human physiology but, similar to CNTs, are also responsible for the cellular uptake of nucleoside analogs used in the treatment of cancers and viral diseases and therefore may play important roles in chemotherapy disposition and response.

3 Gene Organization

The human OATPs are encoded by the *SLCO* genes located on a number of different chromosomes. Members of the *SLCO1* family are clustered in a gene locus on chromosome 12 (Table 1) including a pseudogene related to the *SCLO1B* subfamily [13]. The *SLCO* genes span from 30 to 310 kb in length and consist of 10–18 exons. This gene clustering pattern would suggest these OATPs have arisen through gene duplications of an ancestral gene [14]. In silico analysis predicts many of the OATPs to be expressed as splice variants but lacks experimental verification, tissue distribution, and functional assessment of such isoforms (http://www.genecards.org). However, functional splice variants of rodent Oatp1a3 have been described [15, 16].

In humans, the genes encoding the SLC22 family members tend to be paired or clustered. *SLC22A6* is paired with *SLC22A8* on adjacent segments of chromosomes 11q12.3 to 11q13.2 (Table 2) [17–19], while *SLC22A1–3* are localized within a cluster on chromosome 6q26-7 [20–23]. This paired or clustered pattern is unlike that of many other SLC genes in the larger major facilitator superfamily (MFS). Paired genes tend to be coexpressed and exhibit the most homology, suggesting a common regulatory mechanism for expression and an evolutionary duplication event as the origin of pairing [18, 24].

Of the SLC28 family members, *SLC28A1* was mapped to chromosome 15q25-26 by fluorescence in situ hybridization (FISH) (Table 3) [25]. *SLC28A2* was mapped to the same chromosome but a different locus, chromosome 15q13-14, by radiation hybrid analysis [26, 27]. *SLC28A3*, which encodes CNT3 with only 48 % and 47 % sequence homology to CNT1 and CNT2, respectively, was mapped to chromosome 9q22.2 by FISH and genomic sequence analysis [28].

SLC29A1 was localized to chromosome 6p21.2-p21.1 by FISH analysis [29] whereas *SLC29A2* was mapped to chromosome 11q13 using the same modality (Table 3) [30]. *SLC29A3* encodes ENT3 which possesses a unique N-terminal domain that contains a conserved putative dileucine-based endosomal/lysosomal targeting motif and has been mapped to chromosome 10q22 by genomic sequence analysis [31]. *SLC29A4* has been mapped to chromosome 7p22.1 by genomic sequence analysis [32].

4 Major Polymorphisms

There is now evidence to demonstrate genetic heterogeneity in uptake transporter genes may have important roles in influencing the disposition of transporter-dependent endobiotic and xenobiotic substrates. Within the last decade, numerous polymorphisms have been identified in uptake transporter genes. However, for the most part, studies relating to the clinical consequences of transporter

Table 1 SLCO transporter family

Gene symbol	Protein	Tissue distribution	Cellular localization[a]	Substrates[b]	Important roles	Chromosomal localization
SLC01A2	OATP1A2	Brain, kidney, liver, intestine, eye	BL (ciliary body epithelia) AP (kidney, liver, intestine)	Fexofenadine, deltorphin, ouabain, rosu va statin, methotrexate, imatinib, BSP, T_4, T_3, PGE_2, N-methylquinine, microcystin-LR, E_2G, E_1S, TCA, DHEAS, GCA	CNS distribution, Intestinal uptake, Renal secretion	12p12
SLCO1B1	OATP1B1	Liver	BL	Benzylpenicillin, cerivastatin, atorvastatin, rosuvastatin, pitavastatin, caspofungin, rifampin, atrasentan, valsartan, olmesartan, enalapril, methotrexate, temocaprilat, BSP, SN-38 DHEAS, T_4, T_3, PGE_2, TCA, DHEAS, E2G, E_1S, bilirubin, bilirubin-glucuronides, microcystin-LR	Hepatic uptake	12p12
SLCO1B3	OATP1B3	Liver	BL	Fexofenadine, deltorphin, ouabain, digoxin, rosuvastatin, valsartan, pitavastatin, docetaxel, paclitaxel, CCK-8, rifampin, methotrexate, repaglinide, telmisartan, olmesartan, enalapril, microcystin-LR, BSP, T_3, T_4, E_2G, E_1S, GCA, TCA, DHEAS, DPDPE	Hepatic uptake	12p12
SLCO2B1	OATP2B1	Liver, intestine, brain, placenta,	BL (liver, placenta) AP (intestine)	Benzylpenicillin, pravastatin, fluvastatin, rosuvastatin, fexofenadine, E_1S, PGE_2, BSP, DHEAS,	Hepatic/intestinal uptake	11q13

[a]*BL* basolateral, *AP* apical

[b]*BSP* bromosulfophthalein, *CCK-8* cholecystokinin octapeptide, *DPDPE* [D-penicillamine^{2,5}] enkephalin, E_1S estrone sulfate, E_2G estradiol-17ß-glucuronide, *DHEAS* dehydroepiandrostane sulfate, *GCA* glycocholate, PGE_1 prostaglandin E_1, PGE_2 prostaglandin E_2, $PGF_{2\alpha}$ prostaglandin $F_{2\alpha}$, T_3 triiodothyronine, T_4, thyroxine, *TCA* taurocholate, *SN-38* 7-ethyl-10-hydroxycamptothecin

Table 2 SLC22 transporter family

Gene symbol	Protein	Tissue distribution	Cellular localization[a]	Substrates[b]	Important roles	Chromosomal localization
SLC22A1	OCT1	Liver, brain, intestine muscle, heart, placenta	BL	Metformin, acyclovir, gancylcovir, quinidine, cimetidine, acetylcholine, agmatine, choline, oxaliplatin, imatinib, MPP, TEA	Hepatic uptake	6q26
SLC22A2	OCT2	Kidney, intestine, brain, placenta, lung	BL	Mepiperphenidol, cimetidine, famotidine, ranitidine, memantine, metformin, quinine, debrisoquine, cisplatin, oxaliplatin, choline, acetylcholine, dopamine, epinephrine, histamine, norepinephrine, serotonin, agmatine, MPP, TEA	Renal secretion	6q26
SLC22A3	OCT3	Muscle, liver, placenta, heart, intestine, lung, brain	BL	Atropine, etilefrine, epinephrine, histamine, norepinephrine, agmatine, oxaliplatin, MPP, TEA	Cardiac uptake, Placental translocation	6q27
SLC22A6	OAT1	Kidney, brain, placenta	BL	Cephalosporins, tetracycline, acylclovir, adefovir, cidofovir, methotrexate, gancyclovir, zidovudine, cimetidine, ranitidine, bumetanide, furosemide, ibuprofen, indomethacin, ketoprofen, PAH, α-ketoglutarate, PGE_2, $PGF_{2\alpha}$, cAMP, cGMP, folate, urate	Renal secretion	11q13.1–q13.2
SLC22A7	OAT2	Liver, kidney	BL	Cephalosporins, erythromycin, zidovudine, cimetidine, ranitidine, 5-fluorouracil, paclitaxel, methotrexate, bumetanide, allopurinol, salicylate, cAMP, DHEAS, E_1S, PGE_2, $PGF_{2\alpha}$, PAH, L-ascorbate, α-ketoglutarate	Hepatic uptake	6p21.1–p21.2
SLC22A8	OAT3	Kidney, brain, muscle	BL	Benzylpenicillin, tetracycline, cephalosporins, valacyclovir, zidovudine, cimetidine, ranitidine, famotidine, methotrexate, furosemide, ibuprofen, indomethacin, ketoprofen, salicylate, pravastatin, cAMP, cortisol, PGE_2, $PGF_{2\alpha}$, DHEAS, E_1S, E_2G, TCA, urate	Renal secretion	11q12.3
SLC22A9	OAT4	Kidney, placena	AP	Tetracycline, zidovudine, methotrexate, bumetanide, ketoprofen, salicylate, E_1S, DHEAS, ochratoxin A, PGE_2	Renal uptake	

[a]*BL* basolateral, *AP* apical

[b]*DHEAS* dehydroepiandrostane sulfate, *E_1S* estrone sulfate, *E_2G* estradiol-17ß-glucuronide, *MPP* 1-methyl-4-phenylpyridinium, *PAH* *p*-aminohippurate, *PGE_2* prostaglandin E_2, *$PGF_{2\alpha}$* prostaglandin $F_{2\alpha}$, *TCA* taurocholate, *TEA* tetraethylammonium

Table 3 SLC28/29 transporter families

Gene symbol	Protein	Tissue distribution	Cellular localization[a]	Substrates[b]	Important roles	Chromosomal localization
SLC28A1	CNT1	Liver, kidney, intestine	AP	Gemcitabine, 2′-deoxy-5-fluorouridine, 5-fluorouridine, uridine, cytidine, thymidine	Nucleoside salvage, pyrimidine transport	15q25–26
SLC28A2	CNT2	Liver, kidney, intestine, heart, skeletal muscle	BL	Cladribine, clofarabine, fludarabine adenosine, inosine, uridine	Nucleoside salvage, purine transport	15q13–14
SLC28A3	CNT3	Intestine, pancreas, kidney bone marrow, trachea	AP	Gemcitabine, 2′-deoxy-5-fluorouridine, 5-fluorouridine, fludarabine, cladribine, clofarabine, 6-mercaptopurine, 6-thioguanine, uridine, cytidine, thymidine, inosine, guanosine, adenosine	Nucleoside salvage, purine/pyrimidine tranport	9q22.2
SLC29A1	ENT1	Ubiquitous (liver, heart, testis, spleen, lung, kidney, brain)	AP/BL/IC	Gemcitabine, cytarabine, cladribine, fludarabine, 5′-deoxy-5-fluorouridine, adenosine, guanosine, cytidine, thymidine, uridine	Nucleoside salvage, purine/pyrimidine transport	6p21.2-p21.1
SLC29A2	ENT2	Ubiquitous (skeletal muscle, brain, heart, placenta, pancreas, thymus, kidney)	BL/IC	Gemcitabine, cladribine, fludarabine, clofarabine, 2′,3′-dideoxy cytidine (ddC), zidovudine, , 2′,3′-dideoxy inosine (ddI), uridine, hypoxanthine, adenine, thymine, uracil	Nucleoside salvage, purine/pyrimidine transport Nucleobase transport	11q13
SLC29A3	ENT3	Ubiquitous (placenta, uterus, ovary, spleen, lymph node, bone marrow)	IC	Fludarabine, cladribine, ddC, ddI, zidovudine, adenosine, uridine, adenine	Nucleoside salvage, purine/pyrimidine transport	10q22.1
SLC29A4	ENT4	Brain, heart, liver, skeletal muscle	PM	Adenosine, serotonin, MPP	Amine transporter	7p22.1

[a]*BL* basolateral, *AP* apical, *IC* intracellular membranes, *PM* plasma membrane
[b]*ddC* 2′,3′-dideoxycytdine, *ddI* 2′,3′-dideoxyinsosine, *MPP* 1-methyl-4-phenylpyridinium

pharmacogenetics on drug disposition have only recently become available or initiated. Nevertheless, the clinical relevance of functionally relevant polymorphisms in transporter genes continues to be actively and vigorously studied by various research groups with particular attention to interindividual variability in drug disposition. Indeed, genotype:phenotype correlative studies have the potential to more fully delineate the role of an individual's genetic makeup in determining the response to drug therapy. These types of studies are particularly relevant for oncologists as most chemotherapeutic agents possess narrow therapeutic indices, and thus, the identification of genetic factors that may help predict the interindividual variability in chemotherapy disposition may ultimately help to better tailor individual therapeutic regimens that maximize efficacy while minimizing toxicity.

4.1 SLCO Variants

There has been significant effort in studying polymorphisms in *SLCO* genes as potential determinants of interindividual variability in drug disposition (Table 4). To date, the functional consequences of polymorphisms in the hepatic uptake transporter OATP1B1 have received the most attention. Repeatedly, studies have demonstrated that common polymorphisms in *SLCO1B1* are associated with altered oral drug exposure. One nonsynonymous single nucleotide polymorphism (SNP) is 388A>G (Asn130Asp) [33]. The frequencies of the *SLCO1B1* 388G allele (**1b*) in Caucasians, African Americans, and Asians are approximately 40 %, 75 %, and 60 %, respectively [33–39]. Another common SNP is 521 T>C (Val174Ala) [33]. This allele (*SLCO1B1*5*) has frequencies of approximately 15 %, 2 %, and 15 % in Caucasians, African Americans, and Asians, respectively [33–39]. The 388G and 521C SNPs are in linkage disequilibrium and form the *SLCO1B1*15* haplotype [39].

Several pharmacokinetic studies using the HMG-CoA reductase inhibitor ("statin") pravastatin as a probe for OATP1B1 activity strongly demonstrate that individuals with the *SLCO1B1*5* or **15* genotype have increased drug exposure in comparison to those carrying the reference allele *SLCO1B1*1a* (388A, 521 T). These findings are consistent with in vitro studies indicating the OATP1B1 521C variant has decreased transport function, as a result of decreased cell surface expression, towards a variety of substrates [33, 40–43]. Some studies suggest *SLCO1B1*1b* is a high transport activity genotype since plasma pravastatin levels are lower in subjects carrying this variant than those with the reference allele [36, 44]. However, in vitro studies have not confirmed higher transport activity of the OATP1B1*1b protein [33, 41, 43]. Evaluation of a human liver bank did not correlate *SLCO1B1* genotype with total hepatic OATP1B1 protein expression [40]. Further studies are required to clarify the mechanisms responsible for these in vivo observations. Apart from pravastatin, the pharmacokinetics of a growing number of drugs appears to be dependent on *SLCO1B1* 521 T>C genotype including pitavastatin, rosuvastatin, repaglinide, nateglinide, fexofenadine, atrasentan, valsartan, irinotecan, and ezetimibe (Table 4) [35, 44–51].

Understanding that statin drug levels are at least in part determined by genetic factors, there has been interest in assessing whether *SLCO1B1* genotype also predicts risk for muscle toxicity and cholesterol-lowering effect. Increased systemic drug exposure is a risk factor for statin-mediated myopathies including severe rhabdomyolysis [52] suggesting enhanced drug exposure resulting from *SLCO1B1* variants may similarly elevate risk for such side effects [53]. A recent study, the Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine (SEARCH), randomized over 12,000 patients with prior myocardial infarction to determine whether a daily dose of 80 mg of simvastatin safely produces greater benefits than does a daily dose of 20 mg of simvastatin. The SEARCH Collaborative Group identified an increased incidence of myopathy in those who were taking the higher dose of simvastatin and hypothesized it could be due to genetic variants in drug disposition genes leading to supratherapeutic statin levels [54]. They conducted a genome-wide association study using ~300,000 markers in 85 subjects with myopathy and 90 controls, all of whom were

Table 4 Selected genetic variants in SLCO uptake transporters

Gene	Protein	Polymorphism	Amino acid change	Allele frequency[a]			In vitro function[b]
				CA	AS	AF	
SLCO1B1	OATP1B1	217 T>C	Phe73Leu	2 %	0 %	0 %	↓ E_1S, E_2G, rifampin, rosuvastatin
		245 T>C	Va182Ala	2 %	0 %	0 %	↓ E_1S, E_2G, rifampin, rosuvastatin
		388A>G	Asn130Asp	38 %	63 %	77 %	↔ E_1S, E_2G, rosuvastatin, pravastatin, atorvastatin
		463C>A	Pro155Thr	16 %	0 %	2 %	↔ E_1S, E_2G, rosuvastatin, rifampin
		467A>G	Glu156Gly	2 %	0 %	0 %	↓ E_1S, E_2G, rifampin, rosuvastatin
		521 T>C	Val174Ala	15 %	16 %	2 %	↓ E_1S, E_2G, rifampin, rosuvastatin
		1058 T>C	Ile353Thr	2 %	0 %	0 %	↓ E_1S, E_2G, rifampin, rosuvastatin
		1294A>G	Asn432Asp	1 %	0 %	0 %	↔ E_1S ↓ E_1S, E_2G, rifampin, rosuvastatin ↓ E_1S, E_2G, rifampin, rosuvastatin
		1385A>G	Asp462Gly	1 %	0 %	0 %	↔ E_1S, E_2G, rosuvastatin, rifampin
		1463G>C	Gly488Ala	0 %	0 %	9 %	↓ E_1S, E_2G, rifampin, rosuvastatin
		1964A>G	Asp655Gly	2 %	0 %	0 %	↓ E_1S ↔ E_2G, rosuvastatin, rifampin
		2000A>G	Glu667Gly	2 %	0 %	34 %	↔ E_1S, E_2G, rosuvastatin ↓ rifampin
SLCO1A2	OATP1A2	38 T>C	Ile13Thr	16 %	0 %	2 %	↔ deltorphin II, DPDPE ↑ E_1S, methotrexate
		382A>T	Asn128Tyr	0 %	0 %	1 %	↔ E_1S, methotrexate ↔ deltorphin II, DPDPE
		404A>T	Asn135Ile	0 %	0 %	1 %	↔ methotrexate ↓ E_1S, deltorphin II, DPDPE
		502C>T	Arg168Cys	1 %	0 %	0 %	↓ E_1S, methotrexate
		516A>C	Glu172Asp	5 %	0 %	2 %	↓ E_1S, deltorphin II, DPDPE, methotrexate
		830C>A	Thr277Asn	0 %	0 %	1 %	↔ E_1S, methotrexate
		833A>-	Asn278DEL	0 %	0 %	1 %	↓ E_1S, methotrexate
		841A>G	Ile281Val	0 %	1 %	0 %	↔ E_1S, methotrexate
		968 T>C	Leu323Pro	1 %	0 %	0 %	↔ E_1S, methotrexate
		1063A>G	Ile355Val	2 %	0 %	0 %	↔ E_1S, methotrexate
		2003C>G	Thr668Ser	0 %	0 %	4 %	↔ E_1S, deltorphin II, DPDPE, methotrexate
SLCO2B1	OATP2B1	1175C>T	Thr392Ile		0 %		↓ E_1S
		1457C>T	Ser486Phe		31 %		↓ E_1S
SLCO1B3	OATP1B3	334 T>G	Ser112Ala	78 %			↔ BSP, cholyltaurine
		699G>A	Met233Ile	71 %			↔ BSP, cholyltaurine
		1564G>T	Gly522Cys	2 %			↔ BSP ↓ cholyltaurine

[a] *CA* Caucasian, *AS* Asian, *AF* African–American

[b] *BSP* bromosulfophthalein, *DPDPE* [D-penicillamine^{2,5}] enkephalin, E_1S estrone sulfate, E_2G estradiol-17ß-glucuronide

taking 80 mg of simvastatin daily. The genome-wide scan yielded a single strong association of myopathy with the rs4363657 SNP located within *SLCO1B1* on chromosome 12. This intronic SNP was in nearly complete linkage disequilibrium with the nonsynonymous rs4149056 SNP (521 T>C; $r^2=0.97$), which is associated with increased statin levels in vivo. The odds ratio for myopathy was 4.5 per copy of the C allele and 16.9 in CC as compared to TT homozygotes. More than 60 % of myopathy cases could be attributed to the C variant. Although the mechanisms by which statins cause myopathy is still unclear, OATP1B1 polymorphisms clearly influence risk for toxicity mediated by statins. Because OATP1B1 presents statin drugs to their target in hepatocytes, investigators have examined the role of transporter genetics and subsequent pharmacological effects. In one study, patients with the *SLCO1B1* 521C genotype had reduced lipid-lowering effect by statin drugs than those carrying 521 T [55]. By contrast, there was a lack of influence of *SLCO1B1* genotype to the lipid-lowering response of pravastatin in two studies despite the fact that in one study, the drug levels clearly varied among subject groups [56, 57]. Larger studies will be required to fully clarify the role of *SLCO1B1* polymorphisms in statin toxicity and efficacy.

Only recently has data become available with regard to OATP polymorphisms and interindividual variability in chemotherapy disposition. OATP1B1 variants were found to play an important role in the interindividual variation in methotrexate disposition. Trevino et al. performed a genome-wide analysis of ~500,000 germline SNPs to identify how inheritance affects methotrexate plasma disposition among 434 children with acute lymphoblastic leukemia [58]. Adjusting for age, race, sex, and methotrexate regimen, the most significant associations with methotrexate clearance were found with two SNPs in *SLCO1B1*, rs11045879 and rs4149081. These intronic SNPs were in complete linkage disequilibrium with each other ($r^2=1$) but also in strong linkage disequilibrium with the functional rs4140956 SNP (521 T>C; $r^2>0.84$). The major allele (TT) in rs11045879 was associated with increased methotrexate clearance across treatment regimens and an increased incidence of gastrointestinal toxicity. This seems biologically plausible as increased methotrexate excretion into the GI tract via biliary elimination may increase intestinal exposure to drug and/or metabolites and therefore enhance the risk of GI toxicity.

Polymorphisms in *SLCO1A2* have been identified and variant proteins have been characterized in vitro (Table 4) [59, 60]. Few variants are common with most occurring at a frequency <10 %. Lee et al. identified six nonsynonymous polymorphisms in *SLCO1A2* [60]. Several variants were associated with impaired transport activity in vitro utilizing a recombinant vaccinia-based expression system in HeLa cells, but this was substrate dependent. The 404A>T, 516A>C, and 559G>A variants were associated with impaired transport of the hormone conjugate estrone sulfate, while the 404A>T and 516A>C variants were also associated with impaired transport of the opioid receptor agonists enkephalin and deltorphin II. Western analysis of cell surface expressed OATP1A2 fractions, and confocal immunofluorescence microscopy revealed decreased surface expression, and/or mistrafficking defects may explain, in part, the impaired transport activity of the 404A>T and 516A>C variants.

Badagnani et al. identified and functionally characterized additional polymorphisms in *SLCO1A2* including a rare variant, 833A>-, resulting in early truncation of the OATP1A2 protein, retaining only the first 6 transmembrane domains [59]. They evaluated the interaction of OATP1A2 variants with estrone sulfate and methotrexate in vitro using a *Xenopus laevis* oocyte expression system. Interestingly, the 38 T>C variant, which had demonstrated equivalent transport activity for estrone sulfate, enkephalin, and deltorphin II in the aforementioned study, had ~twofold increased transport activity for estrone sulfate and methotrexate in this study. The 404A>T variant demonstrated equivalent transport activity for estrone sulfate and methotrexate, while the 516A>C variant, along with the 502C>T variant, demonstrated impaired activity for these substrates, and the 833A>- variant demonstrated complete loss of transport activity for both substrates. The underlying reasons for the discrepant results are not clear and may be related to factors such as different expression systems and

times at which uptake was calculated and merits further study, but it is certainly plausible that genetic heterogeneity in *SLCO1A2* may contribute to interindividual disposition in substrate drugs, including the anticancer agent methotrexate.

Similarly, polymorphisms in *SLCO1B3* have been identified (Table 4) [61, 62]. Two common nonsynonymous SNPs, 334 T>G and 699G>A, exist in almost complete linkage disequilibrium with allele frequencies ~80 % in Caucasian populations and ~40 % in African–American populations [63]. Interestingly, in vitro, this double variant was associated with similar kinetic values (K_m and V_{max}) to wild-type OATP1B3 for the antimicrotubule taxane paclitaxel in a *Xenopus laevis* expression system [64]. Furthermore, paclitaxel pharmacokinetics in 90 European Caucasian cancer patients was not associated with OATP1B3 polymorphisms or haplotypes [63]. Conversely, in a separate study, when expressed in Cos-7 cells, the double variant was associated with significantly impaired uptake of testosterone in vitro compared to wild-type OATP1B3 [65]. OATP1B3 was found to be overexpressed in prostate cancer tissues, and patients with the SLCO1B3 variant 334GG/699AA haplotype showed longer median survival (8.5 years vs. 6.4 years; $P=0.020$) and improved survival probability at 10 years (42 % vs. 23 %; $P<0.023$) than patients carrying TT/AA and TG/GA haplotypes. Since OATP1B3 is a known hormone transporter, collectively, these data suggest that the overexpression of OATP1B3 is part of a complex stepwise process of cancer progression, which allows the prostate cancer cells to gain a biological advantage by upregulating testosterone uptake capability and proliferative activity through increased androgen input. The influence of *SLCO1A2* and *SLCO1B3* polymorphisms on drug disposition in vivo remains to be determined.

4.2 SLC22 Variants

OCT1 has been shown to be highly polymorphic in ethnically diverse populations (Table 5) [66–68]. Using model substrates, it was shown that a number of nonsynonymous polymorphisms in *SLC22A1* exhibit reduced activity in vitro. However, until recently, the clinical significance of OCT1 variants had not been investigated. The biguanide metformin is widely used as a first-line therapy for the treatment of type 2 diabetes [69]. Interestingly, metformin was also found to inhibit the growth of cancer cell lines, including breast cancer, using in vitro and in vivo tumor models [70–72]. Metformin is currently being investigated as a therapeutic agent in different clinical settings for all breast cancer subtypes. Metformin has been determined to be a high-efficiency substrate for OCT1 and OCT2 in vitro [73–76]. Moreover, compared with wild-type mice, $Oct1^{-/-}$ mice have reduced metformin distribution to the liver [75]. A recent study demonstrated the importance of OCT1 variants to metformin disposition [77]. Seven OCT1 variants were associated with significantly reduced metformin uptake in vitro when stably expressed in HEK293 cells. Four of the variants, including Ser14Phe, Ser189Leu, Gly401Ser, and 420del, demonstrated significantly reduced V_{max} values compared to the reference wild-type protein. Interestingly, 2 of the 7 functional variants, 420del and Arg61Cys, are relatively common polymorphisms with allele frequencies 19 % and 7.2 %, respectively, in Caucasian populations [68]. Furthermore, 2 of the variants, Ser189Leu and 420del, previously exhibited normal uptake for the prototypical OCT substrate 1-methyl-4-phenylphridinium (MPP^+) in vitro. Finally, OCT1 variants were associated with different responses to metformin in healthy human volunteers. Individuals carrying at least one of four variant genotypes (Arg61Cys, Gly401Ser, 420del, and Gly465Arg) demonstrated significantly higher plasma glucose levels after an oral glucose tolerance test. These data would suggest that polymorphisms in OCT1 may contribute to reduced therapeutic response to metformin clinically. Clinical studies in diabetic and potentially breast cancer patients should be undertaken to further extend these results.

Table 5 Selected genetic variants in SLC22 uptake transporters

Gene	Protein	Polymorphism	Amino acid change	Allele frequency[a]			In vitro function[b]
				CA	AS	AF	
SLC22A1	OCT1	41C>T	Ser14Phe	0 %	0 %	3 %	↑ MPP ↓ metformin
		181C>T	Arg61Cys	7 %	0 %	0 %	↓ MPP, metformin
		253C>T	Leu85Phe	0 %	0 %	1 %	↔ MPP
		262 T>C	Cys88Arg	1 %	0 %	0 %	↓ MPP, TEA, serotonin
		480C>G	Phe160Leu	7 %	2 %	<1 %	↔ MPP, TEA
			Ser189Leu	<1 %	0 %	0 %	↔ MPP ↓ metformin
		659G>T	Gly220Val	0 %	0 %	<1 %	↓ MPP, metformin
		848C>T	Pro283Leu	0 %	0 %	<1 %	↓ MPP, TEA
		859C>G	Arg287Gly	0 %	0 %	<1 %	↓ MPP, TEA
		1022C>T	Pro341Leu	0 %	12 %	8 %	↓ MPP ↔ metformin
		1025G>A	Arg342His	0 %	0 %	3 %	↔ MPP, metformin
		1201G>A	Gly401Ser	1 %	0 %	1 %	↓ MPP, TEA, metformin, serotonin
		1222A>G	Met408Val	60 %	76 %	78 %	↔ MPP, metformin
		1258-60delATG	Met420del	19 %	0 %	3 %	↔ MPP ↓ metformin
		1320G>A	Met440Ile	0 %	0 %	<1 %	↔ MPP
		1393G>A	Gly465Arg	<1 %	0 %	2 %	↓ MPP, metformin
		1463G>T	Arg488Met	0 %	0 %	5 %	↔ MPP, metformin
SLC22A2	OCT2	134insA	Phe45	<1 %	0 %	0 %	↓ MPP
		160C>T	Pro54Ser	0 %	0 %	<1 %	↓ MPP
		481 T>C	Phe161Leu	<1 %	0 %	0 %	↓ MPP
		493A>G	Met165Val	0 %	0 %	<1 %	↓ MPP
		495G>A	Met165Ile	0 %	0 %	1 %	↓ MPP
		808G>T	Ala270Ser	16 %	17 %	11 %	↔ MPP
		890C>G	Ala297Glu	<1 %	0 %	0 %	↓ MPP
		1198C>T	Arg400Cys	0 %	0 %	1.5 %	↓ MPP
		1294A>C	Lys432Gln	0 %	0 %	1 %	↔ MPP
SLC22A6	OAT1	149G>A	Arg50His	0 %	0 %	3 %	↔ PAH, ochratoxin A, methotrexate
		311C>T	Pro104Leu	0 %	0 %	<1 %	↔ PAH, ochratoxin A, methotrexate
		677 T>C	Ile226Thr	<1 %	0 %	0 %	↔ PAH, ochratoxin A, methotrexate
		767C>T	Ala256Val	0 %	0 %	<1 %	↔ PAH, ochratoxin A, methotrexate
		877C>T	Arg293Trp	0 %	0 %	2 %	↔ PAH, ochratoxin A, methotrexate
		1361G>A	Arg454Glu	0 %	0 %	<1 %	↓ PAH, ochratoxin A, methotrexate

[a] *CA* Caucasian, *AS* Asian, *AF* African–American

[b] *MPP* 1-methyl-4-phenylpyridinium, *PAH* *p*-aminohippurate, *TEA* tetraethylammonium

4.3 SLC28 Variants

Coding region SNPs have been recently reported for CNT1, CNT2, and CNT3 (Table 6) [78–81]. Thirteen nonsynonymous SNPs were identified in the *SLC28A1* gene (CNT1) from genomic DNA of 247 ethnically defined individuals [79, 80]. Functional analysis using a *Xenopus laevis* expression system demonstrated that all variants transported thymidine except for the variants Ser546Pro and 1153del, which results in a base pair deletion that causes a frameshift followed by a stop codon. Interestingly, a common CNT1 variant, Val189Ile, with 26 % allele frequency, showed reduced affinity for the anticancer nucleoside analog gemcitabine, suggesting that genetic variation in *SLC28A1* may contribute to variation in systemic and intracellular levels of chemotherapeutic nucleoside analogs.

Table 6 Selected genetic variants in SLC28/29 uptake transporters

Gene	Protein	Polymorphism	Amino acid change	Allele frequency[a]			In vitro function
				CA	AS	AF	
SLC28A1	CNT1	419 T>+TTG	Leu140INS	32 %	18 %	31.50 %	↔ thymidine
		565G>A	Val189Ile	35 %	35.00 %	19 %	↔ thymidine; ↑ IC_{50} gemcitabine
		709C>A	Gln237Lys	19.50 %	28.30 %	14.50 %	↔ thymidine
		1153G>-	Val385DEL	0 %	0 %	3 %	↓↓ thymidine
		1561G>A	Asp521Asn	51 %	3.40 %	10 %	↔thymidine
		1636 T>C	Ser546Pro	0 %	0 %	1 %	↓↓ thymidine
SLC28A2	CNT2	65C>T	Pro22Leu	63 %	45 %	18 %	↔ guanosine
		225C>A	Ser75Arg	67 %	18 %	16 %	↔ guanosine
		734G>C	Ser245Thr	0 %	2 %	23 %	↔ guanosine
		1064 T>C	Phe355Ser	0 %	0 %	4 %	↔ guanosine
SLC28A3	CNT3	338A>G	Tyr113Cys	9 %	9 %	17 %	↔ thymidine, inosine
		982A>G	Ile328Val	0 %	0 %	6 %	↔ thymidine, inosine
		1099G>A	Gly367Arg	0 %	1 %	0 %	↓↓ thymidine, inosine
		1804 T>C[b]	Cys602Arg	ND	ND	ND	↓ uridine, cytidine, thymidine, guanosine, adenosine, gemcitabine, fludarabine
SLC29A1	ENT1	647 T>C	Ile216Thr	2 %	0 %	1 %	↔ inosine, tubercidin, 5-fluorouridine
		1171G>A	Glu391Lys	0 %	0 %	1 %	↔ inosine, tubercidin, 5-fluorouridine
SLC29A2	ENT2	13G>T	Asp5Tyr	0 %	0 %	1 %	↓ inosine, fludarabine, gemcitabine
		93C>A	Asn68Lys	0 %	0 %	1 %	↔ inosine, fludarabine, gemcitabine
		281C>T	Pro94Leu	0 %	0 %	1 %	↔ inosine, fludarabine, gemcitabine
		551–556DEL	Ser184Met, del Gly185 and Val186	1 %	0 %	0 %	↓ inosine, ↔ fludarabine, gemcitabine

[a]*CA* Caucasian, *AS* Asian, *AF* African–American, *ND* not determined
[b]Variant has 1 % allele frequency in Spanish population

Polymorphisms have also been identified in *SLC28A3* (CNT3). Ten nonsynonymous SNPs were identified in *SLC28A3* from genomic DNA from 270 ethnically defined individuals [78]. In general, polymorphisms tended to be rare, indicating that *SLC28A3* is highly conserved. All nonsynonymous variants had similar transport capacities to those of wild-type CNT3 with the exception of a rare variant Gly367Arg. Gly367 is one of the four highly conserved residues in TM8, and conversion to an arginine at this position resulted in an 80 % decrease in transport of both purine and pyrimidine nucleoside substrates, suggesting that TM8 is an important component of the substrate recognition domain.

5 Transport Mechanisms

5.1 OATP Transport

It is generally accepted that OATPs can transport substrates in a bidirectional fashion governed by the solute gradients across the plasma membrane. The mechanisms underlying transport by OATPs have been investigated in some detail, largely focused on understanding driving forces since early in vitro

studies demonstrated lack of stimulation in transport activity by an inwardly directed sodium gradient [82–84]. Studies with rat Oatp1a1 and Oatp1a4 first demonstrated that solute uptake into cells was activated by countertransport with either bicarbonate [85] or reduced glutathione (GSH) [86, 87] with a stoichiometry for GSH/bile acid exchange 1:1 for rat Oatp1 [86]. However, Oatp1-mediated GSH efflux was not dependent on obligate exchange with solute such as bile acids [88]. Interestingly, human OATP2B1 was found to possess pH-dependent transport properties that were solute selective [84, 89]. Extracellular acidification promoted solute uptake, a property of OATP2B1 that bears relevance to the environment in which the transporter is expressed on the apical membrane of enterocytes. This is in contrast to the insensitivity of rat Oatp1 activity by proton gradients [90].

A predominant bile acid efflux function for OATP1B3 in liver has been proposed. The fascinating finding that bile acid transport by OATP1B3 and not OATP1B1 occurs by a GSH cotransport mechanism suggests that OATP1B3 confers hepatocyte protection by limiting the accumulation of toxic intracellular solutes [91]. A 2:1 GSH/bile acid cotransport stoichiometry for OATP1B3-mediated transport was observed. OATP2A1 appears to activate solute uptake by outward exchange with lactate [92]. Overall, it is evident that various OATPs can be defined by different transport mechanisms. Structural modeling of OATP1B3 and OATP2B1 has suggested that OATPs transport solutes across membranes through a rocker-switch type mechanism [93], but the location of solute-binding sites and molecular mechanisms of the transport process are yet unclear.

5.2 OCT/OAT Transport

The basic transport characteristics of OCTs are similar in various species. OCTs transport a variety of organic cations with widely differing molecular structures in an electrogenic manner. Electrogenicity of transport has been shown for the rat transporters Oct1, Oct2, and Oct3 [94–99] and for the human transporters OCT1 and OCT2 [100–102]. OCTs function independently of sodium gradients and are also independent of proton gradients when the effect of proton gradients on the membrane potential is excluded [95, 97, 102, 103]. OCTs are able to transport organic cations across the plasma membrane in a bidirectional manner. In addition to cation influx, cation efflux has been demonstrated for rat and human Octs/OCTs [95, 97, 98, 100, 104].

OATs do not directly utilize energy from ATP hydrolysis for activation of substrate transport. Most members of the OAT family operate as anion exchangers by coupling the uptake of an organic anion into the cell to the release of another organic anion from the cell [8, 105]. OATs utilize existing intracellular to extracellular gradients of anions, such as α-ketoglutarate and lactate, to drive uphill uptake of organic anions against the inside negative membrane potential. In kidney proximal tubular cells, OATs are functionally coupled to sodium-driven mono- and dicarboxylate transporters that establish and maintain the intracellular>extracellular gradients of lactate, nicotinate, and α-ketoglutarate. In the basolateral membrane, the Na^+,K^+-ATPase pumps three sodium ions out that return together with one α-ketoglutarate into the cell via the sodium-dicarboxylate cotransporter 3. α-Ketoglutarate is then exchanged via OAT1 or OAT3 against an organic anion delivered to the cell by the blood. Accordingly, basolateral uptake of organic anions in proximal tubular cells is referred to as tertiary active transport [105]. While OAT1 and OAT3, localized to the basolateral membrane of proximal tubular cells, and OAT4, localized to the apical membrane of proximal tubular cells, have all been demonstrated to be organic anion/dicarboxylate exchangers [106–109], the precise transport mechanism for OAT2 has not been clearly defined. Human OAT2 was reported to transport α-ketoglutarate, but this dicarboxylate did not inhibit OAT2-mediated *p*-aminohippurate (PAH) transport [110]. It has been proposed to be a sodium-independent multispecific organic anion/dimethyldicarboxylate exchanger, but controversy still exists with regard to its specific transport mechanism [111].

5.3 CNT Transport

A number of studies over the past few decades have demonstrated up to seven systems could mediate nucleoside transport in mammalian cells [11]. Five systems demonstrate high-affinity substrate recognition and transport (N1 or cif, N2 or cit, N3 or cib, N4, and N5) [10]. The N1 system primarily is purine selective but also transports uridine, while the N2 system tends to be pyrimidine selective. The N3 and N4 systems are broadly selective, transporting both purine and pyrimidine nucleosides. The N4 system also possesses high specificity towards guanosine [112, 113], while the N5 system is nitrobenzylthioinosine (NBTI) sensitive and transports guanosine [114]. The N1, N2, and N3 transport systems have been identified as the CNT proteins which mediate the unidirectional flow of nucleosides in an active, energy-dependent process coupled to an inwardly directed electrochemical sodium gradient [79, 80, 115, 116]. CNT1, CNT2, and CNT3 correspond to activities N2, N1, and N3, respectively. However, CNT3 has also been shown capable of coupling transport to protons [116]. Proteins responsible for N4 and N5 transport systems have yet to be identified, although it has been postulated they might be either putative polymorphic variants of the three known CNT family members or associated with other nonrelated families of transporter proteins [117].

5.4 ENT Transport

The transport inhibitor nitrobenzylmercaptopurine ribonucleoside (NBMPR) can be used to functionally differentiate ENT1, which mediates equilibrative NBMPR-sensitive (es) transport activity, from ENT2, which mediates equilibrative NBMPR-insensitive (ei) transport activity [118–120]. ENT1 and ENT2 have the capacity to transport both purine and pyrimidine nucleosides although ENT2 also transports some nucleobases such as hypoxanthine [121]. ENT3, which is broadly selective for both purine and pyrimidine nucleosides, appears to function as a transporter in intracellular membranes such as lysosomes [32, 122] whereas ENT4 has been demonstrated to transport adenosine and monoamine neurotransmitters across plasma membranes, primarily in brain and cardiac tissue [123]. ENT3 and ENT4 are insensitive to NBMPR [123]. ENTs mediate facilitated diffusion of nucleosides across membranes bidirectionally according to substrate concentration gradients, although both ENT3 and ENT4 exhibit enhanced activity at acidic pH, suggesting a proton-coupled transport mechanism [122, 124].

6 Protein Structure–Function

6.1 OATP Protein Structure–Function

The OATPs are predicted to be membrane proteins that contain 12 transmembrane (TM) helices that have a characteristic superfamily signature amino acid sequence D-X-RW-(I,V)-GAWW-X-G-(F,L)-L with amino and carboxy termini oriented to the cytoplasmic spaces [14]. Predicted and confirmed *N*-glycosylation sites are found, many conserved between transporters, in extracellular loops 2 and 5. In silico, structural modeling studies with OATP1B3 and OATP2B1 have suggested that the OATPs share features of the major facilitator superfamily (MFS) [93]. According to these models, OATP1B3 is predicted to possess a central pseudo-twofold symmetry axis perpendicular to the membrane plane and a central pore. The pore formed by TM helices 1, 2, 4, 5, 7, 8, 10, and 11 contains conserved basic/polar

residues thought to be important to substrate binding and transport mechanism [93]. In addition, modeling of the large extracellular loop 5 in OATP1B3 revealed similarities to Kazal-type serine protease inhibitors and predict internal disulfide bonds of the present cysteine residues [93]. The relevance of the ten cysteine residues in the fifth extracellular loop of OATP2B1 was examined by mutational analysis [125]. Indeed, mutation of any of the cysteine residues or deletion of the loop itself caused mistrafficking of the protein to the cell surface. Moreover, each cysteine residue was found to be disulfide bridged. Given that the electrostatic potential of extracellular loop 5 is not basic [93], it is not predicted to have functional interactions with solutes, highlighting the important functional role of this domain in membrane insertion.

Elucidating the quaternary structure of OATPs has received little attention despite some evidence that members of this family form homo- or hetero-oligomers. For instance, even under reducing conditions, high molecular weight bands suggesting multimers were present after Western analysis of OATP1A2 protein heterologously expressed in mammalian cells [60]. Cross-linking experiments with OATP2B1 showed that amino groups between two OATP2B1 molecules would have to be minimally 12 Å apart to be consistent with the observed homo-cross-linking found in cells overexpressing the protein [125]. Using immunoprecipitation, attempts to determine whether mouse Oatp1a1 and Oatp1a4 heterodimerize in liver failed to show direct association between the proteins [126].

6.1.1 OCT/OAT Protein Structure–Function

SLC22 family members have a predicted membrane topology composed of 12 α-helical transmembrane domains (TMDs), an intracellular N-terminus, a large glycosylated extracellular loop between TMDs 1 and 2, a large intracellular loop with phosphorylation sites between TMDs 6 and 7, and an intracellular C-terminus [127]. Using site-directed mutagenesis with rat Oct1, 7 amino acids were identified that are involved in cation binding: Trp218, Tyr222, and Thr226 on successive turns of the α-helix that forms the predicted 4th TMD [128], Ala443, Leu447, and Gln448 in the 10th TMD [129], and Asp475 in the middle of the 11th TMD [128, 130]. Two members of the MFS superfamily, the lactose permease LacY and glycerol-3-phosphate transporter GlpT from *E. coli*, were crystallized and their tertiary structure determined [131, 132]. Each demonstrates a large cleft that opens to the intracellular side and is formed by 8 transmembrane α-helices. Based on the tertiary structures of Lac Y and GlpT, a model of the tertiary structure of the TMDs of rat Oct1 with a large cleft was constructed [128]. Importantly, the seven key amino acids that had been previously assigned to the substrate-binding region are located at a similar depth within this cleft. A comparison of substrate size and substrate-binding region in the model suggests more than one compound can bind at the same time. The model of the tertiary structure of the OCTs was further supported by the observation that Cys474 in the 11th TMD of OCT2 that corresponds to Cys474 in rat Oct1 is accessible from the aqueous phase [133].

In rat Oat3 and flounder Oat1, conserved aromatic residues in TMDs 7 and 8 and conserved basic residues in TMDs 1, 8, and 11 are required for transport activity as supported by functional studies in a *Xenopus* expression system [134, 135]. The basic residues in OATs are localized in the positions corresponding to the acidic residues in OCTs, demonstrating the role of these basic residues in the charge specificity of OATs. Leu30 in TMD 1 of OAT1 [136] and Gly241 and Gly400 in TMDs 5 and 8 of OAT4 [137] were shown in vitro to participate in the targeting of the transporters to the plasma membrane. Interestingly, OAT1 was recently shown to exist in the plasma membrane of kidney LLC-PK1 cells as a homooligomer [138]. However, the functional consequences of OAT1 oligomerization are currently unknown.

6.2 CNT Protein Structure–Function

CNTs share a predicted topology based on 13 TMDs [139, 140], consistent with the N-terminus tail facing the cytoplasm and an extracellular C-terminus domain. The membrane orientation of the C-terminus was demonstrated by studying the effect of endoglycosidase F on CNT1, thus demonstrating N-glycosylation at either Asn605 or Asn643, or both [139]. The localization of the N-terminus domain has also been established using appropriate antibodies and immunohistochemical analysis.

Chimeric studies have assisted the identification of structural domains responsible for substrate binding and specificity. Replacement of TMDs 8 and 9 in rat Cnt1 by the corresponding domains in rat Cnt2 turned a transporter that preferred to transport pyrimidine nucleosides into one that preferred to transport purine nucleosides, while substitution of TMD 8 alone resulted in a chimera with Cnt3 substrate selectivity [141]. Moreover, substitution of Ser319/Gln320 in TMD 7 of CNT1 with Gly 313/Met314 of CNT2 permits purine nucleoside transport, converting CNT1 into a CNT3-like transporter, while the additional substitution of Ser353/Leu354 in TMD 8 of this chimera by the corresponding Thr347/Val348 of CNT2 changes the CNT3-like transporter into a CNT2-like transporter [142]. A recent study functionally characterizing 10 conserved aspartate and glutamate residues in CNT1 predicts that residues Glu308, Glu322, and Glu498 are located within a common cation/nucleoside translocation pore [143]. When expressed in *Xenopus laevis* oocytes, the chimera CNT3/1, comprised of the N-terminal half of CNT3 (TMDs 1–6) and the C-terminal half of CNT1 (TMDs 7–13), demonstrated CNT1-like substrate specificity, sodium dependence, and proton independence, indicating the structural features of cation stoichiometry, proton coupling, and binding affinity reside in the C-terminal half of the protein [116]. Furthermore, a substituted-cysteine accessibility method (SCAM) analysis of TMDs 11–13 of CNT3 suggested that only TMDs 11 and 12 may form part of the nucleoside translocation pathway, although TMD 13 may be involved in maintenance of protein function [144].

6.3 ENT Protein Structure–Function

The proposed 11-transmembrane-domain topology of ENT1, including a cytoplasmic N-terminus and an extracellular C-terminus, was confirmed using glycosylation scanning mutagenesis and a variety of antibodies as topological probes [145]. Although ENT1 is N-glycosylated at a single site and ENT2 at two sites, glycosylation is not essential for maintenance of transport activity or proper cell surface targeting [146, 147]. Functional characterization of rat/human ENT chimeras have elucidated critical regions of the proteins involved in substrate and inhibitor interactions. The region encompassing TM3–6 contains residues responsible for sensitivity or resistance to NBMPR [145]. TM1–6 has been demonstrated to be responsible for the ability of ENT2 to transport 3′-deoxynucleosides [148], while TM5–6 has been determined to be a critical region in the ability of ENT2 to transport nucleobases [121].

The functional characterization of point mutations in ENTs has also yielded important structure–function relationships. Reciprocal mutagenesis studies involving ENT1/ENT2 demonstrated that Met33 in TM1 of ENT1 and the corresponding Ile33 residue in ENT2 are important components of the binding sites for coronary vasodilators and nucleosides [149, 150]. Moreover, another mutagenesis study of ENT1 and *C. elegans* Ent1 has shown a residue position in TM11, Leu442 in ENT1, and Ile429 in *C. elegans* Ent1, important for dipyridamole sensitivity [151]. Interestingly, the effect of ENT1 Leu 442 on dipyridamole sensitivity requires prior modification of Met33 to Ile, suggesting a functional interaction between TMs 1 and 11. Mutagenesis studies in ENT1 have similarly identified residues in TM2, Met89 and Leu92 [152, 153]; TM4, Gly154 and Ser160, [152, 154]; and TM5, Gly179 [155], involved in substrate transport and/or NBMPR binding. The TM4 residue Gly154 is notable in that its mutation to the corresponding residue in ENT2, Ser, leads to loss of NBMPR sensitivity [154].

7 Transporters and Substrate Selectivity

Important to our understanding of transporter-mediated substrate disposition is the dynamic interplay between uptake and efflux transporters within any given cells, where the movement of substrate across such cellular compartments may be impeded or facilitated by the localization of transporters on apical or basolateral membranes and the net directional or vectorial movement markedly affected, dependent on the relative expression, activity, and substrate affinity for the individual transporter. Therefore, the net substrate movement across organs such as the liver, kidney, and intestine is highly dependent on not only the complement of transporters but their subcellular localization. Considering that a number of substrates of both uptake and efflux transporters are environmental toxins or dietary constituents, from an evolutionary point of view, transporters currently thought to play important roles in drug disposition appear to have evolved to either enhance toxin elimination or prevent their absorption.

7.1 OATP Substrates

OATPs mediate the sodium-independent transport of a diverse range of amphipathic organic compounds including bile salts, steroid conjugates, thyroid hormones, anionic peptides, numerous drugs, and other xenobiotic substances (Table 1) [14]. OATP substrates are relatively large and range in size from 334 Da (benzylpenicillin) to 1,143 Da (cholecystokinin octapeptide, CCK-8). Some common features of OATP substrates are steroidal or peptidic (linear or cyclic) structural templates. OATP substrates tend to be negatively charged but there are several examples of neutral (digoxin) and cationic (*N*-methyl-quinine) substrates. Understanding of molecular determinants of substrates in their interactions with OATPs has been learned based upon 3D-QSAR and pharmacophore modeling approaches [156, 157]. These studies support the requirement of a hydrophobic region and hydrogen acceptor and donors for OATP substrates. Endogenous substrates for OATPs include hormones such as thyroxine and steroid conjugates, bile acids, bilirubin, and prostaglandins. Several drug classes are substrates for OATPs, such as 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors ("statins"), angiotensin II receptor antagonists, angiotensin II inhibitors, and cardiac glycosides [7]. More recently, anticancer therapeutic agents have been noted to be substrates for various OATP family members, suggesting emerging important roles for OATPs in chemotherapy disposition.

7.1.1 OATP1A2 (OATP-A; *SLCO1A2*)

OATP1A2 was the first human member of this family isolated using in situ hybridization screening from a human liver cDNA library. Interestingly, its expression was noted to be the strongest expression in the brain and kidney [60, 83]. Subsequently, OATP1A2 has been found in the intestine, cholangiocytes, and colon cancer cells, as well as in the human hepatoma cell line HepG2 [158–160]. Its expression in capillary endothelial cells, a component of the blood–brain barrier, suggests a potential important role in the uptake of drugs and neuroactive peptides into the central nervous system [161]. OATP1A2 substrates include endobiotics such as bile salts, steroid conjugates, the thyroid hormones T4, T3, and rT3, and prostaglandin E_2 (PGE_2) and xenobiotics such as bromosulfophthalein (BSP), the opioid receptor agonists [D-penicillamine-2,5] enkephalin (DPDPE) and deltorphin II, fexofenadine, ouabain, rocuronium, and the cyanobacterial toxin microcystin (Table 1) [83, 161–169]. More recently, OATP1A2 was found to be expressed on the apical membrane of intestinal enterocytes, suggesting it plays a role in determining bioavailability of orally administered drugs [170].

Recent data have indicated that OATP1A2 can transport anticancer drugs. Methotrexate, a folate antimetabolite used in patients with malignant and autoimmune diseases, was transported by OATP1A2 in vitro using a *Xenopus laevis* oocyte expression system [59]. Interestingly, transport was sensitive to extracellular pH as acidic pH stimulated methotrexate uptake by as much as sevenfold. Another study demonstrated in vitro transport mediated by OATP1A2 for the orally administered tyrosine kinase inhibitor imatinib mesylate [171]. Because OATP1A2 is normally expressed in the intestine, it would be tempting to speculate that it is an important transporter that facilitates the intestinal absorption of imatinib.

Interestingly, OATP1A2 has been recently found to be overexpressed in breast carcinoma tissues when compared to normal breast tissue [172]. Microarray analysis demonstrated that the expression of OATP1A2 in breast carcinoma samples was most closely correlated to pregnane X receptor (PXR), a nuclear receptor importantly involved in the regulation of drug disposition genes. In a subsequent study from our laboratory, treatment of breast cancer cells (T47D) in vitro with the PXR agonist rifampin induced OATP1A2 expression in a time-dependent and concentration-dependent manner [173]. We identified a PXR response element in the human OATP1A2 promoter located ~5.7 kb upstream of the transcription initiation site. As OATP1A2 is able to transport hormone conjugates such as estrone sulfate and estradiol glucuronide, induction of OATP1A2 expression mediated by PXR may play an important role in breast cancer pathogenesis by enhancing the intracellular accumulation of hydrophilic estrogen metabolites and promotion of cell proliferation.

7.1.2 OATP1B1 (OATP-C; *SLCO1B1*)

This uptake transporter primarily expressed in liver is considered to be a major pathway for the hepatic extraction of many drugs and endogenous compounds. Initially cloned by several groups from human liver [82, 160, 174, 175], OATP1B1 shares 80 % amino acid identity with OATP1B3, but only 65 % identity with its rat and mouse ortholog Oatp1b2 [14]. OATP1B1 is expressed at the basolateral membrane of hepatocytes [82, 160, 174, 175]. OATP1B1 has been functionally characterized in vitro using multiple heterologous expression systems including *Xenopus laevis* oocytes [168, 174], HEK293 cells [82, 160, 175], and HeLa cells [33]. OATP1B1 has remarkably broad substrate specificity and includes endobiotics such as bile salts, conjugated and unconjugated bilirubin, BSP, steroid conjugates, the thyroid hormones T4 and T3, eicosanoids, cyclic peptides, and drugs such as benzylpenicillin, methotrexate, HMG-CoA reductase inhibitors, and rifampicin (Table 1) [41, 43, 82, 160, 168, 174–180].

Data regarding the role of OATP1B1 in chemotherapy disposition is emerging. Irinotecan is a derivative of camptothecin, an antitumor alkaloid isolated from *Camptotheca acuminate* and an inhibitor of topoisomerase I, and is converted by carboxylesterase to its active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38). Nozawa et al. demonstrated that OATP1B1 transported SN-38 in vitro when expressed in HEK293 cells or *Xenopus laevis* oocytes [42]. Interestingly, OATP1B1 did not transport irinotecan or SN-38 glucuronide. Furthermore, it had previously been suggested that OATP1B1 was capable of transporting methotrexate in vitro [181]. A recently characterized transgenic mouse model with functional expression of human OATP1B1 in mouse liver evaluated the in vivo role of OATP1B1 in the disposition of methotrexate [182]. The AUC for IV methotrexate in SLCO1B1 transgenic mice was 1.5-fold decreased compared with wild-type mice. In addition, the amount of methotrexate in the liver was markedly higher (~twofold) in the SLCO1B1 transgenic mice compared with wild-type mice, resulting in 2- to 4-fold higher liver:plasma ratios of methotrexate. Combined with a recent report demonstrating the importance of SLCO1B1 polymorphic variants to methotrexate renal clearance in a genome-wide association study from children with acute lymphoblastic leukemia [58], it would strongly support the notion that OATP1B1 plays important roles in the pharmacokinetic and pharmacodynamic disposition of methotrexate in vivo.

7.1.3 OATP1B3 (OATP8; *SLCO1B3*)

Similar to OATP1B1, OATP1B3 was cloned from human liver [183] and appears to be primarily expressed at the basolateral membrane of hepatocytes [181, 183]. In addition, OATP1B3 is expressed in various human cancer tissues as well as in different tumor cell lines derived from gastric, colon, pancreas, gallbladder, lung, and brain cancers [181]. The pathologic significance of OATP1B3 expression in human cancer tissues remains to be investigated and clarified although a recent study demonstrating overexpression of OATP1B1 in colorectal adenocarcinomas suggested that OATP1B3 reduces the transcriptional activity of p53 with subsequent reductions in transcript and protein levels of its downstream transcription targets, resulting in apoptotic resistance/survival advantage in colon cancer cells [184]. Similar to OATP1B1, OATP1B3 also transports endobiotics such as bile salts, monoglucuronosyl bilirubin, BSP, steroid conjugates, the thyroid hormones T3 and T4, leukotriene C_4 (LTC_4), cyclic peptides, and drugs such as methotrexate, HMG-CoA reductase inhibitors, and rifampicin (Table 1) [40, 61, 168, 176, 178, 181, 183, 185]. However, OATP1B3 also exhibits unique transport properties in that it is able to mediate the cellular uptake of the intestinal peptide cholecystokinin 8 (CCK-8) [186], the opioid peptide deltorphin II [168], and the cardiac glycosides digoxin and ouabain [168].

Taxanes are diterpenes produced by the plants of the genus *Taxus* (yews) that mediate their effects through microtubule inhibition, thereby stopping cell division. The primary route of taxane elimination is cytochrome P450 (CYP)-mediated metabolism in the liver. *Xenopus laevis* oocytes expressing OATP1B3 were capable of transporting the taxanes paclitaxel and docetaxel in vitro [64]. OATP1B3-mediated paclitaxel transport was saturable, time dependent, and highly sensitive to chemical inhibition. Therefore, OATP1B3 may plan an important role in the hepatic uptake and subsequent CYP-mediated elimination of taxanes. Like OATP1B1, OATP1B3 has also been shown to transport methotrexate in vitro using a Xenopus laevis expression system [181]. Furthermore, OATP1B3 stably expressed in a MDCK cell line was much more sensitive to methotrexate than mock-transfected cells when measuring the inhibitory effect of methotrexate on cell growth. As OATP1B3 is expressed in a number of cancer tissues, it may be a desirable drug target as a means of delivering cytotoxic chemotherapy agents intracellularly to precipitate tumor kill.

7.1.4 OATP2B1 (OATP-B; *SLCO2B1*)

OATP2B1 was originally isolated from the human brain [187] and noted to have a near 80 % amino acid sequence identity with its rat ortholog [14]. Although cloned from a brain library, its strongest expression is in the liver, followed by the spleen, placenta, lung, kidney, heart, ovary, small intestine, and brain [160, 168, 188]. In liver, OATP2B1 is expressed at the basolateral membrane of hepatocytes [168]. However, relative to other OATPs expressed in liver, OATP2B1 has more restricted substrate specificity. Thus, its importance in hepatic drug uptake remains to be clarified [14, 89]. Endogenous substrates include PGE_2 and the steroid conjugates dehydroepiandrosterone-3-sulfate (DHEAS) and estrone-3-sulfate, while xenobiotic substrates include BSP, benzylpenicillin, fexofenadine, and statins such as fluvastatin and rosuvastatin (Table 1) [40, 84, 160, 168, 178]. Recent studies suggest the uptake of substrate drugs may be enhanced in cells exposed to acidic pH [89]. Since the physiologic pH, particularly in the proximal portions of the intestinal epithelial cells is acidic, the role of OATP2B1 in the small intestine might differ from that in other tissues [84]. The role of OATP2B1 in the disposition of chemotherapeutic agents remains yet to be defined.

7.2 OCT/OAT Substrates

While larger, lipophilic, and amphipathic organic anions tend to be transported by OATPs, small organic anions and cations are more efficiently transported by members of the organic cation/anion/zwitterions family (SLC22) [189]. As a whole, these family members handle small, amphiphilic organic anions, organic cations, or uncharged molecules of diverse chemical structures with a molecular weight of most compounds of up to 400–500 Da, so-called type I organic anions or cations [128, 190]. Due to their expression in organs of importance for xenobiotic absorption, distribution, and elimination, including the intestine, liver, and kidney, this transporter family also has important roles in limiting exposure to potentially harmful exogenous compounds as well as the disposition of numerous drug substrates.

7.2.1 OCT1 (*SLC22A1*)

Oct1 was first cloned from rat in 1994 [96]. In humans, OCT1 is expressed mainly in the liver on the basolateral membrane of hepatocytes where it mediates the hepatic extraction of many cationic drugs. However, it is also expressed in the heart, skeletal muscle, kidney, placenta, and small intestine [96, 191]. Because OCT1 can mediate bidirectional transport, it also likely participates in the release of organic cations from hepatocytes into the portal circulation. Most OCT substrates are organic cations and weak bases [74, 191], but some uncharged compounds and anions are also transported. OCT1 substrates include the model cations 1-methyl-4-phenylpyridinium (MPP) and tetraethylammonium (TEA); the endogenous compounds choline, acetylcholine, and agmatine; and drugs such as quinidine, quinine, acyclovir, ganciclovir, and metformin (Table 2) [76, 102, 104, 192–195]. Metformin is currently being investigated as a therapeutic agent for breast cancer due to its ability to inhibit growth of cancer cells, including breast cancer, in in vitro and in vivo tumor models [70–72]. Interestingly, Oct1 knockout mice are fertile and have no overt phenotypic defects [75, 196–198]. The tissue concentrations of choline and cimetidine are similar in Oct1 knockout mice and wild-type mice [75, 196]. However, the concentrations of TEA, MPP, and metformin in the liver and small intestine are reduced, indicating a likely key role for OCT1 in the biliary disposition of certain organic cations.

Besides metformin, OCT1 has been found to transport more established anticancer drugs as well. The platinum-based anticancer drug oxaliplatin was demonstrated to be a substrate for human OCT1 [199]. MDCK cells stably transduced with hOCT1 showed increased accumulation and cytotoxicity to oxaliplatin compared to mock-transfected cells in vitro. Furthermore, OCT1 was found to be expressed in multiple colon cancer tissue samples and colon cancer cell lines. Interestingly, the cytotoxicity of oxaliplatin in six colon cancer cell lines expressing OCT1 was significantly reduced by an OCT inhibitor cimetidine, suggesting that OCTs are important determinants of oxaliplatin activity in colorectal cancers.

Imatinib is a tyrosine kinase inhibitor that is effective in the treatment of chronic myeloid leukemia (CML). In the leukemic cell line CCRFCEM, imatinib uptake was temperature dependent and competed by inhibitors of OCT1, including verapamil, amantadine, and procainamide [200]. OCT2 or OCT3 inhibition had no effect, suggesting imatinib influx into the cells is an active process mediated by OCT1. Furthermore, in 70 CML patients treated with imatinib, those individuals with high pretreatment OCT1 expression had superior complete cytogenetic response rates, progression-free survival, and overall survival [201]. Regression analysis indicated that pretreatment OCT1 expression was the most powerful predictor of complete cytogenetic response achievement at 6 months.

7.2.2 OCT2 (*SLC22A2*)

Oct2 was initially cloned from rat [99] and shortly thereafter was cloned from human [102]. OCT2 is expressed mainly in the kidney, but is also found in placenta, thymus, adrenal gland, neurons, and choroids plexus [100, 102, 190, 202–204]. In rats and humans, OCT2 localizes to the basolateral membrane of renal proximal tubular cells [203, 205, 206] and likely mediates the renal excretion of many cationic drugs. OCT2 translocates several cations that are also transported by OCT1. For example, OCT2 transports MPP, TEA, quinine, and metformin with similar K_m values to OCT1 (Table 2) [102, 127]. Uptake by OCT2 could be also documented for choline; the neurotransmitters dopamine, norepinephrine, epinephrine, serotonin, histamine, and agmatine; and drugs such as cimetidine, famotidine, and debrisoquine [127, 193, 207]. Moreover, it is likely that some drugs which undergo glomerular filtration may be reabsorbed in the proximal tubule across the luminal membrane and reenter systemic circulation via OCT2 on the basolateral membrane. Note that human kidneys express an alternatively spliced variant of OCT2 which lacks three C-terminal TMs but is still capable of transporting TEA, MPP, and cimetidine [208]. Oct2 knockout mice demonstrate no significant alteration in terms of levels of TEA in the small intestine, liver, and kidney [197]. However, in Oct1/Oct2 double-knockout mice, TEA secretion in the renal proximal tubule is abolished, suggesting a critical role of these transporters in renal excretion [197].

OCT2 has also been determined to be an important transporter in the disposition of platinum-based anticancer agents. Cisplatin is one of the most widely used platinum-based drugs but its use is limited by its high nephrotoxicity [209]. OCT2 mediates cisplatin uptake in renal proximal tubules, which likely explains its organ-specific toxicity [209]. Combining cisplatin with other substrates that compete for OCT2 potentially offers an effective strategy to decrease nephrotoxicity in the clinical setting. For example, with rat Oct2, cisplatin interacts preferentially with rOct2 and OCT2 inhibitors such as cimetidine and corticosterone blocked the cytotoxicity and transport of cisplatin in rOct2 cells [210]. Interestingly, TEA uptake by OCT1- or OCT3-expressing HEK293 cells was significantly inhibited by cisplatin, but neither transporter mediated significant accumulation of cisplatin [211]. Another study showed that cisplatin causes a significant toxicity in cells transfected with OCT2, but not with OCT1 or OCT3 [199]. Another platinum agent, oxaliplatin, has also been shown to be transported in OCT2-expressing HEK293 cells [211]. However, oxaliplatin but not cisplatin failed to inhibit TEA uptake by OCT2. Interestingly though, oxaliplatin was responsible for significant toxicity both in OCT1 and OCT2 but not in OCT3 expressing cells [199].

7.2.3 OCT3 (*SLC22A3*)

OCT3 has been cloned from human, rat, and mouse [20, 97, 212]. OCT3 is expressed in a number of tissues including skeletal muscle, smooth muscle, liver, placenta, kidney, heart, intestine, spleen, lung, neurons of the brain and sympathetic ganglia, glial cells, and the choroid plexus [204, 213–216]. Model substrates for OCT3 include MPP and TEA and the neurotransmitters epinephrine, norepinephrine, and histamine (Table 2) [127, 212, 217]. Interestingly, tissue levels of MPP were reduced by 75 % in the heart in Oct3 knockout mice compared to wild-type mice [198]. Furthermore, after IV injection of MPP in pregnant females of an Oct3 heterozygote cross, accumulation of MPP in Oct3-/- fetuses was reduced by 65 % compared with wild-type fetuses [198]. These data indicate a likely significant role of OCT3 in the uptake of organic cations and substrate drugs into cardiomyocytes and in the transfer of organic cations and drugs across the placenta.

Oxaliplatin was previously determined to be a substrate for OCT3 as determined in vitro by OCT3-expressing HEK293, but the significance was unclear as it appeared to be a weak substrate [211]. In a follow-up study, OCT3 expression by quantitative mRNA analysis was ~tenfold higher in

colorectal cancer tissue samples versus normal colon tissue in Japanese patients [218]. In addition, OCT3 was more highly expressed in colon cancer cell lines compared to other organic cation transporters. The release of lactate dehydrogenase (LDH), a measure of cytotoxicity, and accumulation of oxaliplatin were increased in OCT3-expressing SW840 cells compared with empty vector-transfected cells [218]. Furthermore, high expressing OCT3 colon cancer cell lines released more LDH and accumulated more platinum after oxaliplatin treatment than low OCT3-expressing cell lines. Taken together, this data suggests OCT3 is an important determinant for the activity of oxaliplatin in colon cancer treatment.

7.2.4 OAT1 (*SLC22A6*)

The kidney and liver are the major route for organic anion elimination, and in the kidney, the translocation of organic anions occurs predominantly in proximal tubular cells [219, 220]. Similar to the liver, the hallmark of the renal organic anion transport system is its multispecific substrate recognition [219–221]. Historically, *p*-aminohippurate (PAH) has been used as a prototypical substrate for this system. The first PAH uptake transporter was cloned and designated OAT1 in 1997 [107, 109, 222]. OAT1 mRNA is expressed predominantly in the kidneys and weakly in the brain. OAT1 protein is localized at the basolateral membrane of renal proximal tubular cells [107]. OAT1 possesses broad substrate specificity including endogenous compounds, such as dicarboxylates, cyclic nucleotides, and prostaglandins, and xenobiotics such as the antibiotics penicillin and cephalosporin; the antivirals adefovir, cidofovir, and acyclovir; nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin and ibuprofen; and loop and thiazide diuretics (Table 2) [8, 19, 107, 194, 207, 223–230]. Studies in OAT1 knockout mice have confirmed the critical importance of this protein in the organic anion secretory pathway of the renal proximal tubule [231].

Methotrexate has been demonstrated to be a substrate in vitro in OAT1- or OAT2-expressing MDCK cells [232–234]. A well-known kidney-associated drug interaction relates to methotrexate. Methotrexate is eliminated primarily unchanged in urine [235]. Interactions between methotrexate and drugs such as NSAIDs, probenecid, and penicillin have been reported and have resulted in severe complications including bone marrow suppression and acute renal failure [236–238]. Like penicillin, we now know that the mechanism behind this interaction is likely due to inhibition of OAT-mediated methotrexate transport by these drugs [239].

7.2.5 OAT2 (*SLC22A7*)

OAT2 was isolated originally from rat liver as a novel transport protein with unknown function [240]. Because of its structural similarities to OAT1, OAT2 was functionally characterized and typical substrates of OAT2 are PAH, salicylate, PGE_2, dicarboxylates, and drugs such as allopurinol and bumetanide (Table 2) [8, 110, 194, 207, 241–244]. OAT2 is expressed predominantly in the liver at the basolateral membrane of hepatocytes [245] and weakly in the kidneys and appears to be involved in the hepatic disposition of some anionic drugs and endobiotics.

Like OAT1, OAT2 has demonstrated methotrexate transport in vitro [233, 234]. However, OAT2 has been shown to transport other chemotherapeutic agents as well. When expressed in *Xenopus laevis* oocytes, OAT2 mediated the transport of 5-fluorouracil and paclitaxel [111]. Uptake was sodium independent and saturable with K_m values 53.8 ± 7.9 nM and 142.8 ± 22.7 nM for 5-fluorouracil and paclitaxel, respectively. Therefore, OAT2 may be responsible, at least in part, for the hepatic uptake of these anticancer drugs in vivo.

7.2.6 OAT3 (*SLC22A8*)

OAT3 was initially isolated from rat [246] and, by mRNA analysis, found to be expressed in the kidneys, liver, brain, and eye. Human OAT3 is mainly expressed in the kidneys and to a lesser extent in the brain [247]. In the kidneys, OAT3, like OAT1, is localized to the basolateral membrane of proximal tubular cells [247], while in the brain, OAT3 is expressed on the brush border membrane of choroid plexus cells [248, 249] and in capillary endothelial cells [250]. In rats, OAT3 was also expressed in additional nephron segments including thick ascending limb of Henle's loop, distal convoluted tubule, and collecting ducts [251–253]. In proximal tubular cells, OAT3 is involved in organic anion secretion, but the physiologic roles of OAT3 in other nephron segments have not been determined. Like OAT1, OAT3 transports PAH, estrone sulfate, prostaglandins E_2 and $F_{2\alpha}$, and various drugs, including the benzylpenicillin, tetracycline, zidovudine, methotrexate, furosemide, NSAIDs, pravastatin, and cimetidine (Table 2) [194, 207, 223, 242, 243, 247, 254–256]. Although OAT3 and OAT1 possess some degree of overlapping substrate specificity, the ability to transport steroids and steroid conjugates such as corticosterone, estrone sulfate, estradiol-17ß-glucuronide, and taurocholate distinguishes OAT3 from OAT1 [8]. The physiologic function of OAT3 appears to be the renal secretion of steroid hormones, their conjugates, and prostaglandins. $OAT3^{-/-}$ mice are phenotypically normal and fertile [249]. Taurocholate, estrone sulfate, and BSP uptake into kidney slices from $OAT3^{-/-}$ mice was reduced, as was fluorescein uptake into cells of the choroid plexus. Thus, a normal development in mice is possible without OAT3 which may be due to the fact that OAT1 provides a backup system. Whether OAT1 or OAT3 transport chemotherapeutic agents other than methotrexate remains yet to be determined. It is also unclear whether OAT1 or OAT3 preferentially transports methotrexate in vivo.

7.2.7 OAT4 (*SLC22A9*)

OAT4 was cloned from the human kidney [257]. OAT4 mRNA is expressed in the kidneys and is localized at the apical membrane of proximal tubular cells [258]. Due to its apical localization, OAT4 is involved either in the absorption of organic anions from the ultrafiltrate or in the secretion of organic anions that were accumulated in the cell by OAT1 and OAT3 localized on the basolateral membrane of proximal tubular cells. In the placenta, OAT4 is expressed on the fetal side of the syncytiotrophoblast cells [259] where it may participate in the release of potentially toxic compounds from the fetus towards the mother and also deliver sulfated precursors from the mother for placental estrogen synthesis [259]. When expressed in *Xenopus laevis* oocytes, OAT4 mediates the transport of estrone sulfate, DHEAS, ochratoxin A, and PGE_2 (Table 2) [257, 260]. Xenobiotic substrates include drugs such as tetracycline, zidovudine, methotrexate, bumetanide, ketoprofen, and salicylate [194, 223, 239, 242, 243, 257, 260].

*7.3 CNT Substrates (*SLC28*)*

The human concentrative nucleoside transporter (CNT) protein family has three members, CNT1, CNT2 and CNT3, encoded by *SLC28A1*, *SLC28A2*, and *SLC28A3* genes, respectively. CNT1 and CNT2 transport pyrimidine and purine nucleosides, respectively, in a sodium-dependent manner, whereas CNT3 has a broader substrate selectivity and the ability to transport nucleosides by both sodium- and proton-coupled mechanisms [10]. Nucleosides are important metabolic precursors in nucleic acid synthesis, being recycled via salvage pathways, and are crucial in normal human physiology for the maintenance of cell and tissue growth [261]. CNTs are also involved in the uptake of most

nucleoside-derived antiviral and anticancer drugs. Nucleosides and nucleoside-derived drugs are hydrophilic molecules and diffuse slowly across cell membranes, thus, requiring active transport. Thus, membrane proteins such as CNTs are necessary to actively concentrate and translocate nucleosides from the extracellular space into the cytoplasm.

7.3.1 CNT1 (*SLC28A1*)

The first CNTs cloned were rat orthologs of CNT1 and CNT2 from rat jejunum and liver, respectively [140, 262]. Subsequently, human CNT1 was isolated from kidney by hybridization cloning and found to be 83 % identical to rat Cnt1 in amino acid sequence [25]. Ritzel et al. detected expression of CNT1 in the intestine, kidney, and liver by Northern blot and RT-PCR analyses [263].

CNT substrate specificity has been determined through a variety of experimental modalities including substrate flux measurements, cross-inhibition studies, and electrophysiology [79, 80, 261, 264]. Human CNTs exhibit high affinity for their substrates with apparent K_m values in the low micromolar range, ranging from 10 to 100 μM typically [10]. CNT1 primarily transports pyrimidine nucleosides (Table 3), and although adenosine binds CNT1 with high affinity, it is not transported [265]. Anticancer nucleoside-derived drugs have been shown to be substrates for CNT1. Gemcitabine, 2′-deoxy-5-fluorouridine (active metabolite of capecitabine), and 5-fluorouridine are good substrates for CNT1 in vitro, whereas 5′-deoxy-5-fluorouridine and the antiviral zidovudine (AZT) are transported with lower affinities [115, 266, 267]. Stavudine, zalcitabine, cladribine, and cytarabine are poorly transported by CNT1 [115, 266].

7.3.2 CNT2 (*SLC28A2*)

Two variants of human CNT2 were isolated simultaneously, with identical amino acid sequence except for a polymorphism at residue 75 (Arg substituted by Ser), and at the amino acid level, CNT2 was found to be 83 % and 72 % identical to rat Cnt2 and CNT1, respectively [26, 263]. Northern blot analysis demonstrated CNT2 expression in the heart, liver, skeletal muscle, kidney, pancreas, and intestine in a distribution that correlates well with the sites of purinergic effects [26]. Human CNT2 primarily transports purine nucleosides, although it also transports uridine (Table 3) [263]. CNT2 has also been shown to transport nucleoside-derived anticancer drugs. CNT2 transports 2′-deoxy- and 5′-deoxy-5-fluorouridine, cladribine, clofarabine, 5-fluorouridine, fludarabine, and didanosine in vitro [266].

7.3.3 CNT3 (*SLC28A3*)

The last CNT subtype cloned was the broadly selective nucleoside transporter CNT3. The human and mouse orthologs were cloned from human mammary gland, differentiated human myeloid HL-60 cells, and mouse liver and showed 79 % identical amino acid to each other [28]. However, CNT3 is only 48 % and 47 % homologous to CNT1 and CNT2, respectively, at the amino acid level, and, uniquely, CNT3 was demonstrated to couple substrate transport to protons [116]. CNT3 demonstrates broader substrate selectivity than either CNT1 or CNT2, transporting both purine and pyrimidine nucleosides (Table 3) [28, 116]. A tissue expression array detected highest expression of CNT3 in the mammary gland, pancreas, bone marrow, and trachea, with substantial expression in various regions of the intestine and modest expression in the liver, lung, placenta, prostate, testis, brain, and heart [28].

In addition to its extensive nucleoside substrate specificity, human CNT3 also exhibits a broader scope of specificity to various therapeutic nucleoside analogs than either CNT1 or CNT2. It efficiently transports both pyrimidine-derived nucleoside drugs, including gemcitabine, 5′-deoxy- and 2′-deoxy-5-fluorouridine, 5-fluorouridine, and zidovudine, and purine-derived nucleoside drugs, such as ribavirin, fludarabine, cladribine, and clofarabine and even nucleobase derivatives such as 6-mercaptopurine and 6-thioguanine [268, 269].

*7.4 ENT Substrates (*SLC29*)*

Most mammalian cells undergo low-affinity, equilibrative nucleoside transport processes, now known in humans to be mediated by members of the SLC29 protein family [11]. Prior to their gene identification, the equilibrative transporters were classified on the basis of their sensitivity to inhibition by nitrobenzylthioinosine (nitrobenzylmercaptopurine riboside, NBMPR), as either es (equilibrative sensitive) or ei (equilibrative insensitive) [270]. In 1996, purification of the archetypal es transporter from human erythrocytes allowed cloning of ENT1 from human placenta [119]. The ei-type transporter, ENT2, was subsequently cloned by virtue of its homology with ENT1 [120]. ENT3 and ENT4 have more recently been identified and characterized resulting from completion of the Human Genome Project [31, 32, 122–124, 271–273]. ENTs facilitate diffusion of nucleosides across membranes bidirectionally in accordance with substrate concentration gradients, although both ENT3 and ENT4 exhibit enhanced activity at acidic pH, suggesting a proton-coupled transport mechanism [122, 124]. In contrast to CNTs, ENTs appear to be widely distributed in the body and have broad substrate specificity.

7.4.1 ENT1 (*SLC29A1*)

Human ENT1 is 78 % and 79 % identical in amino acid sequence to its rat and mouse homologs, respectively [119, 274, 275]. Protein and mRNA expression studies revealed that ENT1 is almost ubiquitously distributed in human and rodent tissues, although its abundance varies between locations [119, 274]. For example, in the human brain, ENT1 is most abundant in the frontal and parietal lobes of the cortex [276]. While primarily expressed at the cell surface, there is also evidence for association of ENT1 with nuclear membranes and endoplasmic reticulum, suggesting it may function in the transport of nucleosides and nucleoside-derived drugs between the cytoplasm and the luminal compartments of these membrane types [277].

ENT1 transports a wide range of purine and pyrimidine nucleosides, with K_m values ranging from 0.05 mM for adenosine to 0.60 mM for cytidine, but is unable to transport the pyrimidine base uracil (Table 3) [119, 278]. ENT1 also has been demonstrated to transport anticancer nucleoside drugs generally well. Gemcitabine, cytarabine, cladribine, fludarabine, and 5′-deoxy-5-fluorouridine are substrates for ENT1 in vitro [279–284]. One rationale for the effectiveness of anticancer nucleoside drugs is that high proliferation rates observed in malignant cells are associated with high levels of es (or ENT1-mediated) transport activity [285]. ENT1 was shown to be more highly expressed in breast cancer cells compared to normal breast epithelia using immunohistochemistry [284]. In a retrospective study, patients with pancreatic adenocarcinoma who had high levels of detectable ENT1 protein had a significantly longer survival after gemcitabine monotherapy than patients with low levels or without detectable ENT1 [286]. Taken together, these data would support the hypothesis that ENT1-mediated nucleoside transport is a determinant of response to nucleoside drugs in some cancers.

ENT1 expression has been correlated with sensitivity to various nucleoside anticancer drugs [280, 281, 284, 285, 287]. ENT1 is considered the primary transporter mediating cytarabine uptake into

human cells and reduced ENT1 function may play a role in resistance to cytarabine [285, 288]. Acute myeloid leukemia (AML) patients whose blast cells showed the lowest rates of cytarabine uptake and ENT1 abundance as measured by the number of NBMPR binding sites demonstrated no response to cytarabine therapy [289]. ENT1 mRNA levels and in vitro cytarabine sensitivities were correlated in patients with resistant childhood AML [290]. Furthermore, AML patients with reduced levels of mRNA encoding 5′-nucleotidases or ENT1 in blast cells at diagnosis had significantly shorter disease-free and lower overall survival with cytarabine treatment [280, 281]. Taken together, these data suggests that ENT1 plays a major factor in cytarabine resistance in AML therapy.

7.4.2 ENT2 (*SLC29A2*)

ENT2 is 46 % identical in amino acid sequence to ENT1 and 88 % identical to its mouse and rat homologs [118, 120, 275, 291]. ENT2 mRNA has been documented in a wide tissue distribution including the brain, heart, placenta, thymus, pancreas, prostate, and kidney but is particularly abundant in skeletal muscle [118, 120]. Immunohistochemical analysis demonstrated cell surface localization of Ent2 in rat cardiomyocytes and basolateral membrane expression of ENT2 in human renal epithelial cells [292, 293]. ENT2 transports a wide range of purine and pyrimidine nucleosides, although with lower apparent affinity than ENT1 except in the case of inosine (Table 3) [118, 120, 278]. Interestingly, ENT2, in contrast to ENT1, is also capable of efficiently transporting a broad range of purine and pyrimidine nucleobases, except for cytosine [118, 121].

Regarding anticancer nucleoside-derived drugs, ENT2 transports gemcitabine, cladribine, fludarabine, and clofarabine in vitro [267, 279, 294]. ENT2 protein expression was significantly correlated with fludarabine uptake as well as in vitro sensitivity of CLL cells to fludarabine, suggesting ENT2-mediated uptake of fludarabine is important for drug sensitivity in CLL patients [295]. Consistent with the weak ability of ENT2 to interact with cytosine and cytidine, ENT2 has a lower apparent affinity for gemcitabine than ENT1 [267]. Of particular note, ENT2 transports 3′-deoxynucleoside analogs used in human immunodeficiency virus (HIV) therapy, 2′,3′-dideoxycytidine (ddC), zidovudine (AZT), and 2′,3′-dideoxyinosine (ddI) [148], and, therefore, represents a key route for cellular uptake of these clinically important drugs used in the therapy of HIV infection.

7.4.3 ENT3 (*SLC29A3*)

ENT3 is only 29 % identical in amino acid sequence to ENT1 but 74 % identical to its mouse homolog Ent3 [31, 122]. ENT3 differs from ENT1 and ENT2 in having a long, 51-residue hydrophilic N-terminal region preceding TM1 which contains a dileucine motif typical of endosomal/lysosomal targeting sequences [296]. ENT3 is predominantly expressed intracellularly and mutation of the dileucine motif to alanine redirects the transporter to the cell surface [122]. RNA dot blot analysis demonstrated wide ENT3 expression, with highest levels in the placenta, uterus, ovary, spleen, lymph node, and bone marrow and lowest levels in the brain and heart [122]. Compared to ENT1, ENT3 is much less sensitive to inhibition by NBMPR. ENT3 has broad specificity for nucleosides and nucleobases but, unlike ENT2, does not transport hypoxanthine (Table 3) [122].

ENT3 has also been demonstrated to transport nucleoside-derived drugs including adenosine analogs used in cancer chemotherapy as well as the antiviral purine and pyrimidine nucleoside drugs ddI, ddC and AZT [122]. Transport activity is strongly dependent upon pH, and the optimal pH of 5.5 likely reflects the location of ENT3 in acidic, intracellular compartments. Fludarabine and cladribine were both transported in vitro using a *Xenopus laevis* expression system [122].

7.4.4 ENT4 (*SLC29A4*)

ENT4 is 86 % identical in amino acid sequence to its mouse homolog [124] but only shares 18 % sequence identity with ENT1 [32]. ENT4 was initially identified as a polyspecific organic cation transporter and designated plasma membrane monoamine transporter (PMAT) [123, 271] but was subsequently found to be a nucleoside transporter mediating pH-dependent adenosine transport optimally at acidic pH and absent at pH 7.4 [124]. Multiple tissue expression RNA arrays suggest that ENT4 is ubiquitously expressed in human tissues but in the rat, the protein is particularly abundant in the heart and brain [124]. Immunohistochemical analysis of isolated cardiomyocytes and cultured endothelial cells revealed ENT4 expression at the cell surface. ENT4 mediates transport of adenosine and serotonin, optimally at pH 5.5 (Table 3) [124]. A role for ENT4 in mediating transport of anticancer nucleoside drugs has not clearly been delineated at this time.

8 Conclusions and Future Perspectives

Uptake transporters such as the SLCO, SLC22, SLC28, and SLC29 families are drug and endobiotic transporters whose importance to pharmacology and physiology is now becoming better established. Yet despite over two decades of study, much remains to be understood. It is of particular relevance to drug discovery, toxicology, and clinical pharmacology to better understand the structure–function–transport energetics relationships among substrates/inhibitors/coupled ions and these transporters for use in the development of drugs with improved tissue targeting and optimized pharmacokinetic and safety profiles. In no field may this be more applicable than medical oncology as the drugs used to treat various malignancies, despite their efficacy, are often associated with narrow therapeutic indices such that the benefits gained from eradicating tumor cells are often counteracted by the potential severe, and even life-threatening, adverse effects from the same agents. A better comprehension of the functional expression and significance of uptake transporters throughout human tissues would provide tools for predicting drug response and clearance. Because many of these transporters are expressed in the tissues of importance to endobiotic and xenobiotic disposition such as the liver, intestine, kidney, and blood–brain barrier, issues of functional redundancy and overlapping substrate specificity make it difficult at times to determine which transporter is the main player for a given substrate. Variations in genes encoding uptake transporters can cause interindividual variations in drug effects or certain pathophysiologic states. Studies regarding the detailed analysis of the consequences of genetic variation, not only limited to single polymorphisms but also to frequent haplotypes of allelic variants and gene–gene interactions, may be of importance to further individualize and optimize treatment regimens in this era of personalized medicine. Understandably, we need to direct our attention to deciphering the physiology and pathophysiology of uptake transporters, especially for those transporters that are least characterized. All these questions will ultimately be answered through the combination of studies at the genetic/molecular level, with animal models including those involving genetic manipulation, and clinical studies aimed at understanding variability in drug response and toxicity.

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High-Throughput Platforms in Drug Metabolism and Transport Pharmacogenetics

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Abstract Although there are now several thousand published studies that have examined the genetic contribution to interindividual variation in drug treatment (i.e., pharmacogenetics), very few have examined the large portions of the genome; rather these have focused on candidate gene and pathway-based study designs. However, multiple large-scale genotyping technologies have recently emerged that allow the researcher to examine pharmacogenetic endpoints ~100–500,000 SNPs at a time. Each genotyping platform is slightly different and applicable to either the clinical setting, wherein the genetic information informs treatment in patients with certain variants, or the research setting, where patients that are treated with certain drugs are either prospectively or retrospectively evaluated for genetic variants that may influence treatment outcomes. The purpose of this chapter is to describe the current study designs in pharmacogenetics, the major findings of these studies that are applied clinically, to provide an overview of commercially available large-scale genotyping technologies, and to discuss how these technologies can be applied in both clinical and research settings. While oncology agents will be the primary focus of this chapter, given that individuals undergoing therapy for cancer are often treated with multiple drugs, it is important to also consider other agents.

Keywords Genotyping • Pharmacogenetics • Platforms • Genes • ADME

1 Introduction

Clinical oncology has witnessed a significant increase in the number of drugs approved for the treatment of malignancies. Since 1990, over 100 drugs have entered the market [1], and there are often multiple options for the prescriber that provide similar efficacy to the general population. Oncology agents are frequently associated with a great degree of interindividual variation in response; there are myriad examples where a drug is not active or causes life-threatening toxicity that interrupts treatment in a significant number of patients. Pharmacogenetics is the use of the genome to identify patients who

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will likely benefit or not benefit from treatment with a particular drug. In some cases, pharmacogenetics has provided a useful means to enhance and prolong lives of patients receiving oncology agents.

Pharmacogenetic studies focus on two types of major endpoints (1) studies aimed at determining genetic relationships between absorption, distribution, metabolism, elimination (ADME), and activation of drugs and (2) those considering receptors, target proteins, resistance, toxicity, and dosing requirements. Historically, large-scale (i.e., those examining >1,000 SNPs) or genome-wide association studies (GWAS; i.e., those examining >100,000 SNPs interspersed across the genome) investigating pharmacogenetics have been hampered because most ADME data comes from clinical trials with small, heterogeneous, and otherwise nonoptimal populations. Indeed only a few large-scale studies have examined activation of drugs (e.g., *CYP2D6* vs. TAM [2], *CYP2C19* vs. clopidogrel [3]), while none have evaluated ADME properties of drugs. Still, some examples of large-scale or GWAS studies examining the latter endpoints have emerged rather recently, with the earliest studies published in 2007 and 2008 [4]. The investigators of these studies collected clinical information from a large number of individuals and focused on a few specific, well-conceived endpoints that are easily and readily measurable.

The human genome provides a new tool for identifying patients who are at risk of inefficacy or toxicity through the use of either somatic or constitutional genetic variations that provide predictive power to the prescriber. Table 1 provides a summary of clinically relevant variants affecting treatment with oncology agents and other commonly prescribed drugs. While much progress has been made in the subject of pharmacogenomics, there are still few studies that have truly explored the genome to maximize the potential for identifying markers that are most strongly related to outcome. Rather, investigators have typically relied upon hypothesis-driven studies evaluating a small number of SNPs in genes that are known to interact with ADME, targets, or clinical outcomes (i.e., candidate gene approaches). Still others have focused on drug pathways to test the interaction of multiple SNPs on the aforementioned endpoints (i.e., pathway-based approaches).

2 Candidate Gene, Pathway-Based, and Genome-Wide Pharmacogenetic Study Design

The candidate gene approach has traditionally been the most widely used design, and studies using this approach have made considerable contributions to the field of anticancer pharmacogenetics. For example, in 2000, a polymorphism in the promoter of the phase II drug-metabolizing enzyme *UGT1A1* was first correlated with toxicity from irinotecan in Japanese patients with cancer using the candidate gene approach [5], and further studies confirmed these results [6–8]. Other examples in anticancer therapy include polymorphisms in *CYP2D6* and tamoxifen pharmacokinetics [9] and clinical endpoints [10, 11], thymidylate synthase gene [12, 13] and *DPD* [14, 15] and fluorouracil toxicity, and *SLCO1B1* variants and irinotecan pharmacokinetics [16, 17] and toxicity [16]. One of the fundamental benefits of this approach is its hypothesis-driven nature. Also, pharmacogenetic candidate gene studies can often be performed with large enough sample sizes to obtain the necessary statistical power. Despite these advantages, this method does have some weaknesses. To maximize efficiency, identifying candidate genes requires a priori knowledge of the nature of the gene–drug interaction. Moreover, selecting polymorphisms to investigate requires knowledge of the effect of the polymorphism on gene expression or function; albeit there are numerous examples of studies that have genotyped without true foreknowledge of a SNPs effect on the expressed protein. Generally, polymorphisms with major effects on drug response are uncommon, while those with minor effects are common [18]. The frequency of polymorphisms with low penetrance may partially explain why the candidate gene approach has failed to replicate correlations of drug response and certain genetic markers [19].

Table 1 Clinically relevant pharmacogenetic relationships

Drug	Gene or variant	Significance
Germline mutations		
Abacavir	*HLA-B*5701*	Strong predictor of IADRs
Allopurinol	*HLA-B*5801*	Strong predictor of IADRs in Chinese (~100 %) although less predictive in those with European ancestry (~20–60 %)
Azathioprine, 6-mercaptopurine	*TPMT*	Very strong predictor of hemotoxicity
Carbamazepine	*HLA-B*1502*	SNP highly associated with carbamazepine-induced Stevens–Johnson syndrome and toxic epidermal necrosis reaction; FDA has recommended testing for all Asian populations
Clopidogrel	*CYP2C19*	Strong predictor of clopidogrel nonresponsiveness, especially with stent rethrombosis
Codeine	*CYP2D6*	Ultrarapid metabolizers are at risk for potentially fatal overdoses, while those carrying poor metabolizing alleles experience less pain relief
Fluoropyrimidines (5-FU, capecitabine, tegafur)	*DPYD*	Results in DPD deficiency with higher risk of toxicity in heterozygotes and variant homozygotes receiving chemotherapy. Can be lethal
Interferon alpha	*IL28B*	In patients receiving IFN alpha for HCV, rs12979860 is associated with sustained virological response and rs8099917 is associated with treatment failure
Irinotecan	*UGT1A1*28*	Homozygotes at increased risk for neutropenia. Variant is indicator for toxicity and response
Isoniazid	*NAT2*	Slow acetylator status associated with a threefold increase in drug-induced liver injury (DILI)
	CYP2E1	Higher CYP2E1 activity (homozygous wild type) may increase the risk of hepatotoxicity
Phenytoin	*Cyp2C9*2, *3*	Variants affect the toxicity and efficacy of phenytoin
Statins	*SCLO1B1*	Associated with statin-induced myopathy
Tamoxifen	*CYP2D6*	Poor metabolizers may experience significantly reduced efficacy, have lower serum endoxifen levels, and in some studies have had higher rates of recurrence. CYP2D6 inhibition (with SSRIs, such as paroketine etc.) further reduces efficacy
Warfarin	*VKORC1*	Over-anticoagulation leading to bleeding. Requires dose reduction
	CYP2C9	Over-anticoagulation leading to bleeding. *2 and *3 alleles require dose reduction
Somatic mutations		
BCR-ABL inhibitors (nilotinib, dasatinib, imatinib)	*BCR-ABL* (*Philadelphia chromosome*)	Drugs have proven efficacy against Philadelphia chromosome + patients only
EGF and EGFR inhibitors cetuximab, panitumumab, erlotinib, gefitinib	*EGFR*	Expression of EGFR receptor target necessary for response. Mutation associated with response to TKI
	KRAS	Activating KRAS mutations are associated with resistance to agents that target EGFR
	PIK3CA	Pathway activated by EGFR receptor which is parallel to KRAS. May be related to cetuximab and panitumumab resistance
BRAF inhibitors (i.e., sorafenib); EGFR inhibitors	*BRAF*	Predictive of response to BRAF inhibitors. Associated with poor response to EGFR inhibitors. BRAF and KRAS mutations are mutually exclusive, so BRAF is only significant in patients with with KRAS. Sorafenib is shown to restore EGFR sensitivity
Alkylating agents inc. temozolomide, BCNU, procarbazine	MGMT expression/ promoter methylation	MGMT reverses DNA methylation. MGMT detection may predict temozolomide resistance in glioblastoma multiforme (GBM); MGMT promoter methylation (silencing) predicts response
RET inhibitor (i.e., vandetanib)	*RET*	Predictor of response to RET inhibitors. Vandetanib was shown to inhibit M918T RET phosphorylation, but was not able to inhibit activation caused by codon 804 RET mutations

IADR immunoallergic adverse drug reaction, *MGMT* DNA repair protein O6-methylguanine-DNA-methyltransferase

These factors have contributed to a multitude of studies with conflicting results and limited clinical application, as is the case with *CYP3A4* [20, 21]. Overall, the candidate approach is best suited for instances where the polymorphisms studied have significant effects on genes known to be involved in the response to the particular drug.

The pathway-based method has also been proven useful in determining anticancer pharmacogenetics. In this approach, variants in several genes known to play a role in a particular drug's pathway are genotyped and then considered together to better understand the genetic basis of drug response. For example, Innocenti et al. demonstrated that polymorphisms in the transporter genes *ABCC1* and *ABCB1* and the drug-metabolizing enzyme *UGT1A1* together help explain the interpatient variation in SN-38, the active metabolite of irinotecan [22]. Similar pathway-based pharmacogenetic studies have investigated docetaxel [23], sunitinib [24], platinum-based agents [25], and doxorubicin and cyclophosphamide [26]. Like the candidate gene approach, the pathway-based approach is still hypothesis driven. Another benefit of this method is that it allows for testing gene–gene interactions, unlike the candidate gene approach [19]. However, testing for interactions often involves more complicated analyses, usually necessitating machine learning techniques and larger sample sizes, and results often contain false positives and false negatives due to low statistical power and multiple testing. Also, the current knowledge base may not be sufficient to select relevant genes or polymorphisms to investigate or to validate any gene–gene interactions identified in the analysis as the mechanism behind gene–gene interactions has yet to be discovered [27].

The genome-wide approach is still new in the field of pharmacogenetics, especially in oncology. To date, there have been only two anticancer pharmacogenetic studies that interrogated 100,000+ SNPs. In the first study, Hartford et al. investigated etoposide-induced secondary leukemia using the Affymetrix (Fremont, CA) GeneChip Human Mapping 100K Set. The researchers genotyped both the germline DNA and secondary leukemic cell DNA from 13 secondary leukemia patients as well as the germline DNA of 169 controls, including 13 matched controls. All genotyped individuals were children with acute lymphoblastic leukemia who participated in one of two treatment protocols that contained etoposide [28]. Though 116,204 polymorphisms were initially investigated, only 64,373 were used because of exclusion due to invariance or low call rates. No single SNP or gene was involved in the majority of cases, though many of the polymorphisms that showed differential distribution patterns between cases and controls were involved in three different pathways: adhesion, Wnt signaling, and regulation of actin [28]. This study did not contain a separate patient validation cohort, though the researchers used available genomic information on 15 HapMap lymphoblastoid cell lines to identify SNPs that correlated with in vitro etoposide-induced gene fusion events [28]. It must be noted that pharmacogenetic studies with cell lines can be confounded by artifactual changes in expression levels of proteins involved in drug pathways [29]. In the second study, Sarasquete et al. explored the genetics behind bisphosphonate-related osteonecrosis of the jaw (ONJ) [30]. Using the Affymetrix GeneChip Mapping 500K Set, the researchers compared the genotypes of 22 patients receiving bisphosphonates for multiple myeloma to the genotypes of 65 matched controls. Though 500,568 SNPs were initially interrogated, 339,972 were used in the final analysis. One intronic SNP (rs1934951) in *CYP2C8* was significantly associated with the development of ONJ, and three other SNPs in *CYP2C8* trended toward an association but were not statistically significant [30]. However, this study contained no validation cohort [30], and the same SNP was not associated with ONJ in men receiving bisphosphonates for bone metastases from prostate cancer [31].

Unlike the candidate gene and pathway-based approaches, the genome-wide method is discovery driven and hypothesis generating and thus may be useful when the etiology of a certain condition is unknown [19], such as bisphosphonate-related ONJ [32]. Like the pathway-based approach, this method also allows for testing gene–gene interactions. However, this method has serious limitations that make it difficult to apply in pharmacogenetic studies. The primary limitation is the large sample size needed to test the number of hypotheses in a genome-wide association study (GWAS) [19]. Many GWASs have been performed to investigate the genetics behind certain diseases, and these studies

now usually genotype thousands of cases and controls. These numbers are often difficult to obtain for most pharmacogenetic studies, with the earliest pharmacogenetic GWASs having genotyped fewer than 400 patients [4]. The difficulty in obtaining enough patients for sufficient power is often augmented by the rarity of healthy controls for many agents, especially in oncology. In addition, the large number of SNPs investigated may result in weak statistical signals and false positives (Type I error). To reduce the impact of Type 1 error, many GWASs employ a two-stage design where a second replication cohort is used to validate signals generated from the first cohort [19], but as stated previously, the numbers needed for this design often exceed those available for pharmacogenetic studies. Most pharmacogenetic GWASs have examined adverse drug reactions (ADRs), such as the previously described studies by Hartford et al. [28] and Sarasquete et al. [30]. This is because ADRs are generally easy to measure, resulting in easily defined cases and controls, and it is often assumed that genetic risk factors will have high penetrance [33]. Still, the risk for some ADRs may be polygenic, have low penetrance, or rely on rare variants that are difficult to detect, complicating the results of a GWAS.

3 Genotyping Platforms

Several companies have produced platforms specifically designed for genotyping genes involved in drug ADME. Some platforms, such as the DrugMEt® pharmacogenetic test developed by Jurilab Ltd (Kuopio, Finland) [34] and the CodeLink P450 platform, originally by Amersham Biosciences and then sold by GE Healthcare (Amersham, UK) [35], have been discontinued, while others have already made an impact in the field of pharmacogenetics. A brief overview of selected genotyping platforms ranging from candidate gene to genome-wide design is therefore provided (for a more comprehensive list of selected platforms, see Table 2).

3.1 AmpliChip

The first platform to obtain FDA approval in 2004 was the AmpliChip® P450 tool, which was developed by Roche Diagnostics Corp. and uses the Affymetrix GeneChip Microarray Instrumentation system [36]. The current version of the platform can identify 27 common alleles in *CYP2D6*, including copy number variations, and 3 common alleles of *CYP2C19* [37]. The platform works by first using Roche's patented PCR technology in two separate reactions to amplify the promoter region and the coding region of *CYP2D6*, a *CYP2D6* duplication-specific product, a *CYP2D6* deletion-specific product, and exon 4 to exon 5 of *CYP2C19*. The amplified products are then pooled, fragmented into 50–200 nucleotide fragments, and labeled on the 3′ end. The labeled fragments are hybridized to the over 15,000 different oligonucleotide probes on the glass surface of the platform. Finally, the platform is scanned by the Affymetrix GeneChip Scanner, which detects the fluorescently labeled DNA fragments hybridized to the probe, and specialized software determines the alleles present [37].

The AmpliChip P450 has already been used in both clinical and research settings, investigating the pharmacogenetics of morphine [38], tamoxifen [39, 40], and a variety of psychiatric drugs [41, 42]. Because of its ease of use and the known impact of *CYP2D6* and *CYP2C19* variants on several drug pathways, the Roche AmpliChip P450 may prove to be a valuable diagnostic tool in the clinic. For example, a 2-year-old boy died after receiving postoperative codeine, a prodrug converted to morphine by *CYP2D6*. It was determined postmortem that the boy had a functional duplication of *CYP2D6* and was thus an ultrarapid metabolizer. Despite receiving the recommended dosage, the boy had a toxic level of morphine in his bloodstream [43]. Perhaps genotyping platforms such as the Roche AmpliChip could be used in the clinic prior to the administration of certain drugs, ultimately reducing

Table 2 Selected genotyping platforms ranging from candidate gene to genome-wide design

Assay	Manufacturer	Genes	Polymorphisms
AmpliChip P450	Roche Diagnostics	2	23
Infiniti Warfarin Assay	AutoGenomics	2	3
Verigene Warfarin Metabolism Nucleic Acid Test	Nanosphere	2	3
eSensor XT-8 System for Warfarin Sensitivity	Osmetech	2	3
PHARMAchip	Progenika Biopharma SA	35	85
CodeLink P450[a]	GE Healthcare	9	110
DMET	Affymetrix, Inc.	225	1,936
48.48 Dynamic Array	Fluidigm	Custom; up to 48	2,304
96.96 Dynamic Array	Fluidigm	Custom; up to 96	9,216
iPlex Gold on MassARRAY platform	Sequenom	Custom	Up to 15,360 per plate
GenomeLab SNPstream	Beckman Coulter	Custom	Up to 18,432 per plate
Illumina ASPE on VeraCode	Illumina	Custom	Up to 72 per plate
Illumina GoldenGate on VeraCode	Illumina	Custom	Up to 384 per plate
Illumina GoldenGate on BeadArray	Illumina	Custom	Up to 1,536 per plate
iSelect HD BeadChip	Illumina	Custom	Up to 200,000 per plate
HumanHap550-Quad + BeadChip	Illumina	Semi-custom	~625,000
Human1M-Duo + BeadChip	Illumina	Semi-custom	~1,200,000
GeneChip Universal Tag Array (3K, 5K, 10K, 25K, 75K)	Affymetrix	Standard or custom	3,000, 5,000, 10,000, 25,000 or 75,000 (standardized only)
Genome-Wide Human SNP Array 6.0	Affymetrix		1,800,000
GeneChip Human Mapping Array (500K, 100K, or 10K)	Affymetrix		500,000, 100,000, or 10,000

[a]These platforms have been discontinued

the tragedies such as the one described here. Moreover, the AmpliChip is well suited for exploratory studies because it provides rather in-depth coverage of *CYP2D6* and *CYP2C19*. For example, it was also used (in part) to genotype individuals treated with tamoxifen leading to significant advances in the understanding of the genetic basis behind tamoxifen inefficacy and toxicity in certain patients [9].

3.2 PHARMAchip

A large-scale platform that was recently developed is the PHARMAchip™ by Progenika Biopharma SA (Vizcaya, Spain). Designed specifically for scanning genes involved in ADME, the PHARMAchip is described as a tool to predict a patient's response to drug therapy [44]. The current version of the platform genotypes 85 allelic variants, including SNPs, indels, and whole-gene deletions and insertions, in 37 genes, including metabolizing enzymes, transporters, targets, and receptors (see reference [34] for a full list of genes). Similar to the AmpliChip platform, the PHARMAchip relies on multiplex PCR to amplify the target sequences and allele-specific hybridization to the platform for allele detection, and the final genotype is determined by Progenika's proprietary software [34]. Because it screens many more genes than the AmpliChip but is limited to genes involved in drug pathways, this platform can be thought of as a pathway analysis with low- to midrange polymorphism coverage. While promising, this new technology has not been extensively tested. The only peer-reviewed literature available is a review of the accuracy of the genotypes ascertained by the platform [34]. To our knowledge, this tool has not been used in a research or clinical setting. While the accuracy of the platform is high, this new tool needs to be further validated before its utility can be accurately assessed.

3.3 DMET

Another tool for interrogating genes involved in drug ADME is the Affymetrix drug-metabolizing enzymes and transporters (DMET) platform that genotypes 1,936 polymorphisms in 225 genes and determines copy number for *CYP2A6*, *CYP2D6*, *GSTM1*, *GSTT1*, and *UGT2B17* (see reference [45] for a full list of genes and a selected list of genes and drug pathways). This platform relies on the molecular inversion probe (MIP) technology to detect variants. For each SNP, a MIP contains two genomic homology regions that flank the nucleotide of interest; one region is located at the 5′ end of the probe and the other at the 3′ end. After these complementary regions bind their target, the space is gap-filled to form a circular probe specific to the SNP. The probe is then cleaved and amplified by using specific primer sites also included in the probe. The linear probes are then labeled, cleaved, and hybridized to the platform. Similar technology is used for detecting indels and triallelic SNPs. For a more detailed description of MIP technology, please see reference [46]. In 2010, the DMET platform received CLIA certification and therefore can be used for FDA-approved genotyping.

One of the unique features of the DMET platform is its balance of breadth and selectivity in the variants it can genotype. By scanning almost 2,000 variants, the DMET platform has greater applicability to exploratory studies than other genotyping tools that were previously discussed. However, the DMET platform remains specific to known drug pathway genes, namely, drug-metabolizing enzymes and transporters, some drug targets (e.g., *VKORC1*), and genes that interact with drug metabolism and transport pathways; thus, it can be considered a pathway analysis genotyping platform with midrange polymorphism coverage.

DMET has already been used to investigate the pharmacogenetics of different agents. The first use of DMET in the literature examined the influence of genetic variants in warfarin dosing, a study that resulted in the identification of a SNP in *CYP4F2* as a determinant of dose requirements [47]. This finding was first shown in a cohort of 436 patients and then replicated in three separate cohorts ($n=61$, $n=269$, and $n=295$), two of which were from different institutions [47]. The clinical relevance of *CYP4F2* has been supported by many studies [48–52], though some studies have not replicated the association [53–57]. It is important to note that these studies have investigated different ethnic populations, so the impact of *CYP4F2* may differ between ethnic groups.

In the second application of DMET, Mega et al. examined the genetics of pharmacokinetic and pharmacodynamic endpoints of clopidogrel in 162 healthy subjects, as well as the genetics behind cardiovascular outcome in 1,477 patients with a history of myocardial infarction after receiving clopidogrel [58]. The investigators investigated 54 polymorphisms in six cytochrome P450 genes (*CYP2C19*, *CYP2C9*, *CYP2B6*, *CYP3A5*, *CYP3A4*, and *CYP1A2*). Mega et al. showed that reduced-function alleles of *CYP2C19* and *CYP2B6* independently correlated with reduced plasma exposure to the active metabolite of clopidogrel and with reduced platelet aggregation in the healthy patient cohort. Reduced-function alleles of *CYP2C19* also correlated with increased risk of death from cardiovascular causes, nonfatal myocardial infarction, nonfatal stroke, and stent thrombosis in the second patient cohort. The relationship between CYP2C19 and clopidogrel has been well validated, and the FDA now includes it on the prescribing information [59].

In the third application of DMET, we investigated the pharmacogenetics of docetaxel-based therapy alone ($n=14$) or in combination with thalidomide ($n=33$) in men with prostate cancer [60]. Ten SNPs in three genes (*PPAR-delta*, *SULT1C2*, and *CHST3*) were associated with response to treatment, and eleven SNPS in eight genes (*SPG7*, *CHST3*, *CYP2D6*, *NAT2*, *ABCC6*, *ATP7A*, *CYP4B1*, and *SLC10A2*) were associated with toxicity [60]. We acknowledge that our small sample size may have led to some false-positive results, but we believe that this exploratory study has produced several candidate genes for further study.

To our knowledge, the DMET array is the largest genotyping platform specifically designed to screen genes known to impact drug metabolism, though several other companies make custom arrays

that are suitable for high-throughput screening in pharmacogenetic studies. While many companies provide customizable genotyping platforms, we will highlight only two such companies: Affymetrix, Inc. and Illumina, Inc. (San Diego, CA), which are the two companies most commonly used in large-scale pharmacogenetic studies so far [4]. In addition to standardized platforms that can genotype 3,000 to 1.8 million markers, Affymetrix offers Custom SNP Kits where the individual investigator may identify between 3,000 and 25,000 SNPs to be genotyped on a single array. These custom arrays use the molecular inversion probe (MIP) technology previously described. Affymetrix claims a minimum of 80 % of selected SNPs will be incorporated into the custom platform, with most platforms containing at least 90 % of requested markers, and a typical delivery time of 8–10 weeks. Using a 20K array, this technology allows for the generation of one million genotypes per day [61], though it is important to note that these platforms cannot be used to scan triallelic SNPs or indels [62]. Despite these limitations, the Affymetrix custom arrays could be used specifically for high-throughput screening of biallelic SNPs in drug-metabolizing enzymes and transporters.

3.4 Illumina Platforms

Illumina also has the capabilities to make custom genotyping platforms that interrogate a range of polymorphisms, anywhere from 1 to 200,000 SNPs. For low-multiplex genotyping assays to interrogate up to 72 loci, Illumina employs allele-specific primer extension (ASPE) to detect indels and SNPs. This technology utilizes allele-specific primers differing at the 3′ end and different capture sequences at the 5′ end, to differentiate the possible alleles. The genomic region containing the SNP is first amplified with PCR, and the allele-specific primers, one for each polymorphism, are then added, and the perfectly complementary primer will preferentially extend. Finally, the labeled targets are then hybridized to VeraCode beads using the primer-specific capture sequences and scanned by the BeadXpress Reader [63].

Illumina employs their GoldenGate technology for low- to midrange genotyping assays of up to 1,536 SNPs. GoldenGate assays use two allele-specific oligonucleotides and one locus-specific oligonucleotide to genotype a single SNP. The allele-specific oligonucleotides differ in their 5′ universal primer sites and their 3′ ends, which recognize the different alleles. The locus-specific primer is composed of a region of genomic complementarity at the 5′ end, followed by an address sequence and a third universal primer region at the 3′ end. After these oligonucleotides are added to genomic DNA, the allele-specific oligonucleotides that match the present allele(s) extend. This product is then ligated to bound locus-specific oligonucleotide. These target sequences are then amplified and fluorescently labeled via universal PCR. Finally, these products are hybridized to the platform using the address sequence to either the iScan or BeadXpress platform [64].

The largest customizable assays offered by Illumina use their Infinium BeadChips. With this technology, an individual investigator can create a completely custom panel that interrogates between 3,000 and 200,000 SNPs or can add up to 60,800 custom SNPs to two standardized products, the Human1M-Duo+BeadChip platform, which scans approximately 1.2 million markers, and the HumanHap550-Quad+BeadChip platform, which scans approximately 625,000 markers [63]. Like Affymetrix, Illumina guarantees an 80 % conversion rate of requested SNPs [65]. To ascertain genotype, the Infinium technology relies on single-base extension of the each probe. First, whole-genome amplification is performed, resulting in a minimum 1,000-fold increase in DNA, and the amplified DNA is then fragmented to approximately 300–500 bases. The fragmented DNA is then exposed to the BeadChip platform, which contains both Infinium I and Infinium II beads that are attached to the 5′ end of 80-mer probes, the last 50 bases of which are used to query the polymorphism. Finally, the probes are enzymatically extended by a single base labeled with either DNP or biotin, and the platform is scanned following immunohistochemical staining. The most common bead type is the Infinium

II, which contains probes that end one base before the SNP in question. Infinium II beads can thus be used to genotype by simple single-base extension, and only one bead type is needed per locus. However, this type of probe cannot be used to interrogate A/T or C/G SNPs. For these rare SNPs, the Infinium I beads, which utilize allele-specific primer extension, must be used. These probes are designed such that the final base on the 3′ end matches the loci in question, and therefore two bead types are needed to interrogate one locus [66]. While this technology can be used to genotype all biallelic SNPs, it cannot be used to interrogate triallelic SNPs.

The Illumina GoldenGate technology has been used specifically to study drug ADME. Lubomirov et al. recently used a customized 1536-SNP platform to investigate genetic contributors to the PK of the anti-HIV drugs lopinavir and ritonavir coformulated together [67]. The platform interrogated both proven and putative functional SNPs in 115 genes known to affect the ADME of antiretroviral drugs in addition to tag SNPs. The investigators also employed a two-stage discovery-validation study design with cohorts of 638 and 117 patients, respectively. The study of the validation cohort resulted in their conclusion that three functional SNPs in drug transporters and one tag SNP in a metabolizing enzyme all affect lopinavir/ritonavir PK. The authors state that similar studies could be used to determine the pharmacogenetics of other drugs [67]. They also suggest that two-stage studies using high-throughput technology to screen polymorphisms in genes affecting drug ADME should be conducted in a variety of drugs, especially those with narrow therapeutic indices and wide interpatient variability, such as anticancer agents.

3.5 Microfluidics

Another technology available for pharmacogenetic studies is microfluidics. One company using microfluidics to miniaturize and automate PCR is RainDance Technologies (Lexington, MA). The RainDance Technologies' RainStorm process utilizes small droplets (1 pL to 10 nL), each of which is a self-contained unit such that, in the case of PCR, each droplet is a separate reaction. Each droplet can then be manipulated on a microfluidic platform that requires no moving parts [68, 69], and the recently released RDT 1000 machine is capable of producing ten million droplets per hour [70]. While this technique has been used in the laboratory for a variety of applications, including a cell-based drug screen [69] and sequencing [71], it has not been used automated for copy number or SNP analysis [72], though RainDance has plans to expand the technology [68]. Fluidigm (San Francisco, CA) also uses microfluidics for many applications, including sequencing and SNP analysis. For SNP genotyping, Fluidigm employs standard TaqMan technology on their dynamic arrays [73]. The largest dynamic array allows 96 samples to be genotyped with 96 primer–probe sets, resulting in 9,216 genotyping reactions per run. Because these reactions occur in such small volumes, the process requires only 5.1 mL of master mix [74]. The major advantages of microfluidics are the speed, precision, and reduced reagent and sample needs. Additionally, whole genome-amplified samples can be sequenced more easily because of the reduction of allele bias [72].

4 Considerations for Drug Metabolism and Transport Pharmacogenetics Using Platforms

Genotyping may be performed for clinical endpoints (such as reducing ADRs or predicting efficacy), exploratory research, or a combination of the two. Ultimately, the primary goal of genotyping will dictate which type of platform is most appropriate. Small-scale platforms, such as the Roche AmpliChip, appear to be better suited for clinical applications and should be used when the gene–drug

relationship is well established (see Table 1). However, small-scale platforms may also be applied in the research setting and are best suited for the hypothesis-driven candidate gene approach or studies that require in-depth knowledge of a patient's genotype. Large-scale, genome-wide platforms, such as those made by Illumina and Affymetrix, are not designed for clinical use, but rather for exploratory scientific studies. Low- to midrange genotyping systems, such as the DMET platform or PHARMAchip, could potentially be used for a combination of scientific studies and clinical endpoints. The platforms are both hypothesis driven and exploratory and can be used well in research. Another benefit of these platforms is that they can be used for the direct comparison across multiple patient populations. However, neither platform is designed for genome mapping, limiting their exploratory utility.

Ultimately the choice of the proper platform will depend on the endpoints set forth by the clinician or investigator and the size of the population (i.e., the study design). It is impossible to directly compare different study designs because so many factors influence the statistical power of a design, including the number of outcomes, the number of SNPs, the allele frequencies, the penetrance of each SNP, the power of the platform to detect the number of SNPs, the population size, and the Type I error level. Nonetheless, there is a significant power advantage to using platforms that interrogate fewer polymorphisms, though Type I error in GWAS can be reduced by careful study design, such as by including a separate validation patient cohort. However, the trade-off in selecting fewer polymorphisms is that some significant variants may be omitted from the study.

Pharmacogenetic testing performed on a larger scale (e.g., DMET) also has significant clinical value. Genotyping hundreds of SNPs in one patient could identify variants affecting the response to many agents, oncologic and otherwise. As with any disease, cancer patients may have comorbidities requiring multiple medicines in addition to their cancer treatment that often result in interactions that cause inefficacy or adverse drug reactions. It is significant that Table 1 includes some of the most commonly prescribed drugs, including statins, clopidogrel, warfarin, and codeine. A platform such as DMET could predict a patient's toxicity and response to many drugs a physician may need to prescribe in the setting of oncology.

Finally, it should be mentioned that genotyping platforms are also used to genotype somatic mutations in tumors, although to our knowledge there are currently no well-developed genotyping platforms that are commercially available in this setting. For example, the ARUP laboratories offer a *KRAS* test (11 mutation sites) that reflexes to *BRAF* analysis if the tumor is found to have a wild-type *KRAS* mutation. This influences treatment with EGFR and BRAF inhibitors (see Table 1); thus an array that could test for mutations in *EGFR*, *KRAS*, *BRAF*, and *PIK3CA* in colorectal cancer tumors could be clinically useful. As further research and development is conducted on somatic mutations, it is expected that arrays detecting somatic mutations will be developed.

5 Conclusion

It is an exciting time in the field of pharmacogenetics. A recent literature search of the keyword "pharmacogenetics" resulted in over 10,000 citations. The growing availability of different genotyping platforms has already aided the field and has the potential for further benefit. The promise of experimental and exploratory genotyping platforms can only be fulfilled when the appropriate technology is selected and necessary statistical power is achieved. Success of genotyping platforms in the clinical setting depends on the polymorphism–phenotype relationship and cost effectiveness of the platform. When matched in the appropriate setting, platforms designed specifically for interrogating polymorphisms in drug-metabolizing enzymes and transporters are poised to make great contributions in personalized medicine.

A challenge to pharmacogenetics is that knowledge of a patient's genotype in key drug-metabolizing enzymes and transporters is not necessarily sufficient for optimizing treatment. As discussed above,

the tumor genotype can also influence a patient's response to a treatment, such as polymorphisms in genes encoding drug targets (e.g., *EGFR* mutations and gefitinib [75–78]). Even when the genotype is known, genetic phenomena such as penetrance, gene–gene interactions, and gene–environment interactions may confound the genotype–phenotype relationship. In addition, clinical factors also affect drug response, such as concurrent therapies or renal and liver function. Thus, the human genome is only one tool for understanding a patient's response to therapy. It is our hope that genotyping platforms such as the ones described here can both identify new polymorphism–clinical endpoint relationships and help in the transition from laboratory observations to more individualized treatment.

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Disclaimer

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Intrathecal Administration

Lindsay B. Kilburn, Stacey Berg, and Susan M. Blaney

Abstract Treatment of central nervous system (CNS) cancers is often hampered by limited CNS penetration of systemically administered chemotherapy. Since most anticancer agents have limited CNS penetration, intrathecal drug delivery was developed in an attempt to deliver anticancer drugs more directly to the CNS. Drugs may be administered by intrathecal or intraventricular routes and may achieve high CSF drug concentrations and more prolonged exposures with relatively small drug doses. Dosing for IT administration is typically based on age rather than BSA since CSF volumes in infants and young children increase at a proportionally greater rate than BSA, reaching nearly adult volumes by age 3. Factors that may lead to obstructed CSF flow or altered CSF production or absorption should be considered prior to IT administration. Toxicities of IT administration are usually self-limiting and include headache, nausea, vomiting and fever or chemical arachnoiditis. This chapter discusses these aspects of IT therapy and also provides an overview of the pharmacology of intrathecally administered anticancer agents including methotrexate, cytarabine, thiotepa, and newer agents and immunotherapies.

Keywords Central nervous system pharmacology • Intrathecal • Intraventricular

1 Introduction

The central nervous system (CNS) is an important site for tumor spread in the form of neoplastic meningitis or intraparenchymal metastasis. Leukemias, especially acute lymphoblastic leukemia (ALL), and lymphomas remain the most common cancers with a predilection for leptomeningeal spread. However, there are many solid tumors that may also disseminate within the CNS including

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breast, gastric, and small-cell lung cancers [1–4]; melanoma [5]; primary CNS tumors such as medulloblastoma and glioma [6, 7]; and childhood tumors such as neuroblastoma, retinoblastoma, and rhabdomyosarcoma [8–11]. Treatment of CNS metastasis can be hampered by the pharmacologic sanctuary effect created by the blood–brain barrier (BBB) and blood–cerebrospinal fluid (CSF) barrier, which often limit the CNS penetration of systemically administered chemotherapy.

Strategies to treat metastatic CNS disease include intrathecal (IT) chemotherapy, radiation therapy, and high-dose systemic chemotherapy. These therapeutic approaches have been used successfully for the prevention and treatment of CNS leukemia. In fact, IT chemotherapy is currently incorporated into all frontline leukemia treatment protocols and is the primary therapeutic modality for the prevention of leptomeningeal dissemination. Unfortunately, however, for most patients with neoplastic meningitis from metastatic solid tumors or for patients with recurrent/refractory CNS leukemias, there are few effective treatments. Ongoing challenges to the successful treatment of these high-risk patients include the limited spectrum of antineoplastic agents that are currently available for IT administration as well as the lack of effective IT combination chemotherapy regimens.

The primary focus of this chapter is to describe the role of IT therapy in the prevention and treatment of neoplastic meningitis, including a brief review of the limitations of systemically administered chemotherapy, an overview of important pharmacologic principles that are relevant to IT administration of anticancer agents, and a review of the pharmacokinetics and toxicities of the most commonly administered IT agents. In addition, we provide an overview of new agents for IT administration that is in earlier stages of preclinical and clinical evaluation.

2 IT Chemotherapy

2.1 Rationale

The BBB and the blood–CSF barrier are natural membrane barriers that, among other physiologic functions, regulate drug entry into and egress from the central nervous system [12, 13]. The BBB, located at the level of the CNS endothelial cell, and the blood–CSF barrier, located in the epithelium of the tiny organs surrounding the ventricles (e.g., choroid plexus, median eminence, area postrema), limit the CNS penetration of toxic substances, including most hydrophilic anticancer agents, from the bloodstream (Fig. 1) [12]. Drug clearance from the CSF generally occurs via passive diffusion. However, in contrast to non-CNS endothelial cells, there are metabolic enzymes and transporters [e.g., multidrug resistance-associated proteins (see ABC Transporters: Involvement in Multidrug Resistance and Drug Disposition Chapter for more information) and organic acid transporters (see Solute Carriers Chapter for more information)], in the blood–brain and blood–CSF barriers that play an important role in the clearance of specific drugs from the CSF [14–18].

Since the cytotoxic activity of many anticancer agents is best correlated with maximal or total exposure, an estimate of CNS drug exposure following systemic drug administration provides insight into whether an agent has potential utility in the treatment or prevention of CNS disease. Because it is difficult to measure drug in the brain interstitial space, drug penetration into the CSF is often used as a surrogate for CNS exposure [14]. CSF penetration is most accurately described by the ratio of the area under the CSF and plasma concentration–time curves (AUC_{CSF}/AUC_{plasma}) (Fig. 2). CSF exposure data are frequently derived from preclinical models, as it is impractical to obtain serial CSF and plasma drug levels routinely after systemic administration in humans. As shown in Table 1, the CSF exposure for the most commonly used anticancer agents is <10 % of the plasma exposure. As a result of this limited exposure, other approaches, such as high-dose chemotherapy and IT drug delivery, were developed to attempt to deliver anticancer drugs to the CNS.

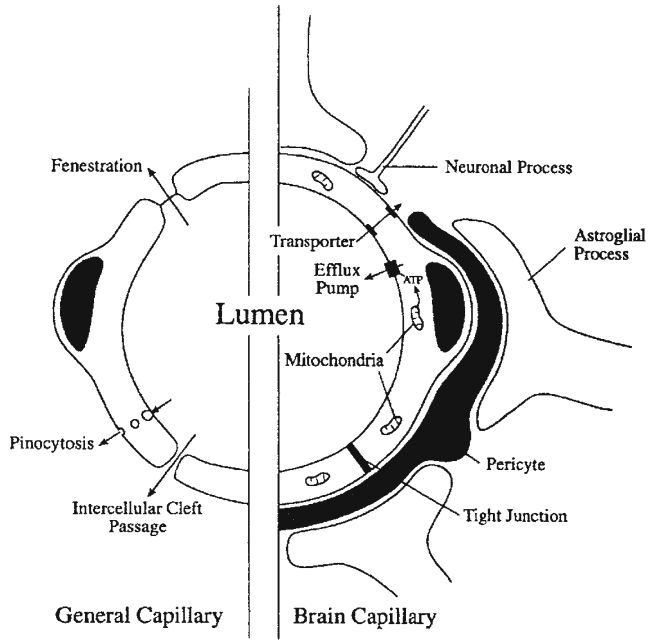

Fig. 1 Differences between brain capillary endothelial cells and endothelial cells in other organs. Brain capillary endothelial cells have tight intercellular junctions and lack fenestrations and pinocytic vesicles. The cytoplasm of the brain capillary endothelial cells is rich in mitochondria that supply energy to the various transport systems for passage of nutrients into the brain and to pump out potentially toxic compounds. Processes from astrocytes, pericytes, and neurons are closely associated with brain capillaries and trophically influence the specialized functions of brain capillary endothelial cells (from [14], with permission)

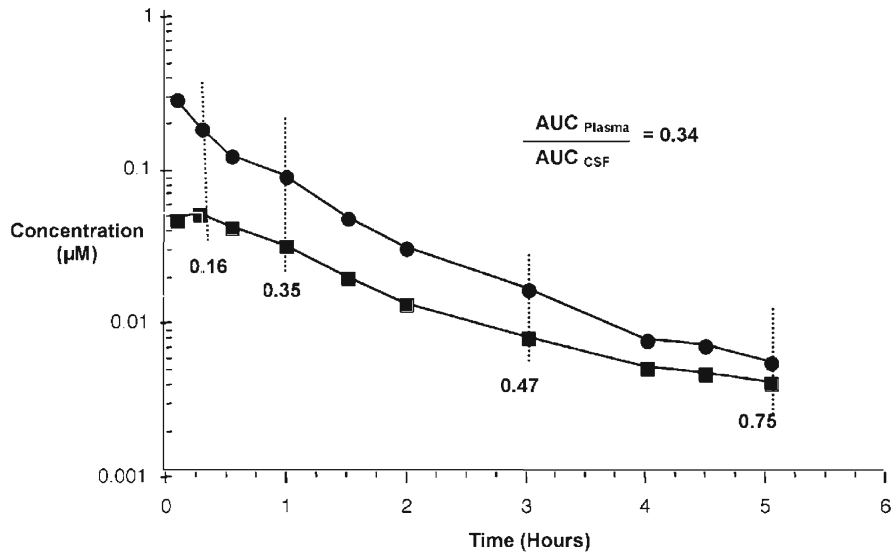

Fig. 2 Plasma (*filled circle*) and CSF (*filled square*) concentration of topotecan in nonhuman primates after an intravenous dose. Ratios of CSF to plasma concentrations at individual timepoints are shown on the graph for the 0.25-h, 1-h, 3-h, and 5-h samples. The CSF/plasma ratios range from 0.16 to 0.75, and the ratio increases over time because of the difference in the shapes of the plasma and CSF concentration vs. time curves. The ratio of drug exposure (AUC) in CSF to plasma is 0.34. Ratios obtained from single-time point measurements at later timepoints overestimate the drug exposure in CSF relative to exposure in plasma (from [19] with permission)

Table 1 Central nervous system penetration of commonly used anticancer drugs

Agent	CSF/plasma ratio (%)
Alkylating agents	
Cyclophosphamide	
Total drug	50
Active metabolite	15
Ifosfamide	
Total drug	30
Active metabolite	15
Thiotepa	>95
Carmustine	>90
Cisplatin	
Free platinum	40
Total platinum	<5
Carboplatin	
Free platinum	30
Total platinum	<5
Antimetabolites	
Methotrexate	3
6-Mercaptopurine	25
Cytarabine	15
5-Fluorouracil	
Bolus	50
Infusion	15
Gemcitabine	7
Antitumor antibiotics	
Anthracyclines	ND
Dactinomycin	ND
Plant alkaloids	
Vinca alkaloids	5
Epipodophyllotoxins	<5
Topoisomerase I inhibitors	
Topotecan	32
Irinotecan	
CPT-1 1 lactone	14
SN-38 lactone	<8
Miscellaneous	
Prednisolone	<10
Dexamethasone	15
L-Asparaginase	ND[a]

For most antineoplastic agents the total CSF drug exposure following administration of a systemic dose is <10 % of the systemic exposure [20]. *ND* not detectable in CSF

[a]Although drug is ND in CSF, CSF L-asparagine is depleted by systemic administration of L-asparaginase

2.2 Systemic Chemotherapy

The primary advantages of systemic therapy compared with IT chemotherapy are that it is technically easier to administer and that it provides more uniform drug distribution throughout the neuraxis. In addition, there is the potential for prolonged CNS drug exposure after protracted infusions, which is of critical importance for cell cycle-specific cytotoxic agents. The primary disadvantage of systemic

chemotherapy is that, because of the limited penetration of most agents into the CSF, high systemic doses of drug may be required to produce cytotoxic CNS exposures. Although a high-dose strategy has been employed effectively with methotrexate, an agent for which calcium leucovorin provides adequate rescue from systemic toxicities [21], this approach is not feasible with most antineoplastic agents, due to systemic toxicities, especially myelosuppression, limit the dose that can be administered.

2.3 IT Drug Delivery

Because the CSF volume is relatively small compared to the plasma volume (150 ml vs. 3,500 ml), high CSF drug concentrations can be achieved using a relatively small drug dose, which minimizes the potential for systemic toxicity. In addition, because CSF drug clearance is often slower than systemic clearance, there is the potential for more prolonged drug exposure at the target site following bolus IT drug administration. Other aspects of IT drug administration that affect CSF drug exposure include relative changes in CSF volume with age, alterations in drug distribution due to alterations in CSF flow resulting from the underlying disease process, and alterations in drug distribution based on site of administration, that is, intraventricular or intralumbar, as well as on patient position following drug administration.

Lumbar puncture is the most common method of IT drug delivery. Children with leukemia routinely receive intralumbar methotrexate, either as a single agent or in combination with cytarabine and hydrocortisone, in addition to their systemic chemotherapy. Although lumbar punctures may cause local pain or discomfort, the procedure is generally well tolerated with the use of local anesthesia. In infants and very young children, the potential for pain and discomfort is reduced further by the use of either conscious or general sedation. Some investigators have attempted to use indwelling lumbar access devices to minimize procedural pain and to ensure drug delivery to the subarachnoid space. However, there is limited oncologic experience with intralumbar access devices. Such devices have inherent risks including bleeding and infection as well as additional risks like catheter breakage or leakage [22]. These devices are not widely used for routine IT drug administration for patients receiving preventative IT therapy.

Although there are pharmacokinetic advantages associated with intralumbar drug delivery, there are also disadvantages to this method. Aside from pain, there are occasional technical challenges with lumbar puncture, and leakage or inadvertent injection into the subdural or epidural space is surprisingly common [23]. In addition, there is heterogeneous drug distribution throughout the neuraxis after IT dosing due to the cephalocaudad flow of CSF. As a result, after intralumbar dosing, some agents may undergo metabolic inactivation or clearance via active transport or bulk flow prior to reaching the ventricles and cerebral convexities, resulting in the potential for inadequate drug exposure at some sites of disease [24–26]. Finally, because drug only penetrates 2–3 mm from the CSF into the surrounding tissue, exposure in bulky leptomeningeal disease or parenchymal metastases is limited [27]. This is of particular concern for solid tumor metastases that may frequently include isolated or disseminated nodular tumor deposits, ranging in size from millimeters to centimeters, within the subarachnoid space or on the cranial or spinal nerve roots [28].

Many of the limitations associated with intralumbar drug delivery can be overcome by the use of an intraventricular access device such as an Ommaya reservoir (Fig. 3). An obvious advantage with an Ommaya reservoir is that injections are more convenient and less painful than intralumbar injections, and drug is less likely to leak outside the subarachnoid space. Intraventricular reservoirs are frequently used in adults with leptomeningeal cancer because of technical difficulties in performing repeated lumbar punctures in patients with spinal stenosis, and are also used in children with nonleukemic neoplastic meningitis or refractory CNS leukemia. The relative ease of intra-Ommaya drug delivery facilitates more effective, less toxic dosing schedules (see below). Finally, CSF drug distribution throughout the neuraxis is theoretically faster and more uniform after intraventricular

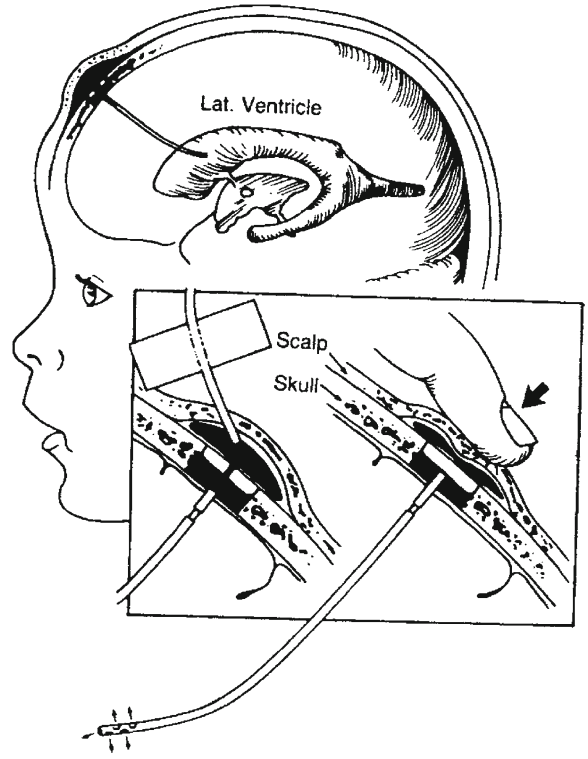

Fig. 3 Diagram of an intraventricular drug delivery system consisting of a subcutaneously implanted Ommaya reservoir attached to a catheter, the tip of which sits in the lateral ventricle (reprinted with permission from [29])

injection, as drug distribution is not against gravity. This improvement in distribution may result in better drug exposure and increased clinical responses [30, 31]. The obvious disadvantage of an Ommaya reservoir is that it requires a neurosurgical procedure for placement as well as the associated inherent risk of infection with an indwelling device.

The classic example of an IT dosing approach facilitated by the placement of an Ommaya reservoir is the "concentration times time" ($C \times T$) dosing schedule, which involves administration of relatively small but frequent consecutive doses of methotrexate or cytarabine (Fig. 4). The goal of $C \times T$ dosing is to maximize efficacy, by maintaining drug concentrations above a cytotoxic threshold for a prolonged period of time, while minimizing toxicity by avoiding high peak concentration-associated neurotoxicity (Fig. 5) [34, 35]. Several studies of the $C \times T$ approach have suggested improved therapeutic results for intraventricular vs. intralumbar chemotherapy in patients with recurrent CNS leukemia or lymphoma [32, 33]. The $C \times T$ regimen was found to be as efficacious as the standard regimen and was associated with less neurotoxicity in a study of 19 patients with meningeal leukemia who were randomized to receive 12 mg/m^2 of IT methotrexate twice weekly vs. $C \times T$ methotrexate (1 mg every 12 h for 6 doses) [33]. In another study, the complete response duration following $C \times T$ dosing was 15 months for 14 of 15 patients (93 %) with refractory meningeal leukemia or lymphoma [32].

Common toxicities of IT drug administration include headache, nausea and vomiting, and fever, often referred to collectively as chemical arachnoiditis. These symptoms are usually self-limited. Neurotoxicity may be increased in young children or when IT therapies are given with concomitant radiation therapy. A recent report, however, suggested increased spinal cord toxicity in adolescents compared with younger and older patients [36].

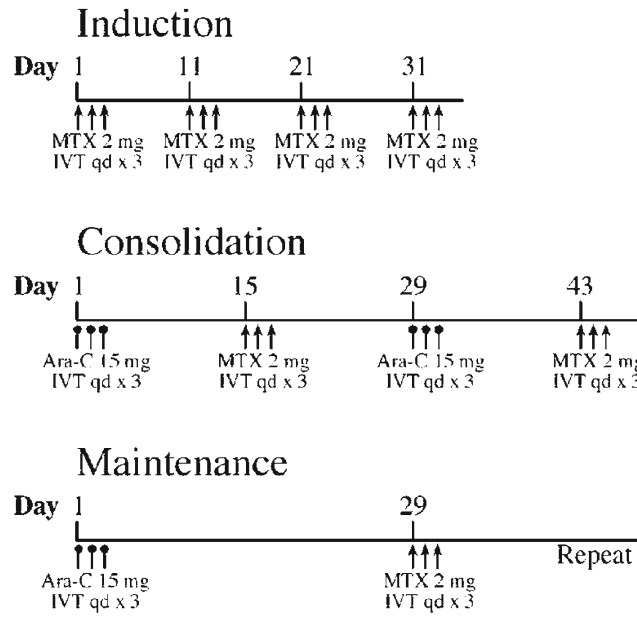

Fig. 4 Schematic diagram of the concentration × time ($C \times T$) regimen for methotrexate and cytarabine. *MTX* Methotrexate, *ara-C* cytarabine, *IVT* intraventricular, *qd* daily. Note: doses are for patients >3 years of age (reprinted with permission from [32])

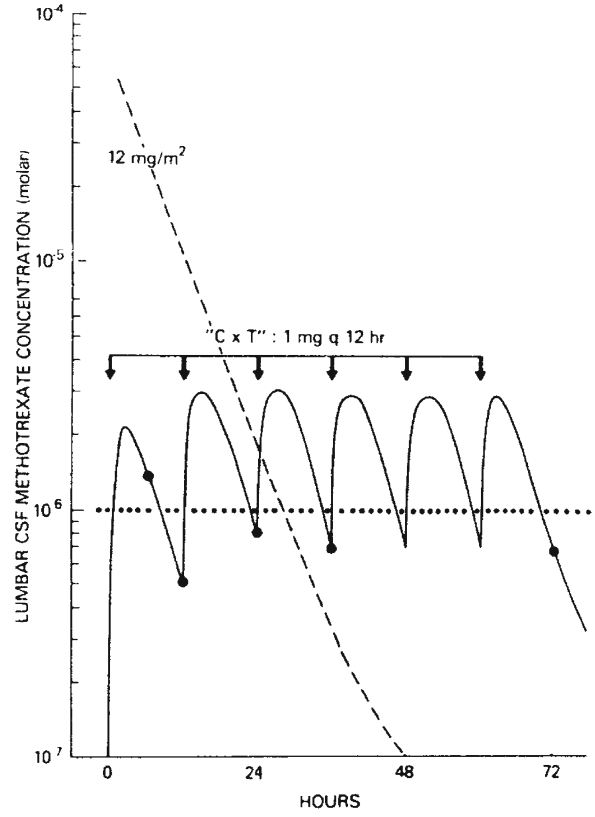

Fig. 5 Comparison of CSF methotrexate concentrations during intraventricular $C \times T$ therapy (*solid line*) and after intraventricular injection of a single 12-mg/m^2 dose. *Horizontal dotted line*, approximation of the therapeutically effected methotrexate concentration (reprinted with permission from [33])

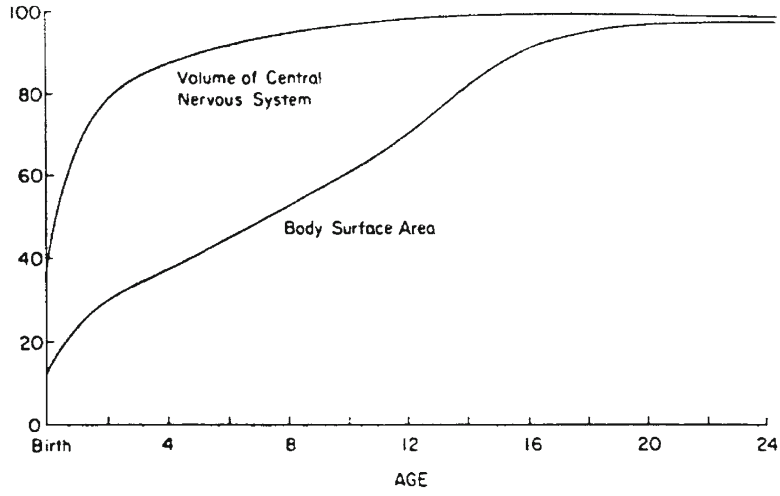

Fig. 6 Relationship between body surface area and CNS volume as a function of age. The CSF volume increases at a more rapid rate than BSA, reaching adult volume by 3 years of age (reprinted with permission from [37])

Table 2 Dosage regimens for intralumbar methotrexate and cytarabine based on patient age

Patient age (years)	Methotrexate dose (mg)	Cytarabine dose (mg)
<1	6	12
1	8	16
>2	10	20
≥3–9	12	24
≥10	15[a]	30

[a]Some investigators do not exceed methotrexate doses of 12 mg for patients ≥3 years of age

3 Factors Affecting Drug Exposure and Distribution After IT Administration

3.1 Age-Based Dosing Recommendations for IT Agents

Whereas most systemically administered anticancer agents are dosed based on body surface area (BSA) or weight, dosing for IT anticancer agents is based on patient age [37, 38]. This is because the CSF volume of infants and young children increases at a proportionally greater rate than BSA (Fig. 6). As a result, the CSF volume of infants and children is large relative to their BSA. In fact, by 3 years of age the CSF volumes of adults and children are essentially the same. Thus, dosing practices based on age rather than BSA avoid "underdosing" infants and "overdosing" adolescents and adults. As a result, age-based dosing recommendations for the commonly used IT agents are standard (Table 2). The direct advantages of age-based dosing for IT agents in children with CNS leukemia included less neurotoxicity, less variability in drug concentrations, and a reduction in the incidence of CNS relapse [37, 38].

3.2 Alterations in IT Drug Distribution

Unobstructed physiologic CSF flow and normal rates of CSF production and resorption are required for optimal drug distribution following IT administration. Nonuniform drug distribution throughout the neuraxis may compromise efficacy, as a result of inadequate drug exposure, or may increase the risk of local or delayed toxicities, owing to increased local drug exposure. Even in the absence of bulky leptomeningeal or parenchymal CNS disease, patients with neoplastic meningitis may have significant alterations or obstruction to normal CSF flow [39–41]. Neuroimaging modalities, such as computed tomography (CT) or magnetic resonance imaging (MRI), are less sensitive in evaluating alterations in or obstruction to CSF flow than radionuclide CSF flow studies. Therefore, consideration should be given to obtaining a radionuclide CSF flow study, such as Indium-111 DTPA or ^{99m}Tc-DTPA scan, prior to the initiation of IT chemotherapy, especially in patients with neoplastic meningitis from solid tumors. In some circumstances, focal CSF flow abnormalities can be readily restored through the use of local radiation to relieve the obstruction [40]. IT chemotherapy should not be administered to patients with significant abnormalities in or obstruction to CSF flow.

3.3 Patient Position After IT Drug Administration

Patient position after IT drug administration may also have a major impact on CSF drug distribution, especially after intralumbar drug dosing, since the drug must mix with CSF then flow throughout the CSF space in order to reach all potential sites of meningeal disease. In nonhuman primates that were maintained in either an upright or a prone position for 1 h after intralumbar methotrexate dosing, peak ventricular methotrexate concentrations and ventricular drug exposure were up to tenfold greater in animals that remained prone. In addition, there was less variability in ventricular methotrexate levels when the animals were prone [42]. A reasonable recommendation therefore is for patients to remain prone or in Trendelenburg position for an hour after intralumbar chemotherapy administration.

4 Pharmacology of Standard Anticancer Agents for IT Administration

4.1 Methotrexate

4.1.1 Intralumbar Methotrexate

Intralumbar methotrexate is the most commonly used drug for IT administration. Interval administration of IT methotrexate for presymptomatic treatment of leptomeningeal leukemia, a standard component of frontline protocols for acute lymphocytic leukemias and many lymphomas, has been instrumental in decreasing the risk of CNS relapse [43, 44]. IT methotrexate is also effective in inducing CNS remissions in patients with overt CNS leukemia [45–47] and is commonly used for palliative treatment of solid tumor neoplastic meningitis, although the utility of methotrexate for most solid tumors is limited due to its restricted spectrum of antitumor activity.

The initial detailed pharmacokinetic studies of methotrexate were performed prior to the development and routine implementation of age-based dosing guidelines. These studies revealed that after an intralumbar dose of 12 mg/m^2, CSF methotrexate elimination is biphasic with half-lives of 4.5 and 14 h [48]. Average methotrexate levels in lumbar CSF exceed 10 μM at 6 h and fall to 0.1 μM by 48 h, while

ventricular CSF levels average only 10 % of simultaneous lumbar levels [31, 48]. Drug elimination occurs primarily via bulk CSF resorption, although a nonspecific transport mechanism exists [49].

As was previously discussed, intraventricular methotrexate administration may overcome many of the limitations associated with intralumbar dosing. Pharmacokinetic studies following an intraventricular methotrexate dose of 6.25 mg/m^2 reveal that peak ventricular methotrexate concentrations exceed 200 μM and remain above 0.2 μM for 48 h. In addition, there is good distribution throughout the neuraxis; lumbar CSF methotrexate is detected within 1 h, and lumbar CSF levels exceed ventricular levels within 4 h after intraventricular dosing [31].

4.1.2 Toxicities of IT Methotrexate

The neurological toxicities associated with IT methotrexate can be characterized as acute, subacute, or delayed [50]. Acute toxicities are not uncommon and occur within several hours to a few days after drug administration. Commonly observed toxicities include headache, stiff neck, back pain, vomiting, fever, and lethargy. These adverse events may occur in isolation or may be associated with a CSF pleocytosis, indicative of a chemical arachnoiditis [51]. Subacute toxicities occurring days to weeks after administration are relatively uncommon and are not always reversible. Subacute toxicities include paraplegia, myelopathy, or encephalopathy, with concomitant symptoms of weakness, ataxia, cranial nerve palsies, seizures, alteration of mental status, or coma [2, 8, 51]. Leukoencephalopathy, a chronic progressive demyelinating process, is a late neurotoxicity that may occur months to years after treatment. Leukoencephalopathy has most commonly been observed in patients who received IT methotrexate combined with craniospinal irradiation and/or high-dose methotrexate [35, 51–53].

Plasma drug exposure after IT methotrexate is approx 100 times less than CSF exposure [48]. However, plasma levels of methotrexate exceed 0.01 μM for twice as long after IT administration as after an equivalent systemic dose. Nonetheless, systemic toxicity is minimal, demonstrating one of the advantages of regional chemotherapy.

Accidental overdose of IT methotrexate is a rare but life-threatening event that most often occurs when a patient inadvertently receives the dose of methotrexate that was intended for intravenous administration. If untreated, the resultant overdose causes overwhelming acute neurotoxicity with subsequent severe morbidity or death. Treatment for such an overdose should include immediate CSF drainage, ventriculolumbar perfusion, and administration of systemic leucovorin and steroids [54]. In addition, glucarpidase (carboxypeptidase-G2), a more specific antidote that converts methotrexate to an inactive metabolite, has been developed. Evaluation of glucarpidase in a preclinical nonhuman primate model demonstrated that after a methotrexate overdose of 50 mg (equivalent to 500 mg in humans), glucarpidase successfully reduced CSF methotrexate concentrations 400-fold within 5 min of administration [55]. Widemann and colleagues recently confirmed these observations in seven patients who received accidental IT methotrexate overdoses (median dose 482 mg). CSF methotrexate concentrations decreased in all but one patient by >95 % following glucarpidase administration. Furthermore, all patients recovered completely with the exception of impaired memory in two [56]. While this agent remains investigational, its emergent use should be considered in any patient with IT methotrexate overdose.

4.2 Cytosine Arabinoside

Cytosine arabinoside (cytarabine, or ara-C) is the second most commonly used IT agent. Like methotrexate, cytarabine is primarily used in the treatment and prevention of leptomeningeal leukemias and lymphomas. Cytidine deaminase, a ubiquitous enzyme in the liver and blood, rapidly converts

systemically administered cytarabine to an inactive metabolite, ara-U [57, 58]. However, cytidine deaminase levels in human CSF are markedly lower than plasma levels. As a result, there is minimal conversion of cytarabine to ara-U following IT dosing [58–60]. Thus, there is a marked reduction in cytarabine clearance after IT compared with systemic dosing. Cytarabine clearance after IT dosing is essentially that of CSF bulk flow, that is, 0.4 ml/min [59], while plasma clearance after intravenous dosing approximates 1,000–3,600 ml/(min m^2) [61]. Thus, there is a tremendous pharmacokinetic advantage for prolonged CSF exposure with IT cytarabine administration.

As with methotrexate, the duration of neoplastic cell exposure to cytotoxic concentrations of cytarabine is an important determinant of response. After intraventricular administration of a 30-mg cytarabine dose, elimination from CSF is biphasic with half-lives of 1 and 3.4 h [59]. Peak CSF concentrations are >2 mM and exceed 1 μM for more than 24 h. Plasma concentrations after a dose of IT cytarabine are undetectable.

In a simulated schedule of ara-C administered using a $C \times T$ dosing approach, 30 mg daily for 3 days, cytotoxic concentrations were achieved for more than 72 h compared with approximately 24 h after a single larger dose of 70 mg [59]. A $C \times T$ approach is the preferred approach for attaining sustained CSF levels when the standard cytarabine formulation is utilized. Another alternative is use of a sustained-release cytarabine formulation, cytarabine liposome, approved for use in the treatment of lymphomatous meningitis (discussed below).

4.2.1 Toxicities

The most common toxicity of cytarabine after IT administration is chemical arachnoiditis [51]. Less commonly, seizures, paraplegia, peripheral neuropathy, and encephalopathy have been reported [51, 62–64]. Leukoencephalopathy has been reported with combined IT therapy and radiation [65].

4.3 Cytarabine Liposome

Cytarabine liposome, a sustained-release formulation of cytarabine for IT administration, was specifically developed to maximize the therapeutic efficacy of this S-phase-specific agent by prolonging CSF exposure [66]. Pharmacokinetic studies after a single dose demonstrate that this novel formulation improves drug distribution throughout the neuraxis. Following intraventricular delivery, lumbar CSF concentrations are equivalent to ventricular concentrations within 6 h. In addition, the terminal CSF half-life of cytarabine liposome is nearly 40-fold greater than that of standard cytarabine (141 vs. 3.4 h) [67]. Thus, an obvious advantage of this agent is that it can be administered much less frequently than standard cytarabine but still provide prolonged CSF exposure [68].

Randomized multicenter trials comparing cytarabine liposome to methotrexate in adults with lymphomatous meningitis demonstrated that there was a trend to improvement in neurological progression and median survival in the cytarabine liposome arm. Likewise, in adults with neoplastic meningitis due to solid tumors, there was a trend toward increased time to neurological progression in the cytarabine liposome-treated patients [69].

Studies in children demonstrate that despite equivalent CSF volumes, pediatric patients ≥3 years of age appear to tolerate a somewhat lower dose of cytarabine liposome than adults. The recommended treatment dose for adults is 50 mg, while in the phase I pediatric study the maximum tolerated dose (MTD) was 35 mg [70]. At higher doses, children experience protracted headaches. Despite the lower dosage recommendation, cytarabine liposome appears to be an active agent in children with refractory leptomeningeal leukemia with seven of nine patients experiencing an objective response.

Pharmacokinetic studies at the pediatric recommended phase II dose of 35 mg demonstrated that 8 days after cytarabine liposome administration, four of five patients still had free cytarabine CSF levels ≥0.4 μM [71].

The primary disadvantage of cytarabine liposome compared to standard cytarabine is the toxicity profile. Cytarabine liposome must be given with concomitant oral dexamethasone for approximately 5 days to prevent chemical meningitis. Otherwise, the toxicity profile of IT cytarabine liposome plus dexamethasone is very similar to that of the standard cytarabine formulation. Acute toxicities include fever, headache, back pain, nausea, and encephalopathy [67].

4.4 Thiotepa

Thiotepa is a lipid-soluble alkylating agent that has been administered by the IT route to children and adults with neoplastic meningitis. Thiotepa is rapidly converted to an active metabolite, TEPA, after intravenous administration, and both thiotepa and TEPA readily and extensively penetrated into the CSF after systemic dosing. In contrast, after IT administration there is no intra-CSF conversion to TEPA, and CSF clearance exceeds bulk flow by almost ninefold [72]. Therefore, there is not a pharmacokinetic advantage for this agent after regional drug administration.

Clinical studies of IT thiotepa have also failed to provide compelling evidence for its use. In a randomized prospective study evaluating IT methotrexate vs. IT thiotepa in adults with neoplastic meningitis, the overall toxicity and efficacy of these agents were essentially identical and overall quite dismal. The median survival for the methotrexate group was 15.9 weeks vs. 14.1 weeks for the thiotepa group [73]. Likewise, in a retrospective study of 15 children with neoplastic meningitis who received IT thiotepa in combination with other therapy, the median survival was only 15.1 weeks [74].

5 New Agents for IT Administration

The limited number of antineoplastic agents that significantly penetrate the blood–brain and blood–CSF barriers and the paucity of agents that are available for IT administration impose significant limitations on the development of strategies to successfully prevent or treat solid tumor neoplastic meningitis or refractory CNS leukemia. Combination chemotherapy regimens are an integral component of successful treatment regimens for many systemic cancers. However, similar strategies cannot be readily employed for neoplastic meningitis because effective IT combination regimens are not available. Therefore, we must identify additional agents suitable for IT administration or agents with favorable toxicity profiles and high CSF/plasma exposure ratios. We have used a nonhuman primate model to evaluate candidate agents for IT administration. In this section we summarize the current status of ongoing clinical trials with these novel agents that hold promise for future study.

5.1 Topotecan

Topotecan is a water-soluble topoisomerase I poison that has demonstrated objective antitumor activity against a variety of adult and pediatric malignancies including non-small-cell lung cancer, ovarian carcinoma, leukemias, and rhabdomyosarcomas. This spectrum of antitumor activity and lack of

neurological toxicity after systemic administration led to a series of preclinical studies that demonstrated the feasibility of IT drug delivery. In a phase I study of IT topotecan, arachnoiditis characterized by fever, nausea, vomiting, and headache with or without back pain was the dose-limiting toxicity in two of four patients enrolled at the 0.7 mg dose level. The MTD was subsequently defined as 0.4 mg. Six of the 23 evaluable patients had evidence of benefit manifested as prolonged disease stabilization or response [75]. A pediatric phase II trial of IT topotecan was subsequently initiated by the Children's Oncology Group with the primary study endpoints of response rate in patients with CNS leukemia in second or greater relapse and response rate and progression-free survival in children with leptomeningeal medulloblastoma. In this study IT topotecan was active in patients with recurrent CNS leukemia but not in patients with leptomeningeal dissemination of medulloblastoma or solid tumors. Overall, the drug was well tolerated [76]. A Pediatric Brain Tumor Consortium study evaluating a protracted dosing schedule to optimize CSF exposure is currently underway.

5.2 Mafosfamide

Cyclophosphamide is a widely used alkylating agent that has a broad spectrum of antitumor activity against many pediatric and adult cancers. However, cyclophosphamide requires oxidation by hepatic microsomal enzymes to express activity and thus is not a candidate for IT or other regional therapeutic approaches. Mafosfamide is a cyclophosphamide derivative that does not require hepatic activation for tumoricidal effect. The spectrums of antitumor activity for mafosfamide and cyclophosphamide are essentially identical. Thus, mafosfamide was deemed an excellent candidate for further study.

Preclinical in vitro studies with mafosfamide, using a representative panel of tumor cell lines with a predilection for leptomeningeal spread, were performed to define an optimal cytotoxic exposure. Preclinical studies in a nonhuman primate demonstrated the feasibility of this dosing approach and provided data for a safe starting dose [77]. A phase I study of IT mafosfamide as a single agent was completed and dose-limiting toxicity with rapid infusion was headache at 5 mg. This was improved with prolonged infusion to 20 min [77], but with prolonged infusion, headache again was dose limiting at 6.5 mg, the MTD. In a phase 1/2 study using IT mafosfamide in an attempt to delay CNS radiation in very young children with newly diagnosed brain tumors, the mafosfamide dose was further escalated with the use of steroid premedication. The MTD for mafosfamide in this study was 14 mg with dose-limiting toxicity of irritability presumed be related to headache. CSF mafosfamide concentrations were above the target concentrations after intraventricular dosing in all patients evaluated at all dose levels. Ventricular concentrations after intralumbar dosing exceeded the target concentration in all but one patient [78]. The preliminary results of the efficacy portion of the trial suggest that the PFS rates of patients in the study (48 %) were at least equivalent to those observed in historical studies [79].

5.3 Busulfan

Busulfan is a dimethanesulfonyloxyalkane that functions as a cell cycle-nonspecific alkylating agent. Clinical busulfan use is generally restricted to preparative regimens for bone marrow transplant because of its profound systemic toxicities, including severe myelosuppression and pulmonary fibrosis. Historically, regional busulfan administration was limited by its poor solubility in aqueous solutions [80]. However, a microcrystalline formulation of busulfan with greatly enhanced aqueous solubility, Spartaject busulfan, was developed specifically for IT administration. In a phase I study in children with neoplastic meningitis, the dose-limiting toxicities were vomiting, headache, and

arachnoiditis. Pharmacokinetic data demonstrated that CSF concentrations of busulfan ranged from 50 to 150 μg/ml and declined to <1 μg/ml within 5 h of infusion. IT Spartaject busulfan was well tolerated overall and the recommended dose for future phase 2 studies is 13 mg [81].

5.4 Gemcitabine

Gemcitabine (2′,2′-difluorodeoxycytidine or dFdC), a deoxycytidine analog, has a broad spectrum of clinical antitumor activity against a variety of tumors with potential for leptomeningeal spread. Similar to cytarabine, gemcitabine is rapidly deaminated to an inactive metabolite following intravenous administration, thereby limiting CSF gemcitabine exposure [82, 83]. After IT administration in the nonhuman primate model, CSF exposure was eight times that achieved in the plasma after a 400-fold higher dose administered IV [84]. These findings led to a phase I study in adult and pediatric patients with neoplastic meningitis. Gemcitabine was administered via Ommaya reservoir or lumbar puncture at three dose levels. However, two patients developed significant neurotoxicity, transverse myelitis, and somnolence. Due to the degree of these dose-limiting toxicities, the study was closed and further development of gemcitabine for IT use was not recommended by the study group [85].

5.5 Monoclonal Antibodies

A relatively newer group of anticancer agents include monoclonal antibodies. These agents do not adequately cross the BBB, but several recent studies and reports have described their use for IT administration.

5.5.1 Rituximab

Rituximab is a humanized monoclonal antibody against the CD20 antigen expressed on many lymphoma cells. In a nonhuman primate model, rituximab administration via Ommaya reservoir was well tolerated. High CSF concentrations were initially achieved followed by distribution into a CSF–brain compartment [86]. Several authors have published case reports of complete responses with rituximab IT administration in patients with neoplastic meningitis [87–93]. Based on these preclinical and clinical reports, a phase I study was subsequently undertaken in adults with recurrent CNS and intraocular lymphoma. Patients were treated at three dose levels by intraventricular administration. CSF levels achieved at all dose levels were comparable to serum levels achieved with systemic therapy. Dose-limiting toxicities occurred in both patients treated at the 50 mg dose level and included hypertension, chest pain, nausea and vomiting, tachypnea, and diplopia. Minor toxicities included paresthesias and leukoencephalopathy. The 25 mg/dose was well tolerated and the recommended phase II dose. Meningeal responses were seen in six of ten patients, with one patient demonstrating a brain parenchymal response [94]. Further studies to evaluate the incorporation of intra-CSF rituximab with other therapies are now underway.

5.5.2 Trastuzumab

Trastuzumab is an antihuman epidermal growth factor receptor-2 (HER-2) antibody that is approved for the treatment of high-risk and refractory breast cancer. Trastuzumab does not cross the BBB.

There are a number of reports of IT administration alone or in combination with other agents (thiotepa, methotrexate) in the treatment of patients with leptomeningeal spread of breast cancer [95–101]. A variety of doses and schedules have been used ranging from 20 to 30 mg weekly to 50 mg every 3 weeks. Therapy was well tolerated with several patients demonstrating response or prolonged stable disease. One patient continued on therapy for 2 years [100]. These results suggest that further studies to define the optimal IT trastuzumab dose and explore CSF pharmacokinetics should be undertaken.

5.6 Radioimmunotherapies

In addition to monoclonal antibodies that are utilized as antitumor agents through their direct action on important receptors, there are increasing reports of the linkage of monoclonal antibodies to radioiodine in an attempt to concentrate radiation at tumor in the CSF and leptomeninges. I-131 has been linked with antibodies to a variety of tumor-associated antigens in the treatment of a variety of solid and primary CNS malignancies with LM dissemination including neuroblastoma, retinoblastoma, melanoma, glioma, ependymoma, and medulloblastoma [102–108]. More recent publications focus on antibodies to the GD2 ganglioside, 3F8, and B7H3, an immunomodulatory cell surface glycoprotein, both of which are expressed on a variety of tumors. Preliminary reports suggested radiation localization with this approach to sites of bulky disease, although in the phase I study of I-131-3F8 variations seemed better correlated to variable CSF flow than to known tumor deposits [105]. Clinical responses with clearance of CSF have been reported even in heavily pretreated patients. In these studies patients were usually pretreated with potassium iodide, liothyronine, antipyretic, with or without steroid, antihistamine, narcotic, and antiepileptics. Toxicities were generally reversible and included headache, fever, emesis, hypothyroidism, as well as dose-limiting hyponatremia and chemical meningitis. Incorporation of these agents into a treatment regimen for patients with recurrent CNS neuroblastoma has yielded promising results although the regimen also included craniospinal radiation so the impact of the IT therapy is difficult to ascertain [104]. However, preclinical data does suggest enhanced effect with the combination of external beam radiation therapy and compartmental radioimmunotherapy. Monoclonal antibodies targeting newer tumor targets as well as utilizing different radioisotopes are now in development.

6 Conclusion

Significant advances in the treatment and prevention of CNS leukemias and lymphomas have been a direct result of a better understanding of CNS pharmacology and the development of effective therapeutic strategies to circumvent the limitations imposed by the blood–brain and blood–CSF barriers. Unfortunately, the treatment and prevention of leptomeningeal metastases from solid tumors and the treatment of recurrent CNS leukemia remain unsatisfactory. Further advancements in the treatment of these devastating entities require continued preclinical and clinical research efforts to identify new agents and combination regimens for IT administration and to evaluate and incorporate new treatment strategies such as IT delivery of monoclonal antibodies or immunotherapies. Strategies that utilize combined systemic and IT therapies may provide improved clinical outcomes [78, 100, 104]. Furthermore, correlative clinical pharmacology studies to maximize efficacy and minimize toxicity are of paramount importance in ensuring that optimal dosing strategies are developed.

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Microdialysis

Austin J. Combest and William C. Zamboni

Abstract Limited tumor penetration could be a contributing factor to treatment of solid tumors when chemotherapy is administered systemically. Microdialysis is a technique utilized to evaluate tumor extracellular fluid disposition of anticancer agents and factors affecting delivery and removal of anticancer agents. Microdialysis utility has been demonstrated when evaluating the disposition of carrier-mediated agents (e.g., liposomes and PEG conjugates), gene therapy, and antisense oligonucleotides, and angiogenesis inhibitors. Future potential for microdialysis includes therapeutic drug monitoring and assessment of surrogate markers for response and toxicity. This chapter will discuss the advantages of microdialysis, the methodology and study design, and application to preclinical and clinical pharmacokinetic and pharmacodynamic studies.

Keywords Microdialysis • Intratumoral extracellular fluid • Pharmacokinetics • Therapeutic drug monitoring

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1 Introduction

1.1 Issues Related to Drug Delivery in Solid Tumors

Major advances have been made in the use of cancer chemotherapy [1]. However most patients, especially patients diagnosed with solid tumors, fail to respond to initial treatment or relapse after an initial response [1]. Thus, there is a need to identify factors associated with lack of response and to develop new treatment strategies that address those factors. The development of effective chemotherapeutic agents for the treatment of solid tumors depends, in part, on the ability of those agents to achieve cytotoxic drug concentrations or exposure within the tumor [2, 3].

It is currently unclear why within a patient with solid tumors there can be a reduction in the size of some tumors while other tumors can progress during or after treatment, even though the genetic composition of the tumors is similar [4]. Such variable antitumor responses within a single patient may be associated with inherent differences in tumor vascularity, capillary permeability, and/or tumor interstitial pressure that result in variable delivery of anticancer agents to different tumor sites [2, 3]. However, studies evaluating the intratumoral concentration of anticancer agents and factors affecting tumor exposure in preclinical models and patients are rare [3, 5, 6]. In addition, preclinical models evaluating tumor exposure of anticancer agents and factors affecting tumor exposure may not reflect the disposition of chemotherapeutic agents in patients with solid tumors due to differences in vascularity and lymphatic drainage [2, 3]. Moreover, it is logistically difficult to perform the extensive studies required to evaluate the tumor disposition of anticancer agents and factors that determine the disposition in patients with solid tumors, especially in tumors which are not easily accessible. Thus, there is impending need to develop and implement techniques and methodologies to evaluate the disposition and exposure of anticancer agents within the tumor matrix.

The need to develop and readily gain information on the tumor disposition of agents may become more important with the increasing number of tumor-targeting approaches, such as gene and antisense therapy, polyethylene glycol (PEG)-conjugated agents, and liposomal delivery [7, 8]. In addition, methodology and study designs used to develop classic cytotoxic anticancer agents, such as platinum, taxane, and camptothecin analogues, may not be appropriate for the new generations of anticancer therapy, such as angiogenesis inhibitors, antiproliferative agents, and signal transduction inhibitors [7, 9]. As these agents may not induce classic toxicities or any toxicity, it may be difficult to recommend a dose for future trials using the standard Phase I dose escalation methods and end points (i.e., maximum tolerable dose and dose-limiting toxicities). Alternatively, defining the dose for Phase II studies could be based on the dose that achieves exposures associated with pharmacologic modulation, optimal biological exposure, or cytotoxicity results from in vitro studies [3, 10]. Historically, investigators have compared in vitro IC_{50} values with plasma concentrations in patients as a means to determine if sufficient exposure has been reached in clinical studies. However, the inherent tumor characteristics which influence tumor penetration and high intra- and intertumoral variability in tumor exposure make this comparison highly unreliable [2, 3, 11], especially when the ratio of tumor exposure to plasma exposure may be approximately 0.2–0.5 [3 , 12–15]. Thus, comparing the in vitro exposures and plasma exposures in patients results in an overestimation of drug exposure in the tumor extracellular fluid (ECF), and thus, the required exposure for effect may be insufficient. The use of methodologies that measure the exposure of anticancer agents within the tumor may improve the level of information needed to make informed decisions during the drug development process.

1.2 Methods to Measure Drug Disposition in Tumors and Tissue

Until recently, drug uptake into tissues and tumors has been described indirectly based on modeling from plasma pharmacokinetics or measured directly from tissue biopsies. As stated above, modeling of tumor exposure based on plasma exposures without incorporation of factors representing tumor heterogeneity is unreliable [2, 3, 11]. The use of tissue or tumor biopsies is associated with several problems. Obtaining serial biopsies is most often logistically impossible, highly invasive, and associated with patient discomfort [3, 16, 17]. Thus, biopsies are usually only available for a single time point or measurement. Measurements of drug concentrations from biopsies are measured in tissue or tumor homogenates, where it may be difficult to control ex vivo catabolism and differentiate between various forms of the drug. Several new advanced techniques, such as magnetic resonance imaging (MRI), positron emission tomography (PET), and microdialysis, have been developed to quantify the concentrations of anticancer agents in vivo [16–18]. However, the use of MRI and PET is complicated by the lack of ability to differentiate between different forms and metabolites of a drug, availability, chemical synthesis of effective probes, and cost [17, 18]. Microdialysis to evaluate the disposition of anticancer agents in tumors and surrounding tissue, on the other hand, is a methodology that has several advantages over other existing methods [2, 7, 19, 20].

1.3 Introduction and Advantages of Microdialysis

Microdialysis is an in vivo sampling technique used to study the pharmacokinetics and drug metabolism in the blood and ECF of various tissues [19–21]. The use of microdialysis methodology to evaluate the disposition of anticancer agents in tumors is relatively new [3, 5, 6]. Microdialysis has been used to evaluate the tumor disposition of 5-fluorouracil, capecitabine, and epirubicin in patients with primary breast cancer lesions and carboplatin, aminolevulinic acid, and methylaminolevulinate in patients with melanoma [5, 6, 22–25]. Microdialysis has also recently been used to determine intratumoral concentrations of methotrexate in patients with high-grade gliomas and cisplatin in patients with oral cancer [26, 27]. These studies depict the clinical utility of microdialysis in evaluating the tumor disposition of anticancer agents in patients with accessible tumors. Microdialysis is based on the diffusion of nonprotein-bound drugs from interstitial fluid across the semipermeable membrane of the microdialysis probe [19–21]. A schematic representation of a microdialysis probe in subcutaneous tissue or tumor is depicted in Fig. 1. Microdialysis provides a means to obtain samples from tumor ECF samples from which a concentration versus time profile can be determined within a single tumor [3, 5, 6, 20].

Microdialysis provides several advantages over autoradiographic studies of tumor biopsies as a method to evaluate anticancer drug concentrations in tumor tissue. With microdialysis techniques it is possible to obtain serial sampling of anticancer drugs from the ECF of a single tumor with minimal tissue damage or alteration of fluid balance [3, 19, 20]. The microdialysis probe can remain in peripheral or central nervous system (CNS) tissue for up to 72 h without complications, such as increased risk of infection, inflammation, or alteration in probe recovery. Prolonged microdialysis sampling at the tumor site has also been conducted for up to 48 h without complication [24]. Samples can be immediately obtained and analyzed from a single probe that allows for the real-time evaluation of physiologic, pharmacologic, and pharmacokinetic changes [19, 28–30]. In addition, a single microdialysis probe can simultaneously sample several analytes of interest, thus allowing for the measurement of drug concentrations and pharmacologic end points that are required for pharmacodynamic studies. Furthermore, the drug concentration can be measured specifically rather than quantitating radioactivity, which may be nonspecific. Because of the pore cutoff size (20 kDa) of the

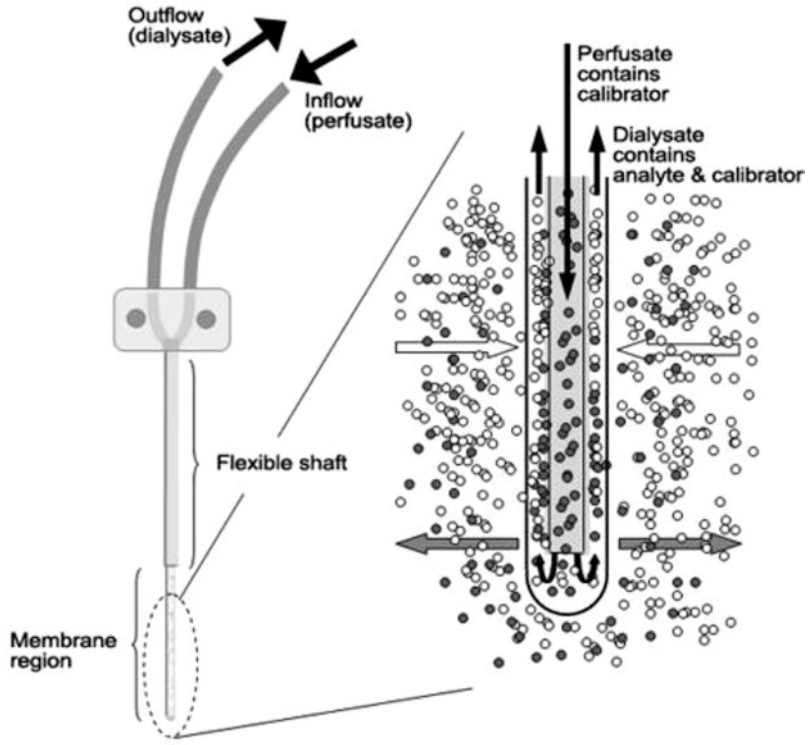

Fig. 1 Schematic of a commercial microdialysis probe with a visual representation of osmosis occurring at equilibrium (reproduced from Ref. Chandra S. Chaurasia, Markus Müller, Edward D. Bashaw, et al. AAPS-FDA Workshop White Paper: Microdialysis Principles, Application, and Regulatory Perspectives Report From the Joint AAPS-FDA Workshop, November 4–5, 2005, Nashville, TN. *The AAPS Journal* 2007; 9 (1) Article 6)

semipermeable membrane, the use of microdialysis allows for the differentiation between liposomally encapsulated drugs, conjugated drugs, protein-bound drugs, and active-unbound drugs in the tumor ECF [7, 31]. Using microdialysis techniques, serial sampling of the nonprotein-bound, active form of anticancer agents can be obtained from a single site in a brain tumor, peripheral tumor, or surrounding tissues. In addition, multiple microdialysis probes can be placed in a single tumor to evaluate intratumoral variability of the analyte of interest [3, 12]. Thus, the data obtained with microdialysis techniques may more closely reflect the disposition of the active form of the drug within the tumor ECF [12, 19, 32].

2 Microdialysis Methodology and Study Design

2.1 Microdialysis System and Setup

The principles of microdialysis sampling have been reviewed in detail previously [20, 21, 32, 33]. In brief, a short length of hollow dialysis fiber is continuously perfused with a physiologic solution. The presence of the analyte of interest in the ECF and its absence in the perfusate leads to a concentration gradient across the dialysis membrane. The analyte diffuses through the dialysis membrane and

is collected for analysis. This process is performed in vivo through the use of a microdialysis probe that is implanted into tissue and continuously perfused with a physiologic solution at a low flow rate (0.5–10 μL/min). After the probe is implanted into tumor tissue, substances are filtered by diffusion from the extracellular space through the semipermeable membrane into the perfusion medium and carried via microtubing into the collection vials.

Commercially available microdialysis probes, microperfusion pumps, and microfraction collectors are available. The type of microdialysis probe used depends on the site or tissues of interest (e.g., subcutaneous tumor or tissue, brain, or liver), size of the tumor, and the analyte of interest [3, 12]. A microdialysis probe (CMA 20, Stockholm, Sweden) with a molecular cutoff of 20 kDa, membrane length of 4 mm, and outer diameter of 0.5 mm is the standard used for most pharmacokinetic studies of drugs in peripheral tissue and tumors [3, 5, 6, 12]. The molecular weight cutoff (i.e., 20kDa) of the semipermeable membrane of probe prevents albumin-bound drug from crossing the membrane. However, small plasma protein, such as α_1-acid-glycoprotein, can pass through the semipermeable membrane. Thus, depending on the protein-binding characteristics of a drug, the recovery may not be limited to unbound drug. The microdialysis probe is perfused by a microperfusion pump and dialysate samples are collected by the microfraction collector. Ringer's solution (USP) is the standard perfusion solution because it is similar to the makeup of ECF. Alternatively, 0.9 % NaCl (USP) can be used for tissue and CNS studies.

2.2 Microdialysis Methodology and Study Design

2.2.1 In Vitro Calibration

Only a fraction (i.e., 10–50 %) of the analyte can cross the probe's semipermeable membrane, and the percent that crosses can vary between probes, drug type, and flow rate [20, 21, 32, 33]. Thus, prior to in vivo studies, it is standard to characterize the transfer rate, relative recovery, and the optimal flow rate of the drug and probe used in the studies. The recovery of drug across the membrane is concentration independent [3, 20, 21, 32, 33]. The objective is to use the lowest flow rate that achieves sufficient recovery of the analyte that can be detected by the analytical system. High flow rates should be avoided due to the propensity to alter the fluid balance in the tumors. The flow rate and collection interval are then modified to attain the needed sample volume required by the analytical system.

In vitro calibration studies are performed by placing a microdialysis probe in a beaker that contains a clinically relevant concentration of the drug or analyte of interest. The probe is perfused at various flow rates (e.g., 0.5, 1, 2, 3, 8, and 16 μL/min), and dialysate samples are collected every 10–25 min based on the required sample volume for the assay. The probe is allowed to reach equilibrium prior to sample collection at each flow rate. The time required to reach equilibrium is based on the flow rate and length of the microdialysis tubing. In vitro recovery is calculated as follows:

$$\text{In vitro recovery} = \frac{\text{Perfusate conc}_{\text{OUT}}}{\text{In vitro solution conc}}.$$

2.2.2 Microdialysis In Vivo Study Design and Procedures

Microdialysis probes can be placed in any accessible tumor and tissue. However, areas of the tumor with pooled blood should be avoided to prevent false results. Probe placement can be confirmed by ultrasound or after tumor or tissue removal in animal studies. Dual probe studies can also be performed to evaluate the intratumoral disposition of the analyte or drug.

After probe placement, a short period (i.e., 45–60 min) is allowed for probe and tumor ECF equilibration prior to the start of calibration [3, 6, 12, 19]. Although use of microdialysis technique results in less tissue damage compared to other sampling methods (e.g., biopsy), insertion of the microdialysis probe into the tumor does induce some tissue damage and immune reactivity. Thus, samples collected immediately after probe insertion may not reflect basal tumor conditions due to acute tissue damage and changes in blood flow associated with probe insertion. Therefore, it is necessary to allow time for the probe and tumor ECF to equilibrate prior to the start of the probe calibration studies.

After probe placement, calibration and washout procedures are performed. Because of variability in recovery for various probes at various sites, the calibration procedure is performed to determine the extent of recovery for each probe at each site. The washout period is performed to remove any drug introduced into the ECF during retrocalibration. The length of the washout period is determined by concentration of drug introduced in the ECF during calibration and the $t_{1/2}$ of the drug in the ECF. After the washout period, the drug is administered or the procedure is started, and the sample recovery procedure is performed.

2.2.3 In Vivo Calibration and Recovery

In vitro recovery may be substantially different from the in vivo (i.e., tumor ECF) recovery [19–21]. In addition, recovery can vary between probes, drug type, flow rate, and tissue or tumor site. An in vivo microdialysis study is a dynamic process in which substances are continuously removed from the tumor ECF by diffusion into the probe. Consequently, the concentration of drug in the perfusate does not reach equilibrium with the tumor ECF. However, under constant conditions (i.e., perfusate flow rate) a steady-state percent recovery, which represents a constant fraction of the ECF concentration, will be reached. Thus, the in vivo recovery value is determined for each probe in each tumor or tissue and is specific for that single procedure. This provides the advantage of accounting for processes that affect recovery in tissues and tumors. The in vitro recovery values can be calculated by retrodialysis calibration, reference or marker compound, and point of zero net flux methods [20, 21, 33, 34].

Retrodialysis calibration method can be used to estimate the steady-state percent recovery [3, 12–14, 29, 33, 34]. Retrodialysis quantification of in vivo recovery is based on the principle that the diffusion process across the microdialysis semipermeable membrane is equal in both directions. Therefore, the analyte of interest can be included in the perfusion medium, and the disappearance from the perfusate into the tumor ECF is used as an estimation of in vivo recovery. In vivo recovery is calculated as follows [3, 33]:

$$\text{In vivo recovery} = \frac{\text{Perfusate conc}_{\text{IN}} - \text{Perfusate conc}_{\text{OUT}}}{\text{Perfusate conc}_{\text{IN}}}. \quad (1)$$

Thus, the estimated drug concentration in the tumor ECF is calculated as follows [3, 21, 28]:

$$\text{Estimated tumor ECF conc} = \frac{\text{Measured microdialysis sample}}{\text{In vivo recovery}}. \quad (2)$$

One limitation of the retrodialysis method is the time required to perform the calibration studies (i.e., 4–5 samples over 1–1.5 h) and washout (3–4 samples which lasts approximately 1 h). Alternatively, if the retrodialysis calibration studies could be performed at the same time as the samples are collected, the ratio of sample number to study duration could be increased. This can be performed by using a reference or marker compound that has the same recovery characteristics as your analyte of interest. This process occurs by placing the reference compound in the dialysis solution during sampling of the analyte of interest. The analyte of interest diffuses from the ECF into the probe

at the same time, rate, and extent as the reference compound diffuses out of the probe and into the ECF. The in vivo recovery of the reference compound is determined using the standard retrodialysis procedure and calculations [Eq. (1)]. The in vivo recovery value for the reference compound is then used to calculate the estimated tumor concentration using Eq. (2).

The point of zero net flux is a calibration method that determines relative recovery of a drug or analyte by varying the concentrations of the drug included in the perfusate solution [33–35]. This procedure is performed by perfusing 4–5 varying concentrations of the drug into the microdialysis probe and measuring the concentration in the outflow dialysate. Plotting the difference between the inflow perfusate drug concentration and the outflow dialysate drug concentration as a function of the perfusate drug concentration results in a line with a slope that is equal to the relative recovery and an x-intercept that is equal to the steady-state tissue ECF concentration of the drug.

2.3 Online/Real-Time Analysis

A potential clinical implementation and advantage of microdialysis methodology is the real-time determination of drug concentration in tissue and tumors, measures of pharmacologic effect, and physiologic function [19, 28, 29]. The ability to link the microdialysis sampling system directly to an analytical system allows for the measurement of pharmacokinetic and pharmacodynamic end points within minutes of obtaining the sample. Thus, medical and pharmacologic interventions can be performed and modifications can be made immediately.

Leggas and colleagues developed a rapid and simultaneous system that measured the inactive carboxylate and active lactone forms of topotecan using an online microdialysis system linked to a microbore high-performance liquid chromatography system [28]. This system allowed for the continuous injection of small amounts of samples, the direct measure of both forms of the drug without the loss of sensitivity, and the additional benefit of fast and automated analysis without the additional sample processing required for pharmacokinetic studies of camptothecin analogues [36, 37]. This system is very versatile and could be used for other camptothecin analogues and anticancer agents [38, 39]. The advantages of this system in pharmacokinetic studies of anticancer agents are the ability to measure the parent compound and metabolites within minutes without disrupting the fluid balance of the tissue, which is especially important in pharmacokinetic studies of drugs in the brain and CSF.

Microdialysis techniques were initially developed to monitor changes in neurotransmitter levels in the brain of preclinical models [40–43]. The use of microdialysis to monitor dynamic changes in glucose and lactate concentrations in the cortex of freely moving rats has accelerated the move to human studies and produced interesting methodological adaptations and results [42]. A schematic diagram of an experimental setup using dual online microdialysis assay in rats is depicted in Fig. 2. The reduction of oxygen levels in the cage led to an immediate rise in lactate and glucose concentrations in the brain. These experiments reported the first temporal relationship between glucose and lactate changes during moderate hypoxia in unanesthetized animals [42]. However, the inability to have analytical instruments required for the detection of specific neurotransmitters, such as lactate and glucose, at the bedside in clinical studies has complicated the need for real-time results. As the function of analytical instruments increase and their size decreases, the use of these systems in clinical practice will increase. Alternatively, delivering the microdialysis sample from the bedside to the analytical lab, as is done with other standard laboratory studies, can produce results in a relatively short period of time.

Tolias and colleagues used microdialysis to evaluate extracellular glutamate in the brains of children with severe head injuries [44]. A microdialysis probe was inserted next to an intracranial pressure bolt in the right frontal area of the brain. Dialysis samples were collected hourly and analyzed for

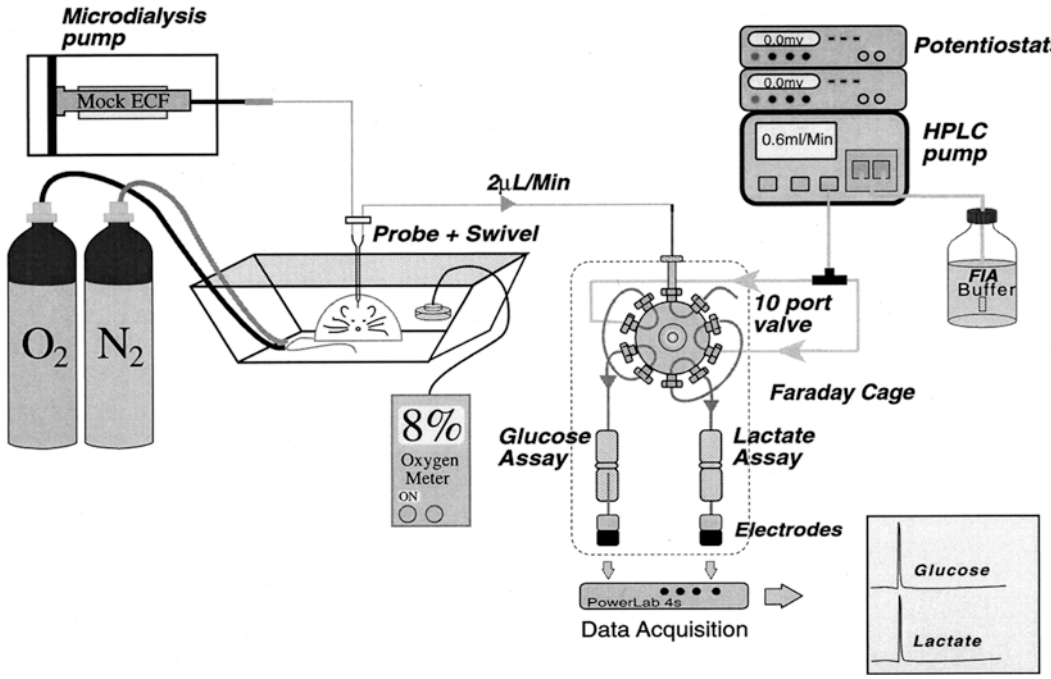

Fig. 2 Microdialysis probe in rat brain with online analytical system. Schematic diagram of the hypoxia experimental setup and dual online microdialysis assay. A ten-port injection valve is shown in the inject position (reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004)

glutamate, glutamine, and various structural amino acids. Clinical monitoring parameters were correlated with amino acid concentrations. A low glutamine to glutamate ratio was associated with increased morbidity. The authors concluded that glutamate metabolism may have a more significant role in the pathophysiology of pediatric head injury than had been recognized. As for the use of microdialysis to generate real-time results, the ability to obtain a sample over a relatively short period of time, send it to the clinical laboratory, and have the results sent back within hours may allow for modifications in the treatment of the patient.

3 Preclinical Microdialysis Studies in Tumor and Tissue

3.1 Preclinical Studies of Tumor and Tissue Distribution

Studies using microdialysis have for the most part involved accessible tissues located near the surface of the skin, with the exception of cerebral tissue. More recently, microdialysis in the pancreas has been performed [45]. In order to determine the feasibility of microdialysis studies in the pancreas, Kitano and colleagues used endoscopic ultrasound-guided fine-needle aspiration, followed by microdialysis probe placement into pancreatic parenchyma to measure 5-FU concentrations in beagle dogs as seen in Fig. 3. Probe placement was successful in six of eight fully anesthetized dogs with concentrations peaking following administration of 5-FU 20 mg/kg and gradually declining within 60 min. The microdialysis probes remained in place for 90 min that included a 10-min equilibration period

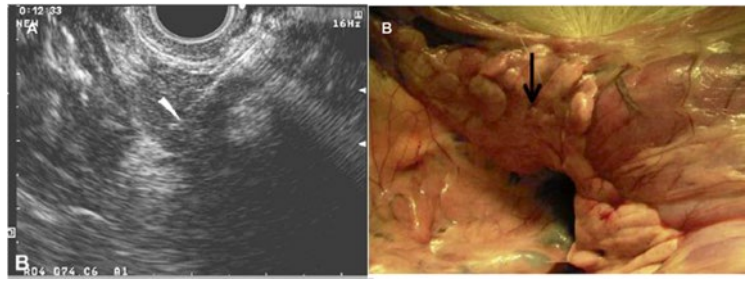

Fig. 3 (**a**) Placement of microdialysis probe in a beagle dog under EUS guidance. (**b**) The pancreas after removal of microdialysis probe (no bleeding was detected)

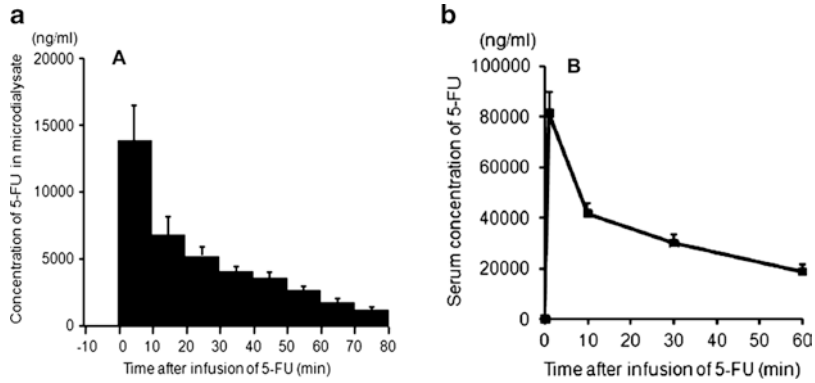

Fig. 4 (**a**) 5-FU concentrations in pancreas ECF. (**b**) 5-FU concentrations in plasma

and eight 10-min sampling times. Concurrent plasma sampling was taken at 1, 10, 30, and 60 min post 5-FU administration. Serum levels were roughly eight times higher than pancreas ECF levels seen in Fig. 4. The ability to measure drug concentrations in the pancreas and pancreatic tumors could greatly improve early phase drug development, giving researchers information on the distribution of the drug into the pancreas.

Studies comparing plasma and tumor ECF exposure associated with response in preclinical models have used microdialysis methodology [3, 14]. Investigators reported a sixfold difference in dose and plasma exposure of topotecan associated with a complete response in mice bearing human neuroblastoma xenografts NB1691 (2.0 mg/kg and 290 ng/mL·h, respectively) as compared to NB1643 (0.36 mg/kg and 52 ng/mL·h, respectively) [46]. However, factors related to the difference in topotecan response in the two neuroblastoma xenograft lines were not identified. Moreover, macro-tumor-related factors affecting sensitivity and the relationship between tumor ECF exposure to topotecan and antitumor activity in the xenograft model had not been established. As a result, the tumor ECF disposition of topotecan using microdialysis methodology was evaluated, and the relationship between topotecan tumor ECF exposure and antitumor response in mice bearing the relatively resistant (NB1691) and sensitive (NB1643) human neuroblastoma xenografts was evaluated [3].

The concentration versus time profiles of topotecan in plasma (top) and tumor ECF (bottom) in NB1643 (–) and NB1691 (---) human tumor xenografts are presented in Fig. 5. There was a 3.5-fold difference in tumor ECF exposure and penetration in NB1643 (25.6 ±19.6 ng/mL·h and 0.15 ±0.11, respectively) and NB1691 (7.3 ±6.1 ng/mL·h and 0.04 ±0.04, respectively) ($p<0.05$), which was

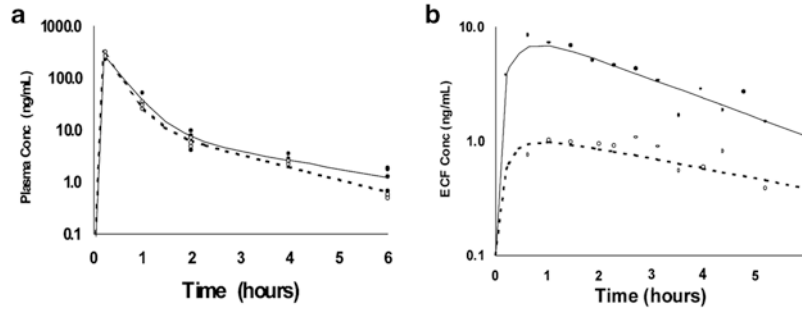

Fig. 5 Topotecan lactone concentration–time profiles in plasma and tumor ECF in resistant and sensitive neuroblastoma tumor xenografts. Representative topotecan plasma (**a**) and tumor ECF (**b**) concentration-time plots in mice bearing NB1691 and NB1643. Individual data points and best fit line of the data are represented for topotecan lactone plasma concentrations (**a**) in mice bearing NB1643 (*hyphen*, *filled circle*) and NB1691 (*continuous dashes*, *open circle*) human neuroblastoma tumor xenografts. Individual data points and best fit line of the data are represented for topotecan lactone tumor ECF concentrations (**b**) in mice bearing NB1643 (*hyphen*, *filled circle*) and NB1691 (*continuous dashes*, *open circle*) human neuroblastoma tumor xenografts. The topotecan lactone tumor extracellular fluid AUC_{0-5} for the representative NB1643 (*hyphen*, *filled circle*) and NB1691 (*continuous dashes*, *open circle*) tumor xenografts were 22.3 and 9.1 ng/mL h, respectively (reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004)

consistent with the difference in sensitivity of these xenografts based on dose and plasma exposure. These results suggest that topotecan tumor penetration may be one factor associated with neuroblastoma antitumor response. These data suggest inherent differences in tumor vascularity, capillary permeability, and/or tumor interstitial pressure between the sensitive and resistant neuroblastoma tumor xenografts. This was the first study reporting a relationship between the exposure of an anticancer agent in tumor ECF and antitumor response.

The significance of ECF as an important exposure for pharmacologic effect of anticancer agents and the inter- and intratumor variability was evaluated in preclinical studies of cisplatin using microdialysis [12]. The relationship between unbound platinum in tumor ECF, total platinum in tumor homogenates, and the formation of platinum–DNA (Pt–DNA) adducts was evaluated after administration of cisplatin in mice bearing B16 murine melanoma tumors. Intratumor variability in platinum disposition was evaluated by placing two probes (A and B) in the same tumor. At the end of the 2-h sample period, tumor tissue was obtained at each probe site and analyzed for total platinum, and bifunctional intrastrand DNA adducts between platinum and two adjacent guanines (Pt–GG), and platinum and adenine and guanine (Pt–AG).

The concentration of unbound platinum in tumor ECF of B16 tumors was detectable from 12 to 120 min after administration. In addition, the concentration versus time profile of unbound platinum in tumor ECF did not follow the plasma concentration versus time profile suggesting that clearance of drug from tumor may be the primary factor affecting drug accumulation within a tumor. The median (range, %CV) area under the concentration versus time curve for ECF (AUC_{ECF}) and tumor penetration were 0.42 μg/mL·h (0.05–1.57, 78 %) and 0.16 (0.02–0.62, 77 %), respectively.

The relationship between unbound-platinum AUC in tumor ECF from probe A and probe B is presented in Fig. 6. The median (range, %CV) AUC_{ECF} from probe A to probe B was 1.9 (1.3–5.5, 55 %). The median (range, %CV) concentration of total platinum obtained at the end of the 2-h microdialysis procedure from probe A to probe B was 1.1 (1.0–2.0, 27%). Using an E_{max} model to describe the relationship between drug exposure and platinum–DNA adduct formation, there was a better correlation between unbound-platinum AUC_{ECF} ($R^2 = 0.69$ and 0.63, respectively) and Pt–GG and Pt–AG compared to total platinum in tumor extracts ($R^2 = 0.29$ and 0.41, respectively). In addition, there was a poor correlation between unbound-platinum AUC_{ECF} and total platinum in tumor extracts ($R^2 = 0.26$).

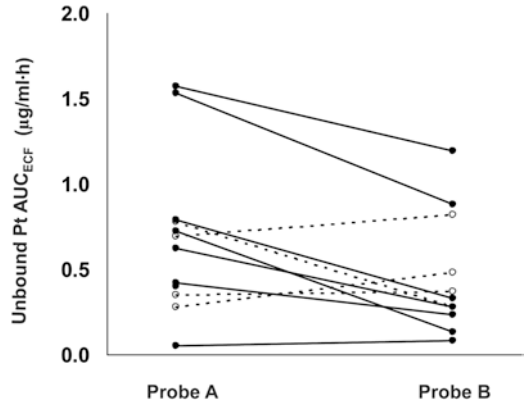

Fig. 6 Low intra- and high intertumoral disposition of cisplatin in murine melanoma tumors. Inter- and intratumoral variability in unbound-platinum AUC_{ECF} in mice bearing B16 tumors and in mice bearing H23 xenografts after administration of cisplatin at 10 mg/kg. In mice bearing B16 murine melanoma tumors, individual AUCs are represented by *closed circle*, and AUCs within the same tumor are connected by *single dash*. In mice bearing H23 human NSCLC xenografts, individual AUCs are represented by ○, and AUCs within the same tumor are connected by *continuous dashes* (reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004)

These results suggest there is relatively high intertumor (approximately a 30-fold range) and low intratumor (approximately a fourfold range) variability in unbound and total platinum in B16 murine melanoma tumors and a poor relationship between unbound and total platinum. In addition, these results suggest unbound platinum in tumor ECF is a better correlate of platinum–DNA adduct formation compared to total platinum measured in tumor extracts.

3.2 Evaluation of Angiogenesis Inhibitors

The angiogenic phenotype is associated with increased tumor neovascularization and hyperpermeability to drugs and other macromolecules [47–49]. Angiogenesis inhibitors could alter the increased tumor vascularization and permeability and have an untoward effect of decreasing tumor exposures of anticancer agents when coadministered with angiogenesis inhibitors. Thus, Ma and colleagues evaluated the tumor disposition of temozolomide administered alone and in combination with the angiogenesis inhibitor, TNP-470 [49]. Temozolomide was administered alone and in combination with TNP-470 to nude rats bearing tumors that differentially expressed low or high vascular endothelial growth factor (VEGF). In both the subcutaneous and intracerebral tumors with high VEGF expression, TNP-470 treatment produced significant reductions in temozolomide tumor exposure and ratio of tumor to plasma exposures. In conclusion, the pharmacodynamic effect of angiogenesis inhibitors on tumor angiogenesis can produce a reduction in tumor concentrations of coadministered anticancer agents. It is increasingly important to understand the pharmacokinetic impact of angiogenesis inhibitors when coadministered with anticancer agents, and additional studies need to be performed to determine the optimal dosing schedules for combination regimens.

3.3 Evaluation of Liposomal Anticancer Agents

The theoretical advantages of encapsulated liposomal drugs are prolonged duration of exposure and selective delivery of entrapped drug to the site of action [50–54]. Major advances in the use of

liposomes as vehicles delivering encapsulated pharmacologic agents and enzymes to sites of disease have occurred over the past 10 years. Moreover, liposomal-encapsulated drugs, such as liposomal doxorubicin (Doxil®), are FDA approved and have documented activity and decreased toxicity [55, 56]. Studies evaluating the disposition and tumor penetration of liposomal and nonliposomal anticancer agents suggest liposomal agents extravasate selectively into solid tumors through the capillaries of tumor neovasculature [54, 57, 58]. However, the mechanisms by which liposomes enter tissue and tumors and release drug are not completely understood. In addition, the liposomes can be engineered to produce a complete spectrum of drug release rates which need to be evaluated in in vivo systems.

SPI-77 (ALZA Pharmaceuticals, Inc.) is cisplatin encapsulated in long-circulating STEALTH® liposome. The disposition of liposomal cisplatin is dependent on the liposomal vehicle [50, 59, 60]. Once the cisplatin is released from the liposome, its disposition follows cisplatin pharmacology. SPI-77 has shown antitumor activity against a wide range of solid tumor xenografts, including murine colon tumors. In a study comparing SPI-77 and cisplatin tumor disposition in mice bearing murine colon tumors, the platinum exposure was several fold higher and prolonged after SPI-77 as compared to cisplatin administration [51]. However, because the platinum exposure was measured in tumor extracts, it is unclear whether the platinum measured was encapsulated, protein-bound platinum, or unbound platinum. In addition, it is unclear whether the platinum exposure was intracellular or extracellular. Thus, it is currently unclear whether SPI-77 releases cisplatin into the tumor ECF or penetrates into the cell as the liposome and then releases the cisplatin intracellularly.

Thus, the tumor disposition of platinum after administration of liposomal formulations of cisplatin (SPI-077) and nonliposomal cisplatin was evaluated using microdialysis in mice bearing B16 murine melanoma tumors [31]. Because of the pore cutoff size (20 kDa) of the semipermeable membrane of the microdialysis probe and the size of the liposome (100 nm), the microdialysis probe was only able to sample unbound platinum and allow the differentiation between liposomal-encapsulated cisplatin and cisplatin released into the tumor ECF.

After administration of cisplatin, the concentration of unbound platinum in tumor ECF was detectable from 12 to 120 min after administration. However, there was no detectable unbound platinum in the tumor ECF after administration of SPI-077 [61]. The results of this study suggest SPI-077 distributes into tumors but releases significantly less platinum into tumor ECF which results in lower formation of platinum–DNA adducts compared to cisplatin. This was the first study using microdialysis methodology to evaluate the tumor disposition of liposomally encapsulated anticancer agents.

The tumor distribution and pharmacokinetic properties of S-CKD602, a STEALTH liposomal formulation of CKD-602, a camptothecin analogue, was compared to CKD-602 in female SCID mice bearing A375 human melanoma xenografts and is shown in Fig. 7 [62]. Microdialysis was used to determine the released fraction of CKD-602 from S-CKD602 in the tumor ECF as compared with nonliposomal CKD-602. Mice were given S-CKD602 at 1 mg/kg of CKD-602 equivalent and CKD-602 at 30 mg/kg. Despite the 30-fold lower dose, S-CKD602 plasma AUC of released CKD-602 (36,905 ng/mL·h) was fourfold higher than nonliposomal CKD-602 (9,117 ng/mL·h). Eighty-two percent of S-CKD602 remained encapsulated out to 75 h following administration. Tumor ECF $AUC_{0–75h}$ of CKD-602 was lower (187 ng/mL·h) following S-CKD602 administration than following nonliposomal CKD-602 ($AUC_{0–\infty}$) administration (639 ng/mL·h), but more importantly, the duration of exposure above 1 ng/mL was 3.6-fold longer following S-CKD602. These results are consistent with antitumor response data of S-CKD602 compared with nonliposomal CKD-602 [3, 10].

3.4 PK Brain Studies in Nonhuman Primates

Nonhuman primates are used as the standard model for the determination of drug penetration into the CNS [10]. These models primarily evaluate the exposure of drug in the CSF of the lateral, fourth ventricle, and lumbar space after intravenous administration. This may provide important information

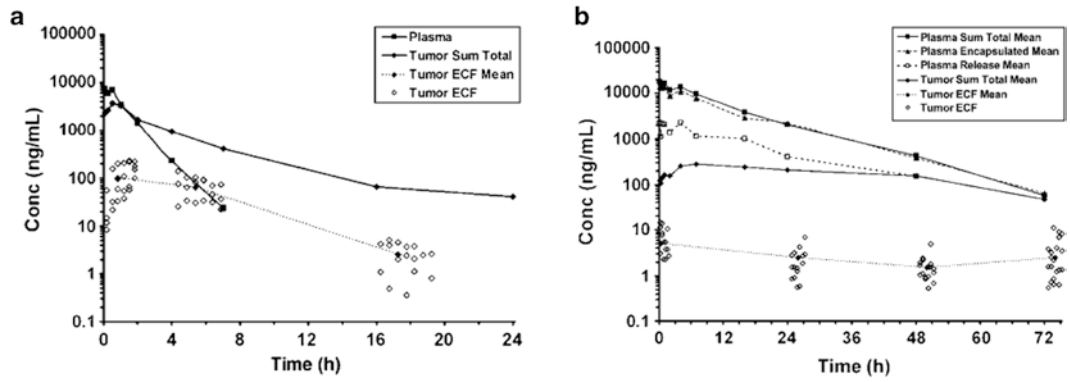

Fig. 7 Concentration versus time profile of CKD-602 in plasma, tumor, and tumor ECF after administration of nonliposomal CKD-602 (**a**) and S-CKD602 (**b**). The plasma and tumor sum total concentrations represent the mean of three mice at each time point. Microdialysis studies ($n = 3$–4 mice per interval) were obtained every 20 min from 0 to 2 h and every 30 min from 4 to 8 h and 20 to 24 h after administration of either CKD-602 (**a**) or S-CKD602 (**b**), ◇, mean tumor ECF concentration at each time point. - - - ◆ - - -, average tumor ECF concentration at each interval. The CV% for the plasma and tumor sum total concentrations at each time point for all samples was <25 %

for the evaluation of cytotoxic exposures in the treatment of embryonal CNS tumors, such as medulloblastoma, leukemia, and bacterial or viral infections that have a high propensity to disseminate throughout the subarachnoid space. However, these exposures may be irrelevant for primary brain tumors that occur in the cerebral cortex [10]. This difference in clinically relevant exposure for primary brain tumors and tumors that spread throughout the subarachnoid space is attributable to various components of the blood–brain barrier at each of these sites [10, 35]. Thus, there is impending need to evaluate the penetration and exposure of anticancer agents in the cerebral cortex.

Fox and colleagues evaluated the exposure of zidovudine in brain ECF as measured by microdialysis in rhesus monkeys [35]. In vivo recovery was tissue dependent and was lower in brain than in blood or muscle. After intravenous administration, the steady-state concentrations of zidovudine in blood, temporalis muscle, and brain were 112 ±64 μM, 105 ± 51 μM, and 14 ± 10 μM, respectively. The steady-state ultrafiltrate concentrations of zidovudine in serum and CSF were 81 ±40 μM and 14 ± 8 μM, respectively. The authors concluded that the CSF and brain ECF concentrations were comparable at steady state. Thus, zidovudine penetration in the brain ECF and CSF is limited to a similar extent, presumably by active transport, as in other species.

4 Clinical Microdialysis Studies

4.1 Clinical Microdialysis Studies in Tissue

In cancer treatment it is currently unclear if it is better to dose chemotherapeutic agents based on body surface area (i.e., mg/m^2), body weight (mg/kg), or fixed doses (i.e., mg). Several studies have shown that dosing anticancer agents based on body surface area does not reduce pharmacokinetic variability [63, 64]. Similarly, Hollenstein and colleagues investigated whether weight-adjusted ciprofloxacin dosing results in comparable concentrations of drug in tissue ECF in obese and lean subjects [65]. Microdialysis was used to sample ECF concentrations of ciprofloxacin in the anterior aspect of the right thigh in age- and sex-matched obese (122 ± 23 kg) and lean (59 ± 9 kg) subjects after an intravenous dose of ciprofloxacin. The tissue penetration was significantly lower in obese patients (0.45 ± 0.27)

as compared to lean patients (0.82 ± 0.36). The authors concluded that the penetration of drug into the ECF of muscle is impaired in obese patients. Therefore, antibiotic doses need not be adjusted for an increase in fat-to-water ratio, and weight-adjusted dosing based on actual body weight will yield adequate tissue levels of ciprofloxacin. Similar microdialysis studies evaluating the exposure of anticancer agents in tissue may help address the optimal method used to calculate doses of anticancer agents.

4.2 Clinical Microdialysis Studies in Tumors

Recently, microdialysis has been modified for use in human drug studies and has provided the opportunity to quantify drug concentrations in tissue and tumors [5–7, 13, 22–27]. Microdialysis has been used to evaluate the ECF disposition of anticancer agents in patients with accessible solid tumors [5–7, 13, 22–27]. The first study that demonstrated the utility of microdialysis in patients with solid tumors was performed by Blochl-Daum and colleagues. The disposition of carboplatin in blood and ECF of tumor and skin were performed in patients with cutaneous malignant melanoma metastases [6]. Microdialysis probes were placed in cutaneous tumors and surrounding skin. The results indicated a rapid but incomplete equilibration between blood and the tumor compartment. Similar results were reported with subcutaneous tissue. The mean ± SD AUC of total (sum of unbound and bound) carboplatin in serum, tumor, and subcutaneous tissue were 1,533 ± 189 μg/mL·min, 853 ± 172 μg/mL·min, and 506 ± 87 μg/mL·min, respectively. There was also significant interpatient variability blood, tumor, and subcutaneous tissue. However, there was greater interpatient variability in tumor and skin exposure as compared to blood. This data suggests that in addition to systemic factors that control blood exposures, there are tumor- and tissue-related factors which add to the variability in the exposure at these sites.

Muller and colleagues evaluated the relationship between 5-fluorouracil (5-FU) exposure in tumor ECF and clinical response in patients with primary breast cancer [5]. Microdialysis probes were placed into the primary tumor and periumbilical subcutaneous adipose layer in patients with breast cancer scheduled to receive neoadjuvant chemotherapy containing 5-FU. In addition, serial blood samples were obtained. The mean ± SD AUC of 5-FU in plasma, tumor, and subcutaneous tissue were 699 ± 75 μg/mL·min, 374 ± 62 μg/mL·min, and 401 ± 151 μg/mL·min, respectively. The pharmacokinetics of 5-FU were similar in tumor and adipose tissue. A high interstitial tumor exposure of 5-FU was associated with increased tumor response, and there was no association between 5-FU exposure in adipose tissue or plasma and tumor response. The authors concluded that the exposure of 5-FU in tumor ECF may predict response in patients with breast cancer. Moreover, this information could be used to optimize dosing and administration schedules to increase the exposure of anticancer agents in tumors and thus improve response.

In a recently published study, clinical tumor response and relapse was assessed to determine if tumor ECF exposure of epirubicin was more predictive than plasma concentrations in 12 patients with primary breast cancer [23]. The authors determined that although there were mean differences in tumor ECF AUC between response categories of complete response (3.7 mg/L·min ± 18; $n = 2$), partial response (4.8 mg/L·min ± 0.7; $n = 5$), and stable disease (3.8 mg/L·min ± 0.8; $n = 5$), the differences were not statistically significant. However, in patients with the highest tumor epirubicin concentrations, one had complete response and the other had partial response, while those with the lowest intratumoral concentrations showed no response, relapsed, and one died from the disease. The tumor penetration of epirubicin measured by the ratio of tumor ECF AUC and plasma AUC was 0.116. There was a lack of correlation between plasma and tumor concentrations that has been described in previous studies. Interestingly, despite similar mean concentrations obtained in tumor and subcutaneous tissue, individual C_{max} and AUC values showed no significant correlation between the two sites. This study as well as other studies published to date evaluating response and intratumoral

concentrations shows, for the most part, an apparent trend to higher tumor exposure in patients with better efficacy but suffer from the limitation of small sample size and lack of power. This warrants a large, adequately powered study to evaluate if the apparent trend to higher intratumoral concentrations in patients correlates with better response.

Muller and colleagues also evaluated the interstitial disposition of methotrexate in patients with primary breast cancer lesions [13]. Microdialysis probes were placed into the primary tumor and periumbilical subcutaneous adipose layer in patients with breast cancer receiving methotrexate as part of a three-drug regimen. The ratio of methotrexate AUC in tumor ECF to plasma was 0.60 ± 0.20. In addition, there was no correlation between methotrexate AUC in tumor ECF and plasma. Unlike the previous study, the exposure of methotrexate in tumor ECF was not associated with response. The lack of a relationship between methotrexate exposure in tumor ECF and response may be associated with variability in transendothelial transfer of methotrexate. This study depicts the importance of not only the disposition of drug in tumor ECF but also the intracellular exposure of anticancer agents as cytotoxic determinants of response.

In another study evaluating methotrexate concentrations in high-grade gliomas, tumor penetration as measured by the ratio of methotrexate AUC in tumor ECF to plasma was 0.28–0.31 in contrast enhancing tumor and 0.032–0.094 in nonenhancing tissue [26]. This study was one of the first to measure cerebral drug concentrations and the first to measure cerebral concentrations of an oncology agent. The large variation in tumor ECF AUC between nonenhancing and enhancing cerebral tissue demonstrates the complexities of predicting the pharmacokinetics of a drug in this compartment once they penetrate the blood–brain barrier. The utility of being able to measure intratumoral pharmacokinetics in drugs such as methotrexate that do not freely penetrate the blood–brain barrier would be extremely important in early phases of development in agents used to treat brain cancer. Agents not detected in tumor ECF at levels at or above the IC_{50} would not be recommended to progress into the next phase of drug development.

The feasibility of prolonged microdialysis sampling was demonstrated by Inge and colleagues in cancer patients being treated with carboplatin [24]. Following local application of lidocaine/prilocaine cream, the microdialysis probe was placed one day prior to carboplatin administration and maintained in place for up to 72 h without complication. The ratio of tumor ECF AUC to plasma was similar to the ratio adipose tissue AUC to plasma and ranged from 0.64 to 1.10 and 0.71 to 1.46, respectively. These results differed from the first study by Blochl-Daum and colleagues. A possible explanation for the unexpected similarity between tumor ECF AUC and plasma AUC was thought to be coadministration of dexamethasone, which can lower the interstitial fluid pressure of tumors. Seeing that a prolonged microdialysis sampling strategy is possible allows for agents with long $t_{1/2}$ and clearances such as carboplatin and oxaliplatin to be measured more accurately. This study also revealed some of the technical pitfalls of microdialysis as two of nine patients did not have evaluable pharmacokinetic data due to [1] leakage of the microdialysis catheter and [2] highly variable and decreasing recovery. The study also revealed that the recovery of microdialysates in the control tissue evaluated (subcutaneous adipose) was fluctuating which raised concerns over if this is the best tissue to use as a control.

5 Pharmacodynamic Studies Using Microdialysis

5.1 Antibiotics

The ability of the microdialysis probe to recover any analyte that is small enough to pass through the semipermeable membrane makes it a useful technique for pharmacodynamic studies [7, 66]. Microdialysis methodology has been used in clinical pharmacodynamic studies of anti-infective

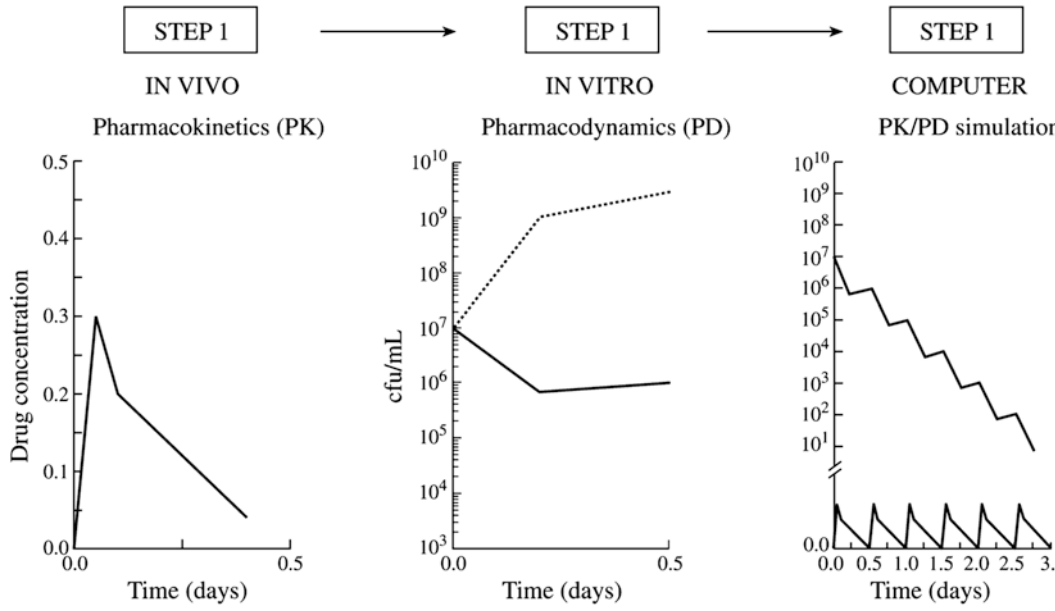

Fig. 8 Study design for in vivo pharmacokinetic and in vitro pharmacodynamic studies of anti-infective agents (reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004)

agents, diabetes, muscle physiology, and brain neurochemistry [7, 43, 66–72]. The specific advantage of microdialysis in the study of anti-infective agents is related to the ability of the probe to measure unbound, pharmacologically active drug in the ECF of tissue, which is the anatomically defined target site for most bacterial infections. In the study of anti-infective agents, microdialysis probes have been placed in subcutaneous tissues, brain, and lung [7, 66]. Microdialysis studies have demonstrated that the concentrations of anti-infective agents in the ECF of subcutaneous tissue may be subinhibitory, whereas the concentrations in the serum may be sufficient for antimicrobial effect. Thus, the use of tissue ECF or serum concentrations as an end point for determining the potential efficacy of anti-infective agents may have a significant impact on clinical decision making. Microdialysis also offers unique opportunities in pharmacokinetic and pharmacodynamic research and the potential to streamline the decision process on the drug development of anti-infective agents and also anticancer agents.

Delacher and colleagues evaluated a combined in vivo pharmacokinetic and in vitro pharmacodynamic approach to simulate the target site pharmacodynamics of antibiotics in humans [67]. This approach was based on the in vivo measurements of interstitial drug pharmacokinetics in tissue and a subsequent pharmacodynamic simulation of the drug concentration versus time profile in an in vitro setting. A schematic illustration of the general concept of the combined in vivo pharmacokinetic and in vitro pharmacodynamic approach is depicted in Fig. 8. Individual concentration versus time profiles of ciprofloxacin were measured in the interstitial space of patients following intravenous administration. Then different isolates of *Pseudomonas aeruginosa* were exposed in vitro to the interstitial ciprofloxacin concentration versus time profile obtained from the in vivo microdialysis experiments. Significant correlations were observed between pharmacokinetic and pharmacodynamic metrics. Moreover, the data were analyzed with an integrated pharmacokinetic–pharmacodynamic model, allowing for a much more detailed evaluation of the data than strictly using minimum inhibitory concentrations. The results of these experiments showed that therapeutic success and failure in anti-infective therapy may be explained by pharmacokinetic variability at the target site, and therefore, this in vivo pharmacokinetic and in vitro pharmacodynamic approach may provide valuable guidance for

drug and dose selection for anti-infective agents. The use of pharmacokinetic drug exposure in the CSF of nonhuman primates to guide in vitro cytotoxicity studies has been used in the development of topotecan for the treatment of medulloblastoma. These procedures and study designs could be combined, along with microdialysis studies of anticancer agents in tumors, as described earlier, to provide information on drug and dose selection of anticancer agents as was performed for the anti-infective agents.

5.2 Brain Neurochemistry

The use of microdialysis probes in neuromonitoring is a new therapeutic opportunity for microdialysis systems [43, 71, 72]. The major value of microdialysis monitoring in severe head injury has been to demonstrate different brain pathophysiologic mechanisms in the living brain and to depict the time course of these changes. Interruption of substrate delivery is a major factor of vulnerability to ischemic damage to the brain in patients with severe head injury, stroke, or subarachnoid hemorrhage. Thus, continuous monitoring of substrate levels in the brain is required to optimize therapy for critically ill patients with brain injuries. Zauner and colleagues evaluated the delivery of oxygen via residual blood as an approach to protecting the brain during ischemia [43]. Therapy was evaluated by continuously measuring brain oxygen, brain CO_2, brain pH, and hourly glucose and lactate concentrations via a microdialysis system. There was an increase in brain tissue oxygen tension and a simultaneous decline in brain lactate during a stepwise increase in inspired oxygen. Although these new monitoring systems and methods are labor intensive and expensive, they can be readily applied in neurosurgical centers.

Disturbed ionic and neurotransmitter homeostases are now recognized as the most important factor contributing to the development of secondary brain swelling after traumatic brain injury. Preclinical studies suggest that posttraumatic neuronal excitation by amino acids leads to an increase in extracellular potassium. Thus, Reinert and colleagues evaluated the relationship between extracellular potassium and high intracranial pressure after severe head injury [71]. An intracranial microdialysis procedure was used to monitor potassium, glutamate, and lactate concentrations in brain ECF. Dialysate potassium concentrations were increased for more than 3 h in approximately 20 % of patients. Moreover, a mean dialysate potassium >2 mM throughout the entire monitoring period was associated with an ICP > 30 mmHg and fatal outcome, as were progressively rising concentrations of potassium in brain ECF. These studies show that microdialysis monitoring of physiologic and pharmacologic targets can be used to predict response in patients.

The relationship between tissue oxygenation and excitatory amino acids in peritumoral edema has also been evaluated during glioma surgery [72]. Microdialysis was used to monitor glutamate and aspartate levels in peritumoral edema during resection of the tumor. Treatment with inspiratory oxygen led to an increase of tissue oxygenation and a decrease in glutamate and aspartate. Future microdialysis studies, such as the study conducted by Blakeley and colleagues [26], could evaluate the exposure of anticancer agents or pharmacologic markers of response in the ECF of a brain tumor after administration of a test dose of drug and prior to surgical resection. These studies could greatly enhance our knowledge of drug delivery and exposure in brain tumors.

The use of microdialysis to determine changes in expression of a substance in order to predict or correlate progression of a disease greatly enhances the utilization of this technique. Real-time analysis of biomarkers also provides the potential to give the clinician more timely information in critical situations, such as traumatic brain injury, to guide in clinical decision making and allowing the clinician to take preemptive rather than reactive steps. In a study with 14 patients following traumatic brain injury, patients were monitored for cerebral metabolites including lactate, pyruvate, glucose, glutamate, and glycerol using microdialysis for a mean of 95 h [73]. Absolute increase in lactate levels, lactate/pyruvate ratio, and elevation in glycerol were all significantly correlated with incidence of

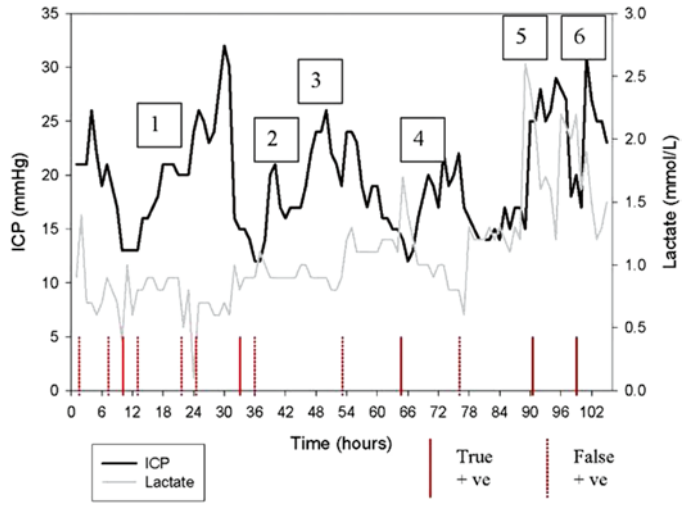

Fig. 9 Depiction of a patient's mean hourly intracranial pressure (ICP) (*upper line*) and hourly lactate levels (*lower line*) measured by microdialysis. This patient had six significant episodes of ICP elevations. The solid vertical lines along the *x*-axis represent the occurrence of a >20 % increase in baseline lactate levels predicting a true positive ICP elevation ($n=5$). The sensitivity is calculated by dividing the number of positive tests (5) by the number of ICP elevations (6) = 83 %. Note that the microdialysis probe remained in place for 102 h

intracranial hypertension, a common complication of severe traumatic brain injury occurring in approximately 50 % of patients seen in Fig. 9. The rise in lactate, lactate/pyruvate ratio, and glycerol levels occurred 2.8 ± 0.30 h, 2.7 ± 0.29 h, and 2.5 ± 0.35 h, respectively, before episodes of intracranial hypertension allowing for preemptive actions. The results of these trials could potentially help create a microdialysis algorithm to guide clinical decision making.

5.3 Transplant

The outcome of liver transplant has improved but there is still a need to develop a tool that would be able to detect complications such as hepatic artery thrombosis and rejection. Microdialysis has become a tool to help monitor for such complications leading from inflammation in both kidney and liver transplantation [74, 75]. Microdialysis probes with a larger cutoff (100 kDa) gives the ability to measure metabolic parameters such as lactate, glucose, pyruvate, glycerol, and inflammatory cytokines such as interleukin (IL)-6, IL-8, monocyte chemotactic protein-1, inducible protein (IP)-10, and complement activation (C5a).

Waelgaard and colleagues evaluated the effect of these metabolic parameters and cytokines following liver transplant in 20 patients. Two microdialysis catheters were inserted into both right and left lobes of the liver and also subcutaneous tissue for 7–10 days. The study showed that for the first time, a considerable increase in IL-8 and C5a occurred in patients with rejection. No changes in these two cytokines were observed for patients with an uneventful post-transplant course. The study also showed that an increase of IP-10 was an independent predictor of success post-transplant. The investigators concluded that the use of microdialysis with a 100 kDa cutoff microdialysis probe shows promise in detecting early diagnoses of complications in patients undergoing liver transplantation.

6 Summary and Future Directions

Microdialysis has been used in the study of neurochemistry, muscle physiology, lipid metabolism, edema, diabetes, traumatic brain injury, antibiotics, transplant, and anticancer agents [7, 22–27, 66, 67, 70–72]. The possible uses of microdialysis in the pharmacokinetic and pharmacodynamic studies of anticancer agents are virtually endless. The use of microdialysis in the development of anticancer agents is based on preclinical and clinical results suggesting that tissue and tumor exposure does not equal plasma exposure, inter- and intratumoral exposure is highly variable, and the exposure of anticancer agents in CSF and CNS lobes is not identical. Future studies should evaluate the correlation between tumor ECF drug exposure and response rather than systemic drug exposure. The advantages of microdialysis in the study of anticancer agents are sampling drug concentrations closer to the target site, as compared to plasma pharmacokinetic studies; obtaining serial samples from a single site within tissue and tumor; measurement of the active-unbound forms of drugs; differentiation between various forms and metabolites of anticancer agents; and simultaneously obtaining samples for pharmacokinetic and pharmacodynamic studies [3, 7, 66]. The disadvantages of microdialysis are that it is invasive and requires in vivo calibration and not all substances will cross the semipermeable membrane. However, as compared to other sampling instruments and methods, these disadvantages are relatively minor.

The use of microdialysis in cancer-related studies will become more important as surrogate markers for response and toxicity are determined and new therapeutic agents are developed. The use of microdialysis can be especially important when evaluating the disposition of carrier-mediated agents (e.g., liposomes and PEG conjugates), gene therapy, and antisense oligonucleotides, and angiogenesis inhibitors [4, 5, 9, 31]. The disposition of carrier agents may be completely different than the parent compound, and the release of drug systemically and in tumor will be important in determining antitumor effect and toxicity. The importance of microdialysis to address these issues is highlighted by the FDA's plan to inquire about methods to define and evaluate carrier systems. The pharmacokinetics and pharmacodynamics of gene therapy agents and antisense oligonucleotides may be drastically different than classical anticancer agents. In addition, owing to analytical assays and detection issues, standard sampling strategies and processing may not be adequate for these agents. Thus, the microdialysis methodology allows for rapid and accurate sampling and separation and may become pivotal in the development of these agents. As the technology of microdialysis advances, the probe will be placed in more logistically difficult organs and tissues. This was seen when investigators evaluated pancreas ECF using a microdialysis probe shaped like a thread. Continuous monitoring of brain, liver, and kidney has been successful using microdialysis evaluating several surrogate biomarkers that can predict response [23, 73, 75]. Microdialysis probes can also be placed in blood vessels and obtain serial samples of unbound drug. These studies can be used to evaluate protein binding and reduce the need for repeated blood sampling and processing. Moreover, the ability to connect the microdialysis sampling instruments to online analytical equipment instruments allows for real-time analysis and may allow for the manipulation and modification of dosing regimens and strategies of anticancer agents.

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Regional Drug Delivery for Inoperable Pulmonary Malignancies

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Abstract Unresectable primary lung cancers and inoperable pulmonary metastases contribute significantly to cancer mortality throughout the world. Isolated lung perfusion and inhalation techniques are potential strategies to enhance delivery of chemotherapeutic as well as biologic agents to the lungs while minimizing systemic toxicities. This chapter reviews current efforts pertaining to regional drug delivery for inoperable pulmonary malignancies.

Keywords Pulmonary metastases • Isolated lung perfusion • Inhalational drug delivery

1 Introduction

Primary and metastatic tumors involving the lungs cause considerable morbidity and mortality in cancer patients. In 2010, approximately 222,500 Americans developed lung cancer [1]. Many of these individuals presented with tumors that were confined to the chest, yet unresectable due to anatomic or physiologic limitations. Currently, median survival of patients with limited-stage small-cell or stage IIIA/B non-small-cell lung cancers treated with chemotherapy and/or radiation approximates 14 months [2, 3]. Whereas most of these individuals die from extrathoracic metastatic disease, a significant number develop life-threatening complications due to uncontrolled growth of their primary tumors. Recalcitrant local disease following definitive induction therapy often precedes the development of systemic metastases in lung cancer patients.

Nearly one third of all patients dying from malignancies of non-thoracic origin suffer from pulmonary metastases [4]. Many patients, particularly those with sarcomas, succumb to uncontrolled pulmonary metastases in the absence of other systemic disease; treatment of these individuals remains controversial. Pulmonary metastasectomy may be beneficial in selected patients. Analysis of more than 5,000 cases entered onto the International Registry of Pulmonary Metastases indicated that survival following pulmonary metastasectomy is contingent on the histology, disease-free interval following resection of the primary malignancy, number of pulmonary nodules, and completeness of resection [5]. Overall, patients with metastatic melanomas do poorly despite complete resections (5-year survival <25 %); in contrast, individuals with germ cell cancers fare much better following pulmonary metastasectomy,

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with 5-year survivals of approximately 60 %. Patients with metastases from epithelial cancers have intermediate survivals. These findings have been confirmed and extended by numerous single-institution studies demonstrating potential efficacy of pulmonary metastasectomy for a variety of tumor histologies in properly selected patients [6–9]. Whereas these data indicate that some individuals with pulmonary metastases can be salvaged by resection alone, the majority of patients either present with or eventually develop multiple metastases that are inoperable. Recurrent disease following complete pulmonary metastasectomy is often attributable to outgrowth of chemo-resistant micrometastases present at the time of initial diagnosis.

Although efficacious for eradication of pulmonary metastases related to lymphoid or germ cell tumors, systemic chemotherapy has not proven to be uniformly beneficial for the treatment of pulmonary metastases secondary to epithelial or sarcomatous malignancies [9–11]. Frequently, systemic toxicities limit optimal dosing of chemotherapeutic agents in patients with these tumors. Conceivably, administration of cytotoxic agents by regional techniques may reduce tumor burden within the lungs while minimizing systemic toxicities in patients with pulmonary metastases. This chapter reviews recent experience pertaining to regional therapy of inoperable pulmonary malignancies.

2 Anatomy of the Pulmonary System

The high frequency of primary and metastatic cancers involving the lungs is attributable to the large surface area of respiratory epithelia at risk for malignant degeneration following carcinogen exposure and the extensive capillary system that entraps circulating cancer cells within the pulmonary interstitium. The lungs are perfused by two circulatory systems [12]. The pulmonary artery (PA) normally delivers all of the output from the right ventricle; although deoxygenated, blood within the PA is sufficient to maintain viability of normal lung parenchyma. The bronchial arterial circulation, emanating from several branches of the descending aorta, provides additional nutrient support to the airway mucosa [13]. Primary lung cancers, as well as metastatic lesions, frequently derive significant, and at times preferential, nutrient support from the bronchial circulation [14–16].

Inhalation- or perfusion-related pulmonary injuries are manifested by desquamation of airway epithelia, alveolar protein accumulation, and edema with or without fibrosis within the interstitial space [17–20]. Depending on the severity of the insult, life-threatening, irreversible interstitial fibrosis may ensue, manifested either as acute respiratory failure or more insidious, restrictive lung disease [21]. The fragility of the pulmonary interstitium, and its limited potential for recovery following severe insults, must be considered when contemplating regional delivery of cytotoxic agents for the management of inoperable pulmonary malignancies.

3 Isolated Lung Perfusion and Other Regional Delivery Techniques

3.1 Nitrogen Mustard and Melphalan Preclinical Studies

Administration of cytotoxic agents by selective lung perfusion was first reported shortly after techniques for cardiopulmonary bypass were established. In 1960, Pierpont and Blades [22] utilized a closed extracorporeal circuit to administer nitrogen mustard to dogs via antegrade [PA, inflow; pulmonary vein (PV), outflow] isolated lung perfusion techniques. Ten of 23 dogs receiving 0.4 mg/kg of nitrogen mustard via 15-min isolated lung perfusion (ILuP) survived the procedure. No washout was used following the perfusion, and three of these ten animals exhibited neutropenia. Histologic changes consistent with acute pneumonitis were evident in the perfused lungs. No dogs survived

Table 1 Concentration of melphalan in perfusate and lung tissue

Drug	Animals	Route	Dose or initial conc.	Lung (μg/g)	References
Melphalan	Rat	ILuP	2 mg	62.2 (34.3)	[25]
		ILuP	1 mg	3.3 (0.09)	
		IV	1 mg	6.9 (1.9)	
Melphalan	Rat	ILuP	0.5 mg	40.9 (3.8)	[30]
		ILuP	1 mg	50.5 (2.6)	
		IV	0.5 mg	0.8 (0.5)	
		IV	1 mg	1.7 (0.2)	

Numbered in parenthesis are standard deviations. *ILuP* isolated lung perfusion, *IV* intravenous infusion

perfusion at higher doses. Subsequently, Jacobs et al. [23] administered escalating doses of nitrogen mustard to dogs via ILuP techniques. In contrast to what was observed by Pierpont and Blades [22], Jacobs et al. [23] noted that doses of nitrogen mustard up to 1.6 mg/kg were tolerated when this agent was administered by 30-min perfusion at flow rates that maintained normal physiologic pressures within the pulmonary arterial system. Creech et al. [24] described techniques for simultaneous bilateral lung perfusion in animals and reported the results of bilateral ILuP in one lung cancer patient as part of a large study involving regional perfusion of a variety of malignancies involving the limbs, pelvis, abdominal viscera, and lungs.

Although additional studies of ILuP with nitrogen mustard were not pursued, a number of preclinical studies have been performed to determine the potential efficacy of the mustard derivative melphalan, for regional treatment of pulmonary metastases. Nawata et al. [25] evaluated the pharmacokinetics and antitumor activity of melphalan administered by ILuP techniques in a rodent sarcoma model. Rats received MCA-induced sarcoma cells via intrajugular vein injection and 7 days later were randomized to receive 1 or 2 mg of melphalan intravenously, 2 mg of melphalan via ILuP (approximately 7–8 mg/kg) administered over 20 min at a rate of 0.5 mL/min, or buffered hetastarch. Seven days following treatment, cohorts of animals were euthanized and pulmonary nodules enumerated. Melphalan concentrations in pulmonary tissues following ILuP were considerably higher than those observed following systemic administration of melphalan (62.2 ± 34.3 μg/g vs. 6.9 ± 1.9 μg/g or 3.3 ± 0.9, respectively) (Table 1). A tenfold reduction in the number of pulmonary nodules was observed in melphalan-perfused lungs relative to lungs from animals receiving intravenous melphalan or hetastarch perfusions. Sixty-seven percent of animals receiving melphalan lung perfusions tolerated contralateral pneumonectomy, compared to 80 % of animals receiving perfusions with hetastarch. No animals survived intravenous administration of melphalan.

Hendriks et al. [26] evaluated the efficacy of melphalan administered by ILuP in a rodent model of adenocarcinoma pulmonary metastases. Median survival of rats receiving unilateral melphalan (2 mg) lung perfusions was 81 ± 12 days compared to untreated animals with bilateral pulmonary metastases (18 ± 1 days) or unilateral metastases (28 ± 3 days) or animals treated with 0.5 mg of melphalan intravenously (37 ± 6 days).

Ueda et al. [27] evaluated long-term pulmonary toxicity of melphalan in a rodent perfusion model. Rats underwent 20-min ILuP with 1 mg of melphalan and were randomly euthanized at monthly intervals for 6 months. In melphalan-treated lungs, perivascular as well as peribronchial edema with septal thickening and interstitial inflammation were observed 30 days following ILuP; all of these changes resolved within 60 days of the perfusion. Transmission electron microscopy revealed minimal proliferation of type II pneumocytes in the perfused lung. Collectively, these experiments suggested that at a dose, which mediates antitumor effects by ILuP, melphalan induces no long-term histologic sequelae in the rodent lung.

Melphalan is often administered in conjunction with tumor necrosis factor-α (TNF-α) during isolated limb perfusion [28, 29]. Hendriks et al. [30] evaluated the effects of melphalan and TNF-α administered by ILuP in a rodent model of pulmonary metastases secondary to colorectal carcinoma. Rats were injected intrajugularly with adenocarcinoma cells and 7 days thereafter were randomized

Table 2 Isolated lung perfusion trials

Author	No. of patients	Agent	Dose	Technique	Duration	Mortality	Response
Hendriks et al. [31]	16	Melphalan	15–60 mg	Closed-circuit antegrade perfusion	30 min	0	N/A
Grootenboers et al. [32]	7	Melphalan	15, 45 mg	Closed-circuit antegrade perfusion	30 min	0	N/A
Johnston et al. [42]	6	Doxorubicin	1–10 mg	Closed-circuit, antegrade perfusion	50 min	0	N/A
	2	Cisplatin	14–20 mg	Closed-circuit, antegrade perfusion	60 min	1	N/A
Burt et al. [43]	8	Doxorubicin	40 mg/m^2	Closed-circuit, antegrade perfusion	20 min	0	N/A
			80 mg/m^2				
Putnam et al. [44]	12	Doxorubicin	60 mg/m^2	Single-pass, antegrade perfusion	20 min	1	1MR
			75 mg/m^2				Four stable disease
Ratto et al. [50]	6	Cisplatin	200 mg/m^2	Closed-circuit, antegrade perfusion	60 min	0	N/A
Schröder et al. [51]	4	Cisplatin	70 mg/m^2	Closed-circuit, hyperthermic, antegrade perfusion	20–30 min	0	N/A
Muller [52]	22	Cisplatin	30 mg/m^2	Torso perfusion	20 min×2	0	1 CR, 12 PR
		Mitomycin	10 mg/m^2				
		Navelbine	25 mg/m^2				
Pass et al. [71]	15	TNF-α	0.3–0.6 mg	Closed-circuit, hyperthermic antegrade perfusion	90 min	0	0
		IFN-α	0.2 mg				
Schrump et al. (unpublished)	8	Paclitaxel	100, 200, and 125 mg	Closed-circuit, hyperthermic retrograde perfusion	90 min	0	Four stable disease

to undergo sham thoracotomy or 25-min ILuP with either saline, melphalan, TNF, or melphalan/TNF. Additional animals received melphalan intravenously; tumor nodules were enumerated 7 days later. In additional studies, animals underwent contralateral pneumonectomy on day 21 and were euthanized 5 days later. Consistent with data reported by Nawata et al. [25], Hendriks and colleagues [30] observed a tenfold reduction in pulmonary metastases in animals receiving melphalan lung perfusions compared to control animals. Pulmonary tissue levels were 30–40-fold higher in perfused animals relative to those receiving comparable melphalan dose intravenously (Table 1). The cytotoxic effects observed following ILuP with 1 mg of melphalan were comparable to those seen after ILuP with melphalan at 2 mg. TNF-α had no appreciable antitumor effects when administered alone and did not appear to potentiate melphalan in this setting. Eighty percent of animals receiving melphalan (2.0 mg)/TNF (200 μg) lung perfusions tolerated contralateral pneumonectomy.

3.1.1 Clinical Trials

In a phase I trial, Hendriks et al. [31] performed 30-min ILuP with melphalan, administered at escalating doses (15, 30, 45, 60 mg) in 300 mL circuit volume using an extracorporeal circuit under normothermic or hyperthermic (42°) conditions (Table 2). Sixteen patients (seven colorectal, five renal, three

Table 3 Melphalan, doxorubicin, and cisplatin concentrations in perfusate, lung, and tumor tissue in patients

Drug	Dose or initial conc.	Perfusate (μg/mL)	N	Lung (μg/g of tissue)	Tumor (μg/g of tissue)	Reference
Melphalan	15 mg	23	12	4.6 (2.7)	5.3 (3.1)	[32]
	30 mg	83	6	3.3 (2.5)	3.6 (3.3)	
	45 mg	94	8	6.1 (2.3)	5.3 (2.6)	
	60 mg	122	3	13.4 (11.5)	8.7	
Doxorubicin	1 μg/mL	0.56[a]	1	0.72	0.64	[42]
	1 μg/mL	0.56[a]	1	0.79	0.25	
	1.5 μg/mL	0.98[a]	1	2.58	0.62	
	3 μg/mL	1.46[a]	1	1.58	2.19	
	5 μg/mL	2.76[a]	1	2.13	1.56	
	10 μg/mL	3.08[a]	1	2.81	2.81	
	40 mg/m^2	4.52[b]	1	0.58	NA	[43]
	40 mg/m^2	3.76[b]	1	4.64	5.03	
	40 mg/m^2	8.48[b]	1	10.1	6.62	
	40 mg/m^2	12.9[b]	1	18.6	14.5	
	80 mg/m^2	27.95	1	57.3	33.5	
Cisplatin	14 μg/mL		1	0.69	1.42	[42]
	20 μg/mL		1	0.68	1.09	
	70 mg/m^2	>100[a]	4	≤98.30		[51]
	200 mg/m^2	>250[c]	6	75	68	[50]

[a]Perfusate peak concentration during the perfusion
[b]Mean perfusate concentration
[c]Calculated from data provided in manuscript

sarcoma, one salivary gland) underwent 21 ILuP procedures followed by complete metastasectomy. Operative mortality was zero; no major systemic toxicities were observed. Grade 3 pulmonary toxicity (pneumonitis) was observed in two patients undergoing normothermic ILuP with 60 mg melphalan. In an extension trial [32], eight additional ILuP procedures were performed in seven patients using 15 and 45 mg melphalan under hyperthermic conditions. Overall, pharmacokinetics of melphalan were linear with dose during ILuP [32]. Considerable interindividual variability was observed, possibly due to different pulmonary blood volumes that added to the initial volume in the circuit. Peak concentrations and area under the concentration curve (AUC) in perfusates at the maximal tolerated dose (MTD) were 108.6 and 53.4 μg/mL, respectively. Normal lung tissue melphalan levels ranged from 1.1 to 26.6 μg/g; tumor tissue concentrations ranged from 0.8 to 11.5 μg/g depending on dose. At the MTD of 45 mg, C_{max} and AUC of perfusates were 101 ± 41 and 53 ± 15 μg/mL, respectively, compared to 0.46 ± 0.37 μg/mL and 6.6 ± 4.7 μg/mL, respectively, in systemic circulations. Normal lung and tumor melphalan concentrations were 6.1 ± 2.2 μg/mL and 5.3 ± 2.6 μg/mL, respectively (Table 3). In this extension trial, three of eight ILuP procedures performed with 45 mg melphalan under hyperthermic conditions were complicated by empyema, postoperative bleeding, or rhabdomyolysis [33]. In total, 29 procedures were performed in 23 patients. After a median follow-up with 62 months, 6 of 23 patients (26 %) were alive and free of disease. Sixteen patients developed recurrent disease, 11 of whom died; 5 of these 16 patients exhibited only extrathoracic disease recurrence. The 5-year overall survival rate was approximately 55 %; median survival time was 84 months. No significant survival differences were noted for patients with carcinomas versus those with sarcomatous lung metastases [34].

Table 4 Doxorubicin concentration in perfusate and lung tissue

Animals	Route	Dose or initial conc.	Perfusate (μg/mL)	Lung (μg/g)	Tumor (μg/g)	Extraction (%)	Reference
Dog	ILuP		0.27–64.1	1.6–65			[35]
Dog	ILuP	1.95 μg/mL		3.9			[36]
	ILuP	2.95 μg/mL		8.8 (1.2)			
	ILuP	4.39 μg/mL		16.9 (1.7)			
	ILuP	5.79 μg/mL		19.2 (0.8)			
	ILuP	7.61 μg/mL		20.6 (4.5)			
Rat	ILuP	80 μg/mL	72.1 (6.9)	107.8 (30.2)		38.3 (13.2)	[37]
	ILuP	160 μg/mL	118.4 (12.1)	172.2 (64.4)		38.9 (15.2)	
	ILuP	320 μg/mL	255.2 (12.8)	498.1 (180.6)		58.3 (13.1)	
	ILuP	480 μg/mL	384.1 (46.2)	418.5 (69)		57 (9.8)	
	ILUP	640 μg/mL	457.6 (32.5)	663.8 (350.2)		41.4 (9.3)	
	IV	5 mg/kg		19.9 (4.4)			
	IV	5 mg/kg		25.5 (1.5)			
Rat	ILuP	80 μg/mL		170.5		5.5	[38]
	ILuP	320 μg/mL		46.2		4.3	
Rat	ILuP	100 μg[a]		13.8 (4.3)		3.9 (2.5)	[41]
		400 μg[a]		58.5 (20.1)		36.9 (10.4)	
		100 μg[b]		2.0 (0.7)		0.8 (0.5)	
		400 μg[b]		5.2 (3.7)		3.2 (3.5)	
Pig	ILuP	50 mg/m^2		21.9			[40]
	IV	50 mg/m^2		3 (0.8)			

Numbers in parentheses are standard deviations. *ILuP* isolated lung infusion, *IV* intravenous perfusion
[a]Free doxorubicin
[b]Liporubicin

3.2 Doxorubicin Preclinical Studies

A series of animal experiments have been conducted to examine the toxicity and potential efficacy of doxorubicin administered by ILuP techniques. Minchin et al. [35] examined the pharmacokinetics of doxorubicin administered by 50-min ILuP in dogs using a closed, oxygenated, extracorporeal circuit; an in-line heat exchanger maintained a physiologic temperature of the perfusate that contained 1–80 mg of doxorubicin in 1 L of whole blood. Uptake of doxorubicin in the canine lung appeared uniform, time-dependent, and saturable, suggestive of either facilitated transport or tissue-binding mechanisms. Maximal tissue to perfusate blood ratios were 10–15 at low doses of doxorubicin; however, with higher perfusate doses, doxorubicin tissue to blood ratios were <2 (Table 4). Doxorubicin was undetectable in the systemic circulation; a systemic to pulmonary circulation leak attributable to bronchial arterial blood flow approximated 10 mL/min.

Baciewicz et al. studied the pharmacokinetics of ILuP with doxorubicin in dogs [36]. Perfusate concentrations ranged from 1.95 to 7.61 μg/mL, and lung concentrations of doxorubicin ranged from 3.9 to 20.6 μg/g (Table 4). A plateau of doxorubicin concentration in lung tissue appeared to be reached at a perfusate concentration of 5.79 μg/mL, suggestive of either saturation of transporters or direct toxicity impeding further uptake.

Weksler et al. [37] evaluated doxorubicin pharmacokinetics of ILuP and intravenous injection in rats. Lung doxorubicin concentrations after ILuP were 107.8–663.8 μg/mL and were significantly higher than those after intravenous doxorubicin (19.9–25.5 μg/mL) (Table 4). Lung doxorubicin concentrations reached a plateau at a perfusate concentration of 255.2 ± 12 μg/mL of doxorubicin. Extraction ratio (the percent of doxorubicin extracted by the lung from the perfusate) appeared to be

related to perfusate concentration, ranging from 38 to 58 %. The optimal perfusate and other pharmacokinetic factors for ILuP using doxorubicin were also investigated in rats [38]. The mean lung concentrations of doxorubicin were <100 and <300 μg/g, respectively, for perfusate concentrations of 80 and 320 μg/mL. Extraction ratios were 5.5 and 4.3, respectively, which were lower than those previously reported by these investigators [37]. The latter study suggested that perfusate concentration and duration of perfusion—but not dose per kilogram or per square meter of body surface area, total infused dose, or the rate of infusion—were the primary factors determining final lung concentrations of doxorubicin [38]. These investigators [39] also observed that rats undergoing single-pass ILuP with 1.6 mg of doxorubicin (320 μg/mL) over 10 min tolerated contralateral pneumonectomy 21 days later. In additional experiments, rats were injected intravenously with MCA-induced sarcoma cells 7 days prior to ILuP with either doxorubicin as described above or normal saline. Three weeks following ILuP, extensive tumor metastases were present bilaterally in all animals undergoing saline lung perfusions and in non-perfused lungs of rats receiving doxorubicin ILuP; no tumor metastases were identified in lungs perfused with doxorubicin; histopathologic analysis revealed moderate interstitial fibrosis in doxorubicin-perfused lungs.

Furrer et al. [40] evaluated the pharmacokinetics and immediate toxicities of doxorubicin (50 mg/m^2) administered either by 15-min single-pass or normothermic recirculating blood perfusion using similar flow rates (~100 mL/min), as well as intravenous systemic infusion in a porcine model. Doxorubicin lung tissue concentrations following single pass were comparable to those observed after recirculating blood perfusion (~18 μg/g vs. 22 μg/g, respectively); in contrast, pulmonary doxorubicin levels were only 3.0 μg/g of tissue following intravenous drug administration. Wet to dry ratios were significantly lower following single pass relative to recirculating blood perfusions, suggesting that doxorubicin administered by single pass induced less acute perfusion-related edema than the same dose of doxorubicin delivered by recirculating blood perfusion techniques.

Yan et al. [41] evaluated distribution of free and liposomal doxorubicin (Liporubicin) administered by ILuP techniques in a rodent pulmonary metastasis model. Briefly, sarcomas were generated in rat lungs, following which either free or liposomal doxorubicin was administered by normothermic, single-pass, antegrade ILuP over 20 min. Heterogeneous drug distribution was observed in the perfused lungs. Liposomal doxorubicin levels in normal lung and tumor tissues were approximately 6-fold and 11-fold lower than free doxorubicin levels following ILuP with 100 μg and 400 μg of doxorubicin, respectively. Furthermore, tumor Liporubicin levels were lower than tumor doxorubicin levels.

3.2.1 Clinical Trials

Several phase I studies have been performed to examine the toxicities and clinical efficacy of doxorubicin lung perfusions in patients with unresectable pulmonary malignancies (Table 2). Johnston et al. [42] treated six individuals with escalating doses of doxorubicin (1–10 μg/mL of perfusate in a closed extracorporeal circuit) via 45–50-min, normothermic ILuP. Three patients underwent unilateral lung perfusions, and three individuals had bilateral simultaneous lung perfusions. Flow rates were adjusted to maintain physiologic pulmonary arterial pressures. Following ILuP, residual perfusate was flushed from the lungs with either blood or low-molecular-weight dextran. Isolated lung perfusion circuits provided excellent separation of pulmonary and systemic circulations even under bilateral simultaneous perfusion conditions. Maximum doxorubicin levels in normal lung equaled or exceeded those observed in tumor tissues following lung perfusion (Table 3) [35, 42]. In two individuals, doxorubicin was detected in mediastinal lymph nodes following lung perfusion, indicating transport of drug through the pulmonary interstitium to the regional lymphatics. One patient developed pneumonia that was fatal. No objective responses were noted in this pilot study in which MTD was not determined.

Burt et al. [43] utilized a closed, oxygenated, extracorporeal circuit to administer doxorubicin via ILuP to eight patients with unresectable sarcomatous metastases. Seven patients were treated at a dose

of 40 mg/m^2, and one patient received 80 mg/m^2 doxorubicin via 20-min perfusion (300–500 mL/min) at ambient temperatures (Tables 2 and 3). Following ILuP, doxorubicin was flushed from the lungs with Hespan. Approximately 14 % of the total dose of doxorubicin in perfusates was extracted by the lungs. Consistent with what was reported by Minchin et al. [35], uptake of doxorubicin in tumors tended to be less than that observed in normal lung tissues (average drug concentrations following ILuP with 40 mg/m^2 of doxorubicin were 11.1 μg/g of tissue for normal lung and 8.7 μg/g of tissue for tumor nodules). A modified toxicity grading system was implemented by these investigators to assess pulmonary toxicity related specifically to drug exposure rather than the thoracotomy procedure itself. Six of the eight perfused patients experienced grade II pulmonary toxicity, defined as >20 % diminution in diffusion capacity for carbon monoxide (DLCO), or dyspnea at rest or with exertion. The single patient receiving 80 mg/m^2 of doxorubicin exhibited complete destruction of the perfused lung resulting in empyema and suppurative pericarditis requiring surgical intervention. Although none of the seven individuals perfused at a dose of 40 mg/m^2 experienced clinically significant pulmonary symptoms, postoperative pulmonary function tests revealed diminished forced expiratory volume in 1 s (FEV_1) as well as DLCO values, indicative of subacute interstitial toxicity.

Putnam et al. [44] treated 12 sarcoma patients with doxorubicin administered via single-pass isolated lung perfusion. Eight patients received 200 mg/mL (approximately 60 mg/m^2) and four patients received 250 mg/mL (approximately 75 mg/m^2) doxorubicin in 1 L of crystalloid solution administered over 20 min. One patient experienced a major response, and four individuals exhibited stabilization of disease. Acute, pulmonary toxicity (interstitial pneumonitis) occurred in two individuals, both of whom were in the high-dose cohort; pneumonitis was fatal in one of these patients. Late pulmonary toxicity evidenced by diminution of ventilation and perfusion was observed in several patients in this study. Although extensive pharmacokinetic data were not published, doxorubicin levels in normal tissues exceeded those in tumor nodules (median 592 μg/g [range 74–2,750] vs. 153 μg/g [range 12–1,294], respectively). These observations, which were consistent with those reported by Minchin et al. [35] and Burt et al. [43], may have accounted for the short- and long-term pulmonary toxicities observed following doxorubicin perfusions in this study.

In a phase I study, Otterson et al. [45] treated 53 patients with inoperable pulmonary malignancies (23 sarcoma, 16 lung cancer, 12 miscellaneous) with escalating doses (0.4–9.4 mg/m^2) of doxorubicin administered via inhalation techniques. Doxorubicin was delivered by an OncoMyst model CDD2A inhalational device, which aerosolized compounds to particles of 2–3 μM and prevented escape of exhaled aerosol. Deposition efficiency of TC99M was used to predict deposition of doxorubicin and predict patient doses. Two of four patients treated with 9.4 mg/m^2 developed dose-limiting pulmonary toxicities. One of eleven patients treated at the 7.5 m^2 dose level experienced >20 % diminution of pulmonary function attributable to study drug. One sarcoma patient exhibited a partial response to therapy, and eight patients had stabilization of disease lasting five or more courses (range 5–15). No pulmonary drug levels were measured in this study. However, systemic doxorubicin levels were, in general, considerably lower than those typically observed following systemic administration of standard doses of doxorubicin.

3.3 Cisplatin Preclinical Studies

ILuP with cisplatin has been evaluated by several investigators. Li et al. evaluated ILuP with cisplatin in a rat lung tumor model [46]. The results demonstrated significantly higher platinum concentrations in pulmonary tumor in rats undergoing ILuP with 0.1 mg/mL of cisplatin than rats receiving a 1 mg intravenous injection (6.7 ± 1.6 vs. 2.5 ± 0.6 μg/g of tissue [$p < 0.05$]) (Table 5). In accordance with the findings of Wang et al. [47], a lower cisplatin level was observed in tumor nodules than in normal lung tissue in the perfused rats, but almost the same levels were seen in the animals treated with

Table 5 Cisplatin concentration in perfusate, lung tissue, and tumor tissue in rats

Animals	Tumor	Route	Dose or initial conc.	Lung (μg/g)	Tumor (μg/g)	Reference
Rat	Sarcoma	ILuP	0.1 mg/mL		6.67 (1.64)	[46]
		IV	1 mg		2.51 (0.60)	
Rat	Sarcoma	ILuP	25 μg/mL	~4	4.76 (0.60)[a]	[48]
		ILuP	50 μg/mL	~11	4.95 (0.80)[a]	
		ILuP	100 μg/mL	~21	4.84 (0.74)[a]	

Data are presented as means and SD. *ILuP* isolated lung perfusion, *IV* intravenous infusion

[a]Concentration at 60 min of ILuP

intravenous cisplatin. These studies suggested that ILuP with cisplatin was pharmacokinetically superior to intravenous injection.

In another study, Li et al. [48] investigated the pharmacokinetics of cisplatin in rat tumor and lung tissues after ILuP using different perfusion times and perfusate drug concentrations. Isolated lungs were perfused over various times with cisplatin at 25, 50, or 100 μg/mL. Total cisplatin concentrations in lung tissues increased significantly with perfusion time and increasing cisplatin perfusate concentration. Cisplatin concentrations in normal lung tissues after a 60-min perfusion ranged from approximately 4–21 μg/g of tissue. However, cisplatin concentrations in the perfused tumor nodules (4 mm in diameter) ranged from 4.17 ± 0.82 to 4.95 ± 0.80 μg/g of tissue and did not change significantly with the perfusion time or perfusate cisplatin concentration (Table 5). Furthermore, cisplatin concentrations in tumor tissue were inversely related to the weight of tumor nodules after ILuP. The results suggest that ILuP may not be beneficial for bulky metastatic disease.

In additional studies, Ratto et al. [49] utilized a porcine model to evaluate the pharmacokinetics of cisplatin administered via 15-min infusion distal to a pulmonary artery tourniquet (stop-flow), 15-min infusion into a lung isolated by tourniquets on the ipsilateral pulmonary artery and pulmonary veins (stop-flow/occlusion), or by 4-h ILuP under normothermic conditions using a closed, oxygenated extracorporeal circuit. Cisplatin (AUC) values in pulmonary tissues were approximately threefold higher in the stop-flow/occlusion animals compared to the stop-flow group [11,538 =/–4,586 μg/(min·mL) vs. 3,658 ± 824 μg/(min·mL), respectively]. Interestingly, lung perfusions with 2.5 mg/kg of cisplatin did not increase pulmonary tissue AUC values relative to those observed following administration of the same dose by stop-flow/occlusion techniques; however, cisplatin AUCs in mediastinal lymph nodes were significantly higher following ILuP compared to stop-flow/occlusion, possibly owing to the duration of drug exposure in the perfusions. Drug uptake in lung tissues and mediastinal nodes following ILuP was dose dependent. Histopathologic analysis revealed no significant differences regarding acute toxicities in pulmonary tissues harvested 4 h after cisplatin administration by any technique.

3.3.1 Clinical Trials

Several phase I studies have been performed to examine toxicities and potential efficacy of cisplatin administered by ILuP techniques (Table 2). Johnston et al. [42] performed total lung perfusion using cardiopulmonary bypass techniques in two patients (one bronchoalveolar lung cancer and one sarcoma). The first patient underwent ILuP with 14 μg/mL cisplatin at 25 °C, whereas the second patient was perfused with 20 μg/mL cisplatin at 40 °C. Perfusion flow rates were adjusted to maintain physiologic pulmonary artery pressures. Perfusion durations approximated 60 min. Peak pulmonary circuit cisplatin levels approximated 10 μg/mL; peak cisplatin levels in systemic circulation ranged from 0.4 to 0.8 μg/mL, indicating a small leak between pulmonary and systemic circuits. Cisplatin levels in a normal lung were ~0.07 μg/g, compared with tumor drug levels of approximately 1.2 μg/g (Table 3). One of the two patients had detectable cisplatin in regional lymph nodes. One of the two patients developed respiratory failure and empyema, dying 81 days after the perfusion.

Ratto et al. [50] administered cisplatin (200 mg/m^2) to six patients with sarcomatous pulmonary metastases via 60-min normothermic ILuP using a closed, oxygenated extracorporeal circuit. Two patients developed reversible interstitial pneumonitis, one of whom required mechanical ventilatory support. No systemic toxicities were observed. Cisplatin levels in normal lung and metastatic lesions were comparable, ranging between 65 and 75 μg/g tissue (Table 3). In all likelihood, the low protein content of the perfusate (approximately 1/7 that of normal serum) enhanced drug delivery during ILuP. Indices of interstitial injury (DLCO, pO_2, and pCO_2) assessed at 10, 30, and 90 days postoperatively were essentially unchanged from baseline values. Response to therapy was not evaluated in this trial.

Schröder et al. [51] performed hyperthermic ILuP with cisplatin in four sarcoma patients. Following metastasectomy, patients underwent isolated lobar or unilateral whole-lung perfusion with 70 mg/m^2 cisplatin administered at a temperature of 41 °C for 20–30 min at a rate which maintained a mean pulmonary artery pressure less than baseline values (approximately 300–500 mL/min). One individual underwent staged bilateral lung perfusions 1½ months apart. Maximal cisplatin concentrations at the completion of the perfusions approximated 98 μg/g of tissue, values which were considerably higher than those observed by Johnston et al. [42]. All patients experienced transient pulmonary toxicity manifested as non-cardiogenic pulmonary edema and desquamation of perfused bronchial mucosa. The one patient who had undergone unilateral whole-lung perfusion exhibited grade II pulmonary toxicity (>20 % decrease of FEV and DLCO relative to baseline values) 3 weeks post-ILuP that gradually resolved over the next 9 weeks. Two additional patients exhibited grade I pulmonary toxicity 3 weeks following ILuP; these toxicities resolved in both patients within 6 weeks of their procedures. One patient undergoing lobar perfusion experienced no clinically significant diminution in pulmonary function. Three of the four patients undergoing metastasectomy and perfusion were alive and free of disease with a median follow-up of 13 months. Collectively, this limited clinical study demonstrated that hyperthermic lung perfusions with cisplatin are feasible in patients with pulmonary metastases.

Muller [52] evaluated the effects of combined regional and systemic chemotherapy for the treatment of inoperable non-small-cell lung cancer. Twenty-two chemo-naive patients underwent 20-min regional perfusion of the thorax with 10 mg/m^2 of mitomycin, 25 mg/m^2 of navelbine, and 30 mg/m^2 of cisplatin. Regional perfusion was accomplished by balloon catheter occlusion of the aorta above the celiac axis and the inferior vena cava at the cavoatrial junction, as well as pneumatic tourniquets on the upper extremities. Three hundred micrograms of GM-CSF were administered intravenously during the perfusion. Thereafter, patients received 250 mg/m^2 of 5-fluorouracil (5-FU) and 20 mg/m^2 of cisplatin via continuous intravenous infusion over 4 days. Two cycles of regional and systemic chemotherapy were administered 4 weeks apart. The overall response rate was 59 % (4.5 % CR, 54.5 % PR). Six additional patients exhibited minor responses. Nearly all patients responding to therapy experienced either improvement or stabilization of pulmonary function. No dose-limiting toxicities were observed during 45 cycles of therapy. Sixteen of twenty-two patients underwent surgery, thirteen of whom had complete (R0) resections. Overall 1-year survivals were 87 % and 68 % for patients with bulky IIIA and IIIB/IV disease, respectively.

3.4 Gemcitabine Preclinical Studies

Several studies have been performed recently to evaluate pharmacokinetics and toxicities of gemcitabine administered by lung perfusion techniques (Table 6). van Putte et al. [53] delivered escalating doses of gemcitabine (20, 40, 80, 160, or 320 mg/kg; approximately 5, 10, 20, 40, and 80 mg, respectively) or buffered starch to rats via 25-min ILuP at a rate of 0.5 mL/min followed by 5-min washout. Pulmonary gemcitabine levels following perfusion/washout with 160 or 320 mg/kg were 1.5 ± 1.6 μg/g and 2.5 ± 1.8 μg/g, respectively. Levels of gemcitabine in systemic circulation were undetectable following ILuP. Lung levels of gemcitabine were 0.2 ± 0.1 μg/g with serum levels of 92.2 ± 63.6 μg/mL following IV administration of 160 mg/kg gemcitabine. In additional experiments, resection of the

Table 6 Concentration of gemcitabine and paclitaxel in perfusate and lung tissue

Drug	Animals	Route	Dose or initial conc.	Perfusate (μg/mL)	Lung (μg/mL)	AUC [μg (h·mg)]	Reference
Gemcitabine	Rat	ILuP	160 mg		1.5 (1.6)		[53]
		ILuP	320 mg		2.5 (1.8)		
		IV	160 mg		0.2 (0.1)		
Gemcitabine	Rat	BFO^{a}_{10}	13.3	2,700	0.62 (0.37)		[55]
		BFO^{a}_{20}	26.7	2,700	0.90 (0.53)		
		BFO^{a}_{30}	40	2,700	0.76 (0.38)		
		BFO^{a}_{40}	53.3	2,700	1.19 (0.77)		
	Rat	BFO^{b}_{10}	5.3	2,700	0.63 (0.13)		[55]
		BFO^{b}_{20}	10.7	2,700	0.94 (0.21)		
		BFO^{b}_{30}	16	2,700	0.97 (0.41)		
		BFO^{b}_{40}	20.4	2,700	1.35 (0.6)		
Gemcitabine	Pig	SPAP	1,250 mg/m^2		2,700 ± 1,800	43,179	[58]
		IV	1,250 mg/m^2		<50	3,180	
Paclitaxel	Sheep	ILuP	40 mg	11.9[a]	15[b]	26.2	[61]
		ILuP	200 mg	69[a]	59.9[b]	78.9	
		ILuP	800 mg	289.8[a]	90.1[b]	183.8	
		IV	200 mg		25.4[b]	73	

[a]Flow rate = 0.5 mL/min
[b]Flow rate = 0.2 mL/min

contralateral (non-perfused lung) was performed 3 weeks following ILuP. Animals (67–100 %) undergoing ILuP at doses of 20–320 mg/kg followed by delayed contralateral pneumonectomy were alive 90 days following lung perfusion. Mortality following pneumonectomy did not appear to correlate with perfusion doses.

In a subsequent study, van Putte et al. [54] further examined toxicity and potential efficacy of gemcitabine delivered by ILuP techniques in a rodent pulmonary metastasis model. Rats with unilateral pulmonary metastases underwent ILuP as described above using gemcitabine at doses of 160 or 320 mg/kg, or buffered starch, whereas animals with bilateral metastases received either a single intravenous dose of gemcitabine (160 or 320 mg/kg) or buffered starch. All rats receiving 320 mg/kg gemcitabine IV compared to 40 % of animals receiving 160 mg/kg IV died within 1 week. The overall survival rate for animals having gemcitabine perfusions was 83 %. Animals undergoing ILuP with either 160 or 320 mg/kg gemcitabine exhibited a twofold increase in pulmonary interstitial fibrosis compared to animals receiving intravenous gemcitabine; the extent of fibrosis induced by perfused gemcitabine was similar for these two doses. Rats with unilateral metastases undergoing ILuP with 320 mg/kg gemcitabine had a median survival time of 38 ± 4 days compared to 28 ± 3 days for animals with unilateral metastases treated with intravenous gemcitabine. These data suggest that gemcitabine administered via ILuP techniques may prolong survival in preclinical animal models yet induces interstitial fibrosis that could be significant in humans.

An additional study [55] was performed to evaluate pulmonary gemcitabine levels following administration of this drug during blood flow occlusion (BFO) for 10, 20, 30, or 40 min. Gemcitabine was delivered at rates of 0.2 or 0.5 mL/min (Table 6). Pulmonary uptake was saturated after 20-min BFO; no significant differences in pulmonary gemcitabine levels were observed using flow rates of 0.5 mL/min relative to 0.2 mL/min. Furthermore, no significant differences in wet to dry ratios were observed between different flow rates and perfusion times. Pulmonary gemcitabine levels were three- to sixfold higher following delivery of drug via BFO compared to IV administration of gemcitabine at the MTD of 160 mg/kg.

In subsequent experiments van Putte et al. [56] examined the effects of delayed washout following ILuP on pulmonary gemcitabine levels in a rodent model. In this study, doses of gemcitabine (1.3–6.7 mg/mL in 25 mL perfusate) were administered via ILuP. An additional cohort of rats underwent 6-min ILuP with 6.7 mg/mL gemcitabine followed by 5-min flush and 30 or 60 min of reperfusion; another cohort of animals had 6-min perfusion followed by delayed cross-clamp release for 30 or 60 min followed by a 5-min flush. Interestingly, whereas pulmonary gemcitabine levels after 30-min ILuP exceeded those observed following 6-min perfusion, the wet to dry ratio (indicative of tissue edema) was higher in the 30-min perfused lung. Tissue drug levels after 6-min perfusion were 70 % of levels observed following 30-min ILuP. Although the lung was not saturated, 6-min perfusion resulted in pulmonary gemcitabine levels of 2.3 ± 0.34 mg/g; these levels were comparable to what had been observed in a previous toxicity study (2.5 ± 1.8 mg/g) using 30-min ILuP with 320 mg/kg (5.3 mg/mL inflow concentration). A linear relationship was observed between perfusate concentration and tissue drug levels, suggesting that uptake of gemcitabine into lung parenchyma occurs primarily by diffusion rather than active transport mechanisms. Overall 43–51 % of the drug in the perfusate was absorbed into the lungs. These findings suggest that decreased ILuP times, with delayed cross-clamp release, result in comparable tissue drug levels with less interstitial edema (hence potentially reduced long-term pulmonary toxicity). Efficacy studies using such modified ILuP techniques have not been published.

In additional studies, van Putte et al. [57] utilized a porcine model to evaluate pharmacokinetics of gemcitabine delivered via selective pulmonary artery perfusion (SPAP) techniques. Briefly 16 pigs underwent SPAP with gemcitabine (1 g/m^2). Three groups underwent SPAP for 2 min with either normal, 50 %, or 90 % reduced pulmonary blood flow. An additional group received systemic administration of a comparable dose of gemcitabine over 30 min. C_{max} and AUC values for 2-min and 10-min SPAP were eleven- and sixfold and two- and threefold higher, respectively, than those observed following IV gemcitabine infusions. Flow reduction led to inhomogeneous pulmonary drug delivery. The relatively high C_{max} and AUC values achieved during SPAP may be attributable to efficient first-pass uptake into the lung, resulting in systemic AUC levels comparable to those observed following systemic administration of gemcitabine. The fact that uptake of gemcitabine after 2-min SPAP was only five- to sixfold higher than IV administration despite a 30-fold difference in drug concentration at the catheter tip suggests that SPAP at 2 min may saturate uptake mechanisms in the lung. SPAP for 10 min appeared to be optimal for pulmonary drug uptake.

In a related study, these investigators [58] used a porcine model to examine pharmacokinetics of gemcitabine (1.25 g/m^2), carboplatin (AUC 5), or both administered by 2-min SPAP followed by 30-min blood flow occlusion to delay drug washout from the lung. Additional animals received similar doses of gemcitabine or carboplatin IV. Gemcitabine and carboplatin lung levels 8 min after completion of SPAP exceeded 2,500 μg/g and ~17 μg/g, respectively, tapering off linearly over the next 20 min. Pulmonary gemcitabine levels, when this drug was administered with carboplatin, were 750 μg/g, suggesting that carboplatin adversely affected gemcitabine uptake. In contrast, carboplatin lung levels, when this drug was perfused with gemcitabine, were somewhat higher (65 μg/g) than when carboplatin was administered alone. Serum levels following SPAP of gemcitabine and/or carboplatin were also somewhat higher than when these drugs were administered intravenously.

3.4.1 Clinical Trials

To date, no data pertaining to regional delivery of gemcitabine for treatment of pulmonary malignancies have been reported.

3.5 Paclitaxel Preclinical Studies

All of the animal and human lung perfusions described thus far have utilized antegrade (inflow via PA, outflow via PV) perfusion techniques that may not be optimal for drug delivery to pulmonary neoplasms, which frequently derive their blood supply from the bronchial arteries [14, 16, 59]. The fact that chemotherapeutic agents administered by selective bronchial artery infusion can mediate significant regression of pulmonary neoplasms [15, 60] attests to the relevance of this circulatory system regarding growth of pulmonary malignancies. By exploiting venous collaterals between the pulmonary and bronchial arterial systems, retrograde perfusion (inflow via pulmonary vein; outflow via pulmonary artery) may enhance the efficiency of drug delivery to primary as well as metastatic tumors in the lung.

Schrump et al. [61] utilized a sheep model to evaluate the feasibility, pharmacokinetics, and immediate toxicities of paclitaxel administered via retrograde, hyperthermic ILuP techniques. Adult sheep underwent 90-min hyperthermic, retrograde ILuP using a closed, oxygenated, extracorporeal circuit with a 3 L perfusate containing crystalloid and packed red blood cells (pRBC) to a final hematocrit (Hct) of 10 and escalating doses of paclitaxel (2–800 mg). Paclitaxel levels in perfused tissues increased with escalating perfusate doses; drug levels in high-dose perfusates declined more slowly, suggesting that uptake of paclitaxel into pulmonary tissues was saturable. The average C_{max} (50 ng/mg) in lung tissues obtained when 200 mg of paclitaxel was utilized in the perfusion (78 μM paclitaxel) was approximately twofold higher than that observed following systemic infusion of the same dose of paclitaxel over 1 h; tissue AUCs under these conditions were relatively comparable (Table 6). The plasma C_{max} and AUC following 1-h infusion of 200 mg of paclitaxel (approximately 150 mg/m^2) in sheep were essentially comparable to those reported by Maier-Lenz et al. [62] following 1-h infusion of 225 mg/m^2 of paclitaxel in cancer patients. When the dose of paclitaxel in the perfusate was increased to 800 mg (approximately 325 μM), C_{max} of paclitaxel in perfused tissues was 86 ng/mL, and AUC was 165 (ng·h)/mg. Paclitaxel levels in the systemic circulation were undetectable at all perfusate doses during the ILuP; following restoration of circulation to the perfused lung (after washout), systemic levels were either undetectable or extremely low, indicating that retained drug was not rapidly released from the perfused lung into the systemic circulation. Histopathologic examination of lung tissues obtained 3 h following completion of the ILuP revealed no pulmonary hemorrhage, alveolar edema, or interstitial thickening. Survival was not evaluated in these experiments, nor did the sheep model allow assessment of antitumor activity of paclitaxel administered in this manner.

In a more recent study, Tanju et al. [63] compared early effects of paclitaxel and docetaxel on pulmonary physiology in rats undergoing ILuP. Briefly, rats underwent ILuP with paclitaxel (140 mg/kg), docetaxel (70 mg/kg), or normal saline delivered at a rate of 0.5 mL/min with perfusion pressures of 20 mmHg. Ventilation pressures, compliance, and blood gases were evaluated 5 min after restoration of pulmonary blood flow, prior to resection of the perfused lung. Ventilatory pressures were higher, compliance was lower, and pO_2 values were lower in drug-treated rats compared to controls. ILuP with docetaxel resulted in less CO_2 retention than paclitaxel. Docetaxel-treated lungs exhibited intra-alveolar hemorrhage and mononuclear cell infiltration without perivascular edema. In contrast, animals perfused with paclitaxel exhibited dense perivascular and intra-alveolar edema. These data suggest that docetaxel might be preferable to paclitaxel for ILuP. Delayed toxicities associated with these agents were not assessed in this study.

3.5.1 Clinical Trials

Schrump et al. conducted a phase I study of hyperthermic, retrograde isolated lung perfusion with paclitaxel in patients with unresectable pulmonary malignancies (Schrump, unpublished). Ten lung

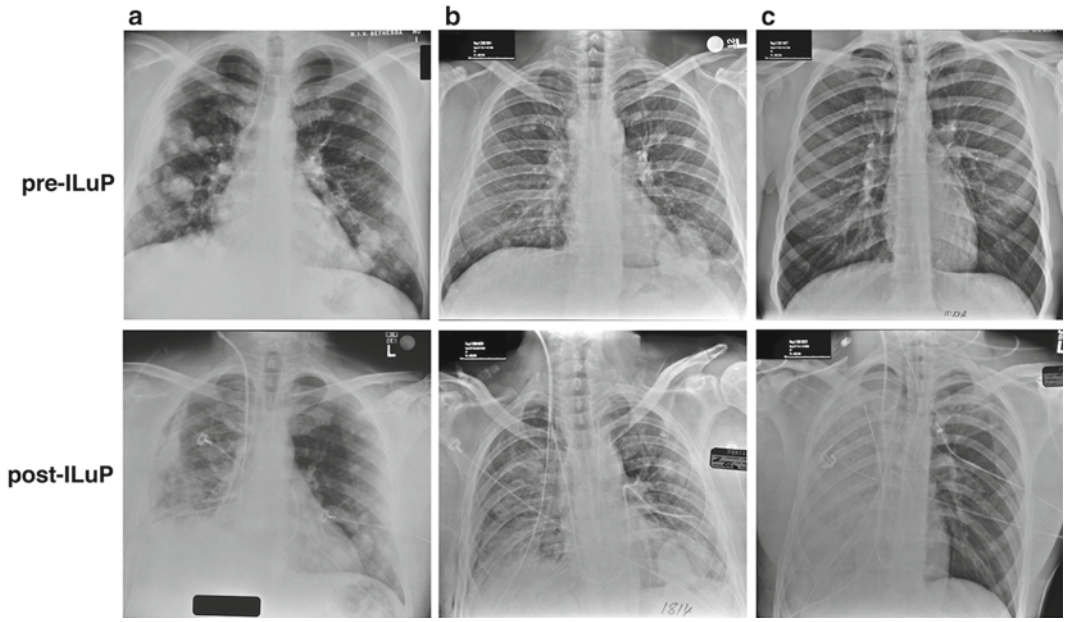

Fig. 1 Preoperative and 24-h postoperative chest X-ray films from three representative patients undergoing hyperthermic retrograde ILuP with 100 mg of paclitaxel (**a**), 125 mg of paclitaxel (**b**), or 200 mg of paclitaxel (**c**). Corresponding pharmacokinetic data for these patients are summarized in Table 7 (*use figure from Fig. 1 of Chap. 21 on page 359 of the first edition*)

perfusions (two left, eight right) were performed in eight patients with refractory pulmonary metastases. Five of those perfusions were performed in the context of complete pulmonary metastasectomy. All patients received intravenous decadron, diphenhydramine, and cimetidine prior to ILuP. Inflow was achieved by a single retrograde cardioplegia cannula placed into the isolated left atrial cuff or by dual cannulation of the ipsilateral superior and inferior pulmonary veins. Outflow was established by cannulation of the ipsilateral main pulmonary artery. Flow rates were adjusted to maintain a pressure of 14–16 mmHg within the pulmonary veins; under these conditions flow rates ranged between 500 and 1,000 mL/min. Temperatures of the perfusate were adjusted via in-line heat exchanger to maintain a temperature of 39.5–41 °C in the lung, assessed by temperature probes placed into the upper and lower lobes.

Prior to initiation of the perfusion, the isolated lung was flushed with 1 L of Ringer's lactate containing 250 μg of prostaglandin E to dilute the pulmonary vasculature. Thereafter, the lung was perfused for 90 min with paclitaxel using a closed, oxygenated circuit containing a dilute blood perfusate (Hct = 10). Following completion of the paclitaxel perfusion, the lung was flushed with 2 L of Ringer's lactate prior to reestablishing normal blood flow to the isolated lung.

No dose-limiting toxicities were observed in three patients undergoing four perfusions with 100 mg of paclitaxel. However, significant pneumonitis requiring mechanical ventilation was observed in three patients perfused with 200 mg of paclitaxel. Although the pneumonitis was dramatically reversible in all three patients, the severity of the acute pulmonary injury warranted dose reduction. As such, three additional patients underwent four lung perfusions with 125 mg of paclitaxel; no pulmonary toxicity was observed in these individuals. Representative chest X-ray films and pharmacokinetic data for three individuals are depicted in Fig. 1 and Table 7. Uptake of paclitaxel in tumor tissue equaled, if not exceeded, that in normal lung parenchyma. Although no objective responses were observed, prolonged disease-free interval was observed in four individuals who underwent ipsilateral metastasectomy at the time of ILuP; these individuals underwent contralateral metastasectomy shortly after ILuP yet recurred in the non-perfused lung.

Table 7 Paclitaxel concentrations in perfusate, plasma, and lung tissues

	C_{max} (mg/L)			Normal lung (ng/mg of tissue)		Tumor (ng/mg of tissue)	
Patient	Dose (mg)	Perfusate	Plasma	90 min	120 min	90 min	120 mg
1	100	30	0.059	6.2	10.9	4.8	9.8
7	125	40	0.1	17.3	12.9	25.6	19.0
6	200	51	0.16	24.4	14.7	26.8	24.2

3.6 TNF-α Preclinical Studies

Although the macrophage-derived cytokine TNF-α exhibits potent antitumor effects [64, 65], systemic administration of tumoricidal doses of recombinant TNF-α is not tolerated in cancer patients [66, 67]. However, due to the effects of TNF on tumor vasculature, this cytokine has been utilized with melphalan in hyperthermic isolated limb and liver perfusions resulting in complete response rates approximating 75 % in melanoma and sarcoma patients [68, 69]. Weksler et al. [70] evaluated the antitumor effects of TNF-α in a rodent perfusion model. Preliminary in vitro experiments revealed that 42 μg/mL of murine or human TNF-α inhibited in vitro proliferation of MCA-induced sarcoma cells by 20–40 % relative to untreated cells. Tumor-bearing rats undergoing ILuP with 420 μg of TNF-α exhibited a five- to sevenfold reduction in the number of metastases in the perfused lung compared to the non-perfused lung. These data suggested that when administered by ILuP techniques, TNF-α can mediate significant antitumor effects without apparent systemic toxicity.

3.6.1 Clinical Trials

In a phase I trial, Pass et al. [71] treated 15 patients with pulmonary metastases from a variety of malignancies by 90-min hyperthermic ILuP using a closed, oxygenated extracorporeal circuit containing 0.2 mg of interferon-α and escalating doses (0.3–0.6 mg) of TNF-α (approximately 7 μg/mL in the highest cohort of patients). There were no operative deaths, and reduction of disease (not meeting criteria for partial response) was observed in three patients; TNF-α levels in pulmonary tissues were not ascertained in this study (Table 2). One patient experienced reversible interstitial pneumonitis requiring mechanical ventilation. FEV and DLCO, as well as ventilation and perfusion in the treated lung, were diminished 10–20 % relative to baseline values 8 weeks following ILuP, suggesting subclinical pulmonary toxicity following ILuP.

3.7 Interleukin-2 Preclinical Studies

Aerosolization is an appealing method for administration of cytokines such as interleukin-2 (IL-2) for inoperable pulmonary malignancies. In a series of dog experiments, Khanna et al. [72] evaluated the toxicities and potential efficacy of either free or liposomal IL-2 in normal dogs. Free IL-2 (5×10^6 units) was administered twice daily by inhalation techniques. Additional dogs received aerosolized saline. Leukocyte counts in bronchoalveolar lavage (BAL) were significantly higher, and immune effector populations including leukocytes and eosinophils were higher in dogs receiving liposomal IL-2 compared to those receiving free IL-2.

An additional canine study [73] was performed to examine the characteristics and distribution of nebulized IL-2 liposomes. The mass median aerodynamic diameter of the liposomes was 1.98 ± 2.02 μm. Aerosolized IL-2 was deposited homogeneously throughout the lungs of

anaesthetized dogs. Approximately 24 h after inhalation, most of the liposomes remained in the lungs, whereas some were taken up into spleen.

In subsequent studies, Khanna et al. [74] treated dogs with pulmonary metastases [7] or spontaneous lung cancers [2] with aerosolized liposomal human IL-2. Two of four dogs with metastatic osteosarcoma exhibited complete regressions lasting >12 months and >20 months. One dog with lung cancer had stable disease for 8 months. Numbers of immune effector cells (eosinophils and lymphocytes) were significantly increased in BAL, and mean BAL effector lytic activity was significantly increased 15 days after commencing IL-2 inhalations compared to pretreatment values. Interestingly, this lytic activity was not evident 30 days following commencement of aerosolized IL-2 therapy. No pulmonary toxicities were observed in this study.

3.7.1 Clinical Trials

A number of trials have been performed to evaluate the toxicity and efficacy of inhaled IL-2 in patients with potentially inoperable pulmonary malignancies. Lorenz et al. [75] treated 16 patients with refractory pulmonary malignancies with five daily administrations of aerosolized IL-2. Reversible, dry nonproductive cough was dose limiting. Mild decreases in pulmonary function tests and pO_2 were seen in all patients. One complete response, one partial response, and one mixed response were observed in 14 patients with metastatic renal carcinomas. Dose-dependent expansion of immune effector cells was observed in BAL fluids. Increased systemic levels of soluble interleukin-2 receptors were observed. No other systemic effects of aerosolized IL-2 were evident. Melichar et al. [76] observed no increase in urinary neopterin levels following inhalational IL-2 therapy in 13 patients with metastatic renal cell carcinoma, consistent with a lack of systemic immune activation.

Huland et al. [77] reviewed results of inhaled IL-2 therapy for nearly 300 patients with pulmonary metastases, 188 of whom had metastatic renal cell carcinoma. A variety of doses and schedules were used for IL-2 administration. Overall, inhaled IL-2 was well tolerated. Among 188 patients with renal cell carcinoma treated at a single European clinic, progression of pulmonary metastases was prevented in nearly 70 % of patients with a median duration of 7 months. Overall survival appeared to be improved relative to historic controls (17.2 vs. 5.3 months).

In a small single-institution study, Enk et al. [78] treated seven melanoma patients with pulmonary metastases with inhalation IL-2 for 6 months. Patients also received periodic bolus administrations of DTIC every 4 weeks. Therapy was well tolerated. No significant systemic toxicities were observed. Six patients developed cough. The overall response rate was 71 %; two patients exhibited complete response, two patients had partial remissions, and one had stable disease. Cough and dyspnea induced by inhaled IL-2, which appears to be consistent with an asthma-like syndrome [79], could ameliorate induction of accessory cell function of alveolar macrophages [80], thereby attenuating antitumor immunity mediated by this cytokine.

4 Summary and Future Directions

Only 3 of 106 patients undergoing ILuP procedures in the aforementioned trials died as a direct result of perfusion (perioperative mortality = 3 %); these data indicate that ILuP can be performed safely in properly selected individuals with unresectable pulmonary neoplasms. Data from Schröder et al. [51], as well as our experience (Schrump unpublished), indicate that cisplatin and paclitaxel lung perfusions can be performed in the context of aggressive pulmonary metastasectomy (including lobar resections) without apparent significant long-term sequelae.

In a recent study Nowak et al. [81] examined alterations of tumor and normal lung tissues following ex vivo ILuP. Briefly, lobectomy and pneumonectomy specimens from lung cancer patients were ventilated and perfused ex vivo using a physiologic crystalloid solution for 10, 60, 90, 120, and 240 min. Perfusions up to 120 min could be performed without disruption of histologic or physiologic parameters. However, perfusions greater than 120 min in duration resulted in progressively severe lung edema, with increased inspiratory and pulmonary artery pressures. Perfusions more than 240 min in duration were associated with loss of cell viability and associated histologic abnormalities; these ex vivo studies using human lungs provide potentially useful information regarding the development of ILuP regimens for inoperable pulmonary malignancies.

In an additional study Schumann et al. [82] evaluated the effects of reperfusion of isolated lungs following low and high perfusion pressures or low and high positive end-expiratory pressure (PEEP) (4 vs. 8 mmHg). Lung weights were lower following reperfusion with low PA relative to high PA pressures (mean 27 vs. 40 mmHg). Pulmonary edema (reflective of total lung weight) was lowest, whereas compliance was highest, and lungs exhibited lowest amounts of alveolar inflammation/destruction when reperfusion was performed using low perfusion pressures and high PEEP. These studies, which were designed primarily for lung transplant purposes, may have direct implications regarding reperfusion techniques used for future ILuP trials for cancer.

At present, the major limitation of ILuP relates to the lack of specificity regarding uptake and cytotoxicity of drugs in normal lung parenchyma relative to tumor tissues; this phenomena has been well established for doxorubicin, and agents such as melphalan or TNF may have limited use in ILuP owing to their potential for inducing significant interstitial injury. Continued efforts should focus on the identification of novel agents that mediate cytotoxicity preferentially in cancer cells. Furthermore, efforts should be undertaken to elucidate the pathophysiology of perfusion-related pneumonitis [83, 84] and to identify agents that can ameliorate such injury. For instance, depletion of tissue plasminogen activator or administration of *N*-acetyl-cysteine attenuates reperfusion injury in transplanted lungs [85, 86]; conceivably, similar strategies could be used to minimize pneumonitis observed following ILuP. In addition, a standardized system should be utilized for all future clinical trials to enable objective assessment of pulmonary toxicities following ILuP. At present, ILuP appears most effective when performed in the context of pulmonary metastasectomy, and future trials should focus on the use of ILuP or inhaled drugs as an adjuvant to aggressive resections. Continued efforts should also be directed toward refining minimally invasive techniques for delivery of chemotherapeutic agents via torso perfusion, SPAP, or selective bronchial artery infusion, as well as the development of inhalation agents for the treatment of inoperable pulmonary malignancies.

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Blood–Brain Barrier and CNS Malignancy

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Abstract Improving treatment outcomes for patients with central nervous system (CNS) malignancies is associated with a series of difficult challenges. The use of enzyme-inducing antiepileptic drugs and glucocorticoids significantly affects the pharmacology of many systemically administered anticancer agents. The blood–brain barrier plays a major role in restricting the delivery of drugs to the CNS and there are a host of drug resistance mechanisms within the blood–brain barrier and brain tumors which further limit the effectiveness of therapeutic agents. This chapter reviews important characteristics of CNS malignancies and focuses on unique aspects of care for neurologic cancers, characteristics of the blood–brain barrier (BBB), drug resistance mechanisms, important drug interactions, and novel approaches to increase drug delivery to the brain tumor tissue.

Keywords Glioma • Glioblastoma multiforme • Blood-brain barrier • Bevacizumab • Temozolomide • Radiation • Drug delivery

1 Introduction

The brain is a frequent site of primary and metastatic tumors. It is estimated that approximately 22,000 new cases of primary brain tumors (<1.5 % of all malignant diseases) and 170,000 new cases of new brain metastases are diagnosed annually in the United States [1]. In the pediatric population, primary brain tumors are the most common solid tumor second only to leukemia in overall tumor frequency in this age group. The prognosis of patients with the most common types of malignant brain tumors remains poor despite significant advances in neuroimaging, microsurgery, and radiation. The current median survival of glioblastoma multiforme patients is about 15 months and survival at 2 years is 26 % [2]. The majority (80 %) of malignant gliomas are known to recur within 2 cm of the original tumor site due to the presence of locally invasive glioma cells, whereas distant metastases are exceedingly rare. For patients with intraparenchymal brain metastases, the median survival after diagnosis varies from 4 to 13 months depending on the clinical scenario. Patients with leptomeningeal

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metastases have a median survival of about 2 months. The outcome for patients with primary central nervous system (CNS) lymphomas and some of the pediatric primary brain tumors is considerably better.

New therapies and novel approaches are clearly needed to treat these cancers. Surgery and radiation for primary and metastatic brain tumors have been optimized during the past decades. As a result, it is unlikely that further advances in these treatment modalities will substantially add to survival in these malignancies. Thus, the development and clinical testing of novel pharmaceutical agents represents the primary route to improve the outcome for patients with brain cancer. However, the development of novel therapies for brain tumors is more complicated as the CNS is in a unique and distinct anatomical compartment, surrounded by cerebrospinal fluid (CSF), shielded by the blood–brain barrier (BBB), lacking lymphatic drainage, and characterized by exquisite sensitivity to chemical or physical intervention. The delivery of therapeutic agents past the BBB is a major factor limiting progress in the development of clinically effective agents in the area of neuro-oncology [3–5].

This chapter will describe the major CNS malignancies as their individual natural histories and sensitivities to therapies are critical in considering novel approaches to each. In addition, the unique aspects of care in the patients with CNS malignancies that impact on trial design and evaluation will be reviewed. The remainder of the chapter will focus on the BBB, drug resistance mechanisms in brain tumors, important drug interactions in neuro-oncology, and novel approaches to increase drug delivery to brain tumor tissue.

2 CNS Malignancies

Primary Brain Tumors

Brain tumors are comprised of primary CNS malignancies and metastatic disease from systemic cancers. The primary brain tumors encompass a diverse range of pathological entities with different natural histories and treatment approaches.

Primary brain tumors are often classified as either gliomas or nongliomas. Astrocytomas, oligodendrogliomas, mixed oligoastrocytomas, and ependymomas are the most common gliomas, which are diffuse and infiltrative making complete surgical resection difficult. Nongliomas consist of medulloblastomas, CNS germ cell tumors, primary CNS lymphomas, and benign tumors such as meningiomas and pituitary adenomas [6]. This chapter will focus primarily on high-grade astrocytomas which are the most common and difficult to treat primary brain tumors.

2.1 Astrocytomas

The World Health Organization (WHO) grading system uses pathologic evidence of tumor cellularity, mitotic rate, endothelial proliferation, and necrosis to grade gliomas. Grade 1 astrocytomas (i.e., pilocytic astrocytomas) are usually well-circumscribed lesions that can often be completely resected. Grade 2 astrocytomas are diffusely infiltrating low-grade tumors with higher cellularity but low mitotic rates. Grade 3 (anaplastic) astrocytomas have more mitoses, while grade 4 astrocytomas (glioblastoma multiforme) have higher mitotic rates, endothelial proliferation, and/or tumor necrosis.

The most common and most aggressive of these tumors in adults is the glioblastoma (WHO grade IV). The current standard of therapy for this cancer is to remove as much tumor as possible without causing neurological consequences followed by concurrent radiation and temozolomide therapy with an

additional 6 months of adjuvant temozolomide [2]. The addition of temozolomide improves the median survival of patients from 12 to 14.6 months and the percent of patients alive at 2 years from 10 to 26 %. Unfortunately, this therapy does not result in patients being cured and survival at 5 years is only 10 % [7]. Optimal therapy for patients with anaplastic astrocytomas (WHO grade III) remains unclear. Clinical trials are currently underway that will formally compare survival with radiation or with radiation with temozolomide as prescribed for patients with glioblastoma. Grade II astrocytomas are diffusely infiltrating and often grow slowly over time and may evolve into higher grade astrocytomas requiring more aggressive therapy. As there is no proven survival benefit from early treatment with radiation, these patients are often followed closely with serial imaging and treatment with radiation is used when it appears that the tumor is becoming more aggressive or impairing function. Surgery is the preferred treatment for pilocytic astrocytomas (WHO grade 1).

2.2 Oligodendrogliomas

Tumors that contain oligodendroglial elements are relatively rare accounting for about 10 % of all primary brain tumors [6]. They are classified as either low grade (Grade II) or anaplastic (Grade III), which are now often defined by a 1p19q co-deletion that is found by examining surgically derived tumor. Mixed oligoastrocytomas contain both oligodendroglial and astrocytic elements. The median survival for low-grade oligodendrogliomas/oligoastrocytomas is 10 years. Surgery and radiation are the primary treatment modalities for these tumors. As early radiation therapy has not been proven to improve survival, these patients are often followed closely and radiation is used when there is documented progression. Temozolomide is usually reserved for the recurrent disease setting. Anaplastic oligodendrogliomas are characterized by high cellularity, nuclear pleomorphism, frequent mitoses, abundant endothelial proliferation, and necrosis. Recent evidence has shown significant benefit in the treatment of anaplastic oligodendroglioma using radiation followed by procarbazine, lomustine, and vincristine (PCV) [8]. This regimen of radiation followed by therapy with PCV has now become the standard of care.

2.3 Ependymomas

Ependymomas are classified as either low grade or anaplastic. The primary treatment for these tumors is surgical resection which can be curative. Radiation is often administered following surgery if there is residual tumor or an anaplastic histology. These tumors are relatively resistant to chemotherapeutic agents [9].

2.4 Medulloblastomas

Medulloblastoma and other CNS primitive neuroectodermal tumors such as pineoblastoma and cerebral neuroblastoma are uncommon in the adult population. Medulloblastomas usually occur in the posterior fossa located either in the cerebellar hemisphere or the vermis and may involve the fourth ventricle. Treatment usually involves surgery, radiation, and chemotherapy. Postoperative radiation to the craniospinal axis with a boost to the site of the primary tumor can improve outcomes and is curative in some patients. As craniospinal irradiation produces considerably more compromise to bone marrow reserve in adults than in children, the role of adjuvant chemotherapy in adults remains controversial. Chemotherapeutic agents with efficacy include platinum-containing agents, etoposide, alkylating agents, and vincristine. Long-term survival is achieved in 60–80 % of children treated for this disease [10].

2.5 Primary CNS Lymphoma

CNS lymphomas constitute about 2–3 % of all brain tumors in patients with an intact immune system. Nearly half of all CNS lymphomas occur in patients older than 60 years of age. The role of surgery is limited to making a diagnosis as this tumor is quite sensitive to radiation and chemotherapy and diffusely infiltrates the brain even involving the eyes and cerebrospinal fluid in 20 % of patients. Whole brain radiation yields a median survival of only 1 year and the rare long-term survivor invariably has severe neurotoxicity as a result of the therapy. As a result, systemic chemotherapy has been used in an effort to reduce the need for radiation therapy. Typical systemic lymphoma regimens, such as cyclophosphamide, vincristine, doxorubicin, and prednisone (CHOP), produce an initial tumor response followed by rapid regrowth with no improvement in overall survival. More recently, high-dose systemic methotrexate has been shown to have a major impact on survival in this disease. Methotrexate administered as a single agent at doses of 8 g/m^2 given every 2 weeks produces high response rates and an overall survival approaching 4 years without apparent neurotoxicity and a long-term cure rate approaching 30 % [11]. Other investigators have added a variety of other systemic agents to a high-dose methotrexate backbone including cytarabine, procarbazine, vincristine, and rituximab with similar results. Despite being a large monoclonal antibody, rituximab also appears to have single-agent activity in patients with recurrent primary CNS lymphoma. Attempts to disrupt the BBB using intra-arterially administered hypertonic mannitol followed by the administration of intravenous and intra-arterial chemotherapy results in similar survivals [12, 13].

2.6 Metastases to the CNS

Intraparenchymal brain metastases are ten times more common than primary CNS tumors and are most commonly associated with cancers of the breast, lung, and melanoma. These lesions usually result from hematogenous spread and are most common at the junction of the gray and white matters. Eighty percent of brain lesions occur in the cerebral hemispheres, 15 % in the cerebellum, and 5 % in the brainstem [6]. Surgery, highly focused radiation, and/or whole brain radiation are the primary therapy options for these lesions. Chemotherapy is of limited use since most tumors that metastasize to the brain either are not sensitive to chemotherapy or have been heavily pretreated with previous agents.

Leptomeningeal metastases occur when tumor cells reach the cerebrospinal fluid as a result of hematogenous dissemination or direct extension from CNS or systemic malignancies. These tumor cells are then carried by cerebrospinal fluid flow throughout the neuroaxis. As a result, treatment needs to be directed to the entire neural axis. Standard treatment now includes radiation therapy to sites of bulk or symptomatic disease and intrathecal chemotherapy designed to treat tumor cells in the cerebrospinal fluid. Intrathecally administered chemotherapy is carried by the flow of CSF fluid to distant regions of the neuraxis and is discussed further in Intrathecal Chemotherapy chapter. As a result, if the tumor causes a disturbance in CSF flow pathways, intrathecal drugs may not be delivered to affected regions of the neuraxis. Systemic administration of high-dose methotrexate or the use of conventional systemic chemotherapy is also considered for this devastating complication of cancer.

3 Unique Patient Care Issues in Patients with CNS Malignancies

Patients with primary brain tumors often present with seizures, peritumoral edema, elevated intracranial pressures, and a variably disrupted BBB. Each presents a challenge to the clinician caring for the patient or the researcher designing novel therapeutic approaches, which is not present in

systemic cancers. The BBB poses a unique therapeutic hurdle in drug delivery and understanding the physiology is crucial in further drug development. Similarly, inherent drug resistance mechanisms including drug transporters and enzymes pose unique challenges in the treatment for CNS malignancies.

3.1 Control of Seizures: Use of Anticonvulsants

Seizures occur in 25–50 % of patients with primary or metastatic brain tumors [14–16]. In general, the use of prophylactic anticonvulsants is not recommended [17]. Seizures are most common in patients with tumors involving the cortical and subcortical cerebral hemispheres. These seizures may be more difficult to control than idiopathic epilepsy and multidrug regimens may be required. Anticonvulsants such as phenytoin, phenobarbital, and carbamazepine induce CYP450 isoenzymes. This was first noted in patients with glioblastoma treated on a phase I/II study with paclitaxel who did not develop the expected alopecia or myelosuppression [18]. Pharmacologic studies in these patients demonstrated that enzyme-inducing antiepileptic drugs (EIAED) altered the CYP450-mediated metabolism of paclitaxel resulting in significantly lower plasma steady-state concentrations of the drug. Subsequent studies have demonstrated lower than expected plasma concentrations of many other anticancer agents including vincristine, teniposide, and irinotecan when used in conjunction with EIAED [19–22]. Since these important drug interactions were recognized, phase I trials have included separate dose escalation arms for patients who are and are not taking concomitant EIAEDs. More recently, clinicians are using non-EIAED, such as levetiracetam, to minimize the risk of drug interactions.

3.2 Control of Brain Edema: Corticosteroids and Anti-VEGF Therapies

Blood vessels supplying high-grade gliomas and brain metastases generally lack the tight junctions found in normal brain. As a result, plasma proteins leak through these vessels bringing water with them and result in peritumoral edema, mass effect, and the associated neurologic symptoms [23]. In addition, intravenously administered contrast agents traverse these abnormal vessels resulting in the contrast enhancement seen in MRI or CT imaging of the brain in patients with aggressive malignancies involving the CNS. An increase in vascular endothelial growth factor (VEGF) in brain tumors also leads to the development of increased peritumoral edema and increased intracranial pressure, which can lead to life-threatening complications. Glucocorticoids are frequently used to restore the integrity of the BBB which results in substantial reductions in brain edema and intracranial pressure. Dexamethasone is the most frequently used glucocorticoid, which can induce CYP450 isoenzyme albeit to a less extent than EIAED. Investigators have suggested that glucocorticoids might decrease the amount of systemic therapy that reaches the brain tumor by restoring the normal physiological properties of the BBB. Animal studies have shown that pretreatment with dexamethasone decreases cisplatin concentrations in normal tissue adjacent to the tumor. However, it does not decrease the concentration of topotecan or cisplatin in brain tumor tissue [24, 25]. Moreover, prolonged use of glucocorticoids may lead to significant side effects including myopathy, Cushingoid features, glucose intolerance, oral and esophageal candidiases, poor wound healing, and peptic ulcers. Bevacizumab, a humanized monoclonal antibody that inhibits VEGF, has recently been used as an alternative to decrease peritumoral edema [26, 27].

3.3 Characteristics of the Blood–Brain Barrier

The CNS is physically and metabolically separated from the systemic circulation by the presence of the BBB. The BBB is a dynamic interface that regulates the exchange of substances between the blood and brain and maintains optimal conditions for neuronal and glial function [28]. It selectively transports nutrients and beneficial endogenous substances into the brain and excludes toxic metabolites. The unique barrier inherent to the CNS is the endothelial cells of the brain capillaries [29]. Cerebral capillaries differ from peripheral capillaries by the specific characteristics of their endothelial cells. The brain capillary endothelial cells maintain brain homeostasis by filtering exogenous compounds and transporting nutrients, ions, hormones, and recruiting immune cells. They lack fenestration and pinocytic vesicles and consist of cerebrovascular endothelial cells interconnected by continuous reinforcement of tight junctions that form a physical barrier [30]. They also contain more mitochondria than the peripheral endothelial cells [31]. The increase in mitochondria is thought to be for the active transport of nutrients to the brain from the blood. These features give a high transendothelial electrical resistance (TEER) in the range of 1,500–2,000 $\Omega \times cm^2$ as compared to 3–33 $\Omega \times cm^2$ in other tissues [32, 33] which leads to low paracellular permeability.

Other features of the BBB include the astrocytic foot processes, pericytes, and perivascular macrophages within a basal lamina that strengthen the BBB [34, 35]. Astrocytes also confer a protective role on the BBB against both hypoxia and hypoglycemia [36, 37].

Transport across the BBB is via various different mechanisms including simple diffusion, facilitated diffusion, receptor-mediated endocytosis, and carrier-mediated efflux pump. Simple diffusion is a type of passive diffusion whereby drugs or endogenous substances travel across the BBB based on a concentration gradient between the blood and the brain without requiring ATP. Generally, drugs that passively diffuse through the BBB are lipophilic [37] and have a molecular weight less than 400–500 Da [38].

Facilitated diffusion is another form of passive diffusion whereby there is a binding of a solute to a transporter on one side of the membrane which triggers a conformational change in the protein resulting in the passage of the substance to the other side of the membrane from a high to low concentration. These solute carriers are composed of 43 families [39] with each solute carrier member transporting a specific substrate such as sugar, amino acid, oligopeptide, organic anion, and organic cation [38]. These transporters also play a critical role in various physiological processes including importing and exporting nutrients, neurotransmitters, and metabolites [40]. Organic anion/cation transport systems are of special importance due to their role in transporting anticancer drugs at the BBB.

Receptor-mediated endocytosis is usually a three-step process that involves receptor-mediated endocytosis at the luminal (blood) side followed by intracellular movement and exocytosis at the abluminal side of the brain endothelial cells [41]. This highly specific energy-dependent transport is responsible for the uptake of many different ligands including hormones, growth factors, enzymes, and plasma proteins. Other implicated receptors include transferrin receptors, insulin receptors, lipoprotein-related protein 1, and lipoprotein-related protein 2 receptors as well as diphtheria toxin receptor [42]. This transport mechanism allows for large molecules to be transported across the BBB, which is potentially a useful mode of delivery for anticancer therapy [43].

Although some features of the BBB are retained, others are significantly altered in brain tumors. Several key assembly proteins of the tight junctions in primary brain tumor capillary endothelial cells were either downregulated or lost [44]. These changes result in the disruption of the tight junctions leading to "leaky" BBB allowing for possible entry of chemotherapy into sections of the tumor, while other parts of the tumor have an intact BBB posing a therapeutic dilemma [45, 46]. Other alterations of the BBB include enlargement of the perivascular space, slight swelling of the basal lamina, and increase in number of fenestrations and pinocytic vacuoles. Some of these fenestrations may be explained by the lack of normal astrocytes supporting the cerebral endothelium within the tumor [37].

Similar to variability in permeability of the BBB, there are regional differences in tumor blood flow. Degree of vascular permeability and capillary density varies not only within a tumor, but also with the type of tumor. There is great heterogeneity in vascular permeability in high-grade gliomas with the outer rim containing many angiogenic vessels, while the central areas tend to have low capillary density leading to the central area of hypoxia and necrosis. The area of brain surrounding the tumor mass that contains the leading zone of the infiltrative glioma cells has relatively normal vascular permeability and density [3].

3.4 Mechanisms of Drug Resistance

3.4.1 Drug Pumps

P-Glycoprotein (P-gp/ABCB1)

P-glycoprotein (P-gp) is a 170-KDa glycosylated membrane protein encoded by the multiple drug resistance 1 (MDR1) gene. P-gp is expressed in healthy tissues including those involved in drug absorption, distribution, and elimination, namely, the small intestine, the BBB, the liver, and kidney [47, 48]. P-gp is mainly expressed in the luminal membrane of brain capillaries [49, 50]. It extrudes substrates back into the circulation after they initially diffuse into the endothelial cell membrane thereby restricting their penetration into the brain. P-gp has also been detected in blood vessels that supply human gliomas and metastatic brain tumors but at reduced levels compared to those at the BBB [51].

3.4.2 Multidrug Resistance-Associated Proteins

Multidrug resistance-associated proteins (MRPs) are predominantly organic anion and neutral organic compound transporters. While they are also ATP-dependent transporters, some require the presence of cofactors for transport [52]. There are multiple subsets of MRP: MRP1 and MRP4 are present in the blood-facing membrane of the human choroid plexus epithelial cells. MRP1, MRP4, and MRP5 have been identified in endothelial cells from brain tumors [53–55]. MRP3 has been detected in glioma capillaries [53, 55] but not in normal brain endothelial cells [56].

In an MRP4 knockout mouse model, topotecan was not only elevated in the brain but also in the CSF emphasizing the important role that MRP4 plays in determining the CNS distribution of the drug [57]. There is growing evidence that increased MRP expression may be a factor in intrinsic or acquired drug resistance in a subset of brain tumors.

Breast Cancer Resistance Protein (BCRP/ABCG2)

Breast cancer resistance protein (BCRP) was originally identified in a breast cancer cell line which exhibited resistance to anthracyclines [58]. Subsequent studies have shown that BCRP is expressed on the luminal membrane of endothelial cells lining the brain [59].

Topotecan is an excellent substrate of BCRP and a moderate substrate of P-gp. de Vries et al. [60] demonstrated the brain to plasma area under the curve ratio was 3.2-fold higher for topotecan in mice with knockout P-gp and BCRP.

In vitro evidence suggests imatinib mesylate may also be effective in the treatment of malignant gliomas owing to its potent inhibition of platelet-derived growth factor receptor (PDGFR-α and

PDGFR-β) [61]. However, clinical studies suggest that imatinib mesylate has minimal activity in the treatment of malignant gliomas in humans [62]. Imatinib can reach intratumoral concentrations similar to those or higher than in plasma in regions of glioblastoma where the BBB is disrupted as indicated by contrast enhancement on magnetic resonance imaging [63]. Even though imatinib may reach the tumor, the activity of BCRP may explain its minimal efficacy in the treatment of malignant gliomas.

Other Transporters

Organic anion transporting polypeptides (OATPs) are sodium-independent, multi-specific anions exchangers that exchange a drug for another ion or molecule. OATP-mediated transport can be bidirectional and depends on local substrate gradients. Among OATP family members, four transporters have been identified at human blood–brain interfaces. OATP1A2 and OATP2B1 are localized at the luminal membrane of brain endothelial cells [54], whereas OATP3A1 is expressed in the choroid plexus [64]. OATP1A2 and 2B1 have been detected in the blood–tumor barrier in gliomas and may affect the availability of chemotherapeutic drugs to tumor cells [54]. OATP substrates are anionic amphipathic molecules with molecular weights greater than 450 Da and a high degree of albumin binding [65].

The organic anion transporters (OATs) are anion exchangers. The localization of most OATs in the brain is unclear although OAT3 and OAT1 are found in epithelial cells of the human choroid plexus [66]. The contribution of individual OATs to the brain disposition of their substrates is currently unknown.

Organic cation transporters (OCTs) are expressed in human brains with localization to neurons and glial cells and not to endothelial cells [67]. OCTs mediate the bidirectional transport of small, hydrophilic, positively charged compounds.

3.4.3 Glutathione-Related Mechanisms

Glutathione and its related enzyme complex are known to protect tumor cells against chemotherapy-induced damage. This system includes glutathione, glutathione-related enzymes including glutathione *S*-transferase, and transporters that export glutathione–drug complexes. The combination of glutathione with chemotherapeutic agents leads to a conjugation reaction that is catalyzed by glutathione *S*-transferase, leading to the formation of less toxic and more water-soluble conjugates. Drug families known to be susceptible to this resistance system include chloroethyl nitrosoureas, platinum compounds, anthracyclines, and phosphamides [68].

The glutathione/glutathione *S*-transferase system has been shown to play a role in drug resistance of gliomas through both a change in glutathione *S*-transferase and altered intracellular glutathione concentrations. A potential relationship exists between survival and the level of expression and subcellular localization of glutathione *S*-transferase in patients with high-grade gliomas [69, 70].

3.4.4 O^6-Methylguanine-DNA Methyltransferase

O^6-Methylguanine-DNA methyltransferase (MGMT) is a single-enzymatic pathway which repairs DNA adducts at the O^6 position of guanine in a stoichiometric fashion. It is self-inactivated and has been termed a “suicide enzyme” [71, 72]. MGMT is associated with resistance to alkylating agents, and modulation of this enzyme has been under investigation for over two decades [71, 73]. MGMT rapidly reverses alkylation, including methylation, at the O^6 position of guanine by transferring the alkyl group to the active site of the enzyme. Expression levels of MGMT vary considerably between organs with relatively low levels in the brain and high levels in the liver

MGMT activity has been extensively studied and has been shown to have an effect in patients treated with the alkylating agent temozolomide in patients with gliomas. Low MGMT levels have been shown to correlate with progression-free survival in temozolomide-treated gliomas, primarily GBM [74, 75]. Immunoexpression of MGMT is associated with poor response in those receiving therapy with radiation and temozolomide, whereas tumors lacking reactivity show marked tumor shrinkage and clinical improvement. Lower MGMT expression is associated with better outcome following temozolomide therapy [76].

MGMT promoter methylation was found to be a favorable prognostic factor in patients with GBM treated with temozolomide lowering the risk of death by 55 % in comparison to patients without MGMT promoter methylation [77]. Patients who do the best are the ones who have methylated MGMT and receive temozolomide [78–81].

3.4.5 Topoisomerase II

Topoisomerase II, which consists of α and β isoenzymes, is involved in negative supercoiling of superhelical DNA, allowing for replication, recombination, and transcription of nuclear DNA. Alteration of the topological state of DNA by topoisomerase II allows accessibility of DNA to drug-mediated alkylation. Topoisomerase IIα causes drug resistance to a number of chemotherapeutic agents including both those directly affecting topoisomerase II and those that do not such as doxorubicin, mitoxantrone, cisplatin, and BCNU. Altered activity is involved in the development of drug resistance in a subset of intracranial tumors including high-grade gliomas and medulloblastomas [82, 83]. The mechanisms by which topoisomerase II can affect drug sensitivity include (1) reduction in topoisomerase II concentration resulting in decreased access of DNA to the effects of chemotherapy, (2) altered topoisomerase II subcellular localization, and (3) mutations in the structural topoisomerase II gene leading to drug-resistant variants (see Table 1).

Table 1 CNS malignancies and clinical pharmacology

Achieving optimal plasma concentrations	Crossing the BBB	Entering and affecting the tumor	Effects on the normal brain	Methods to increase drug delivery	Methods to document drug delivery
EIASD	Integrity of BBB	Local blood supply	Neurotoxicity	High dose systemic Rx	Surgical
Glucocorticoids	MW	Hypoxia		Biochemical disruption of BBB	Microdialysis
	Lipid solubility	Pumps		Intra-arterial therapy	Imaging: MRS, PET, SPECT,
	Charge	Drug resistance mechanisms		Osmotic BBB opening	
	Pumps	Chemotherapy resistant tumors		Intrathecal therapy	
	Protein binding			Intratumoral and intracavitary Rx	
	Agents that restore BBB integrity (glucocorticoids and anti-VEGF therapies)			Convection enhanced delivery	

4 Drug Delivery

4.1 Major Factors Influencing Drug Delivery in Brain Tumors

As is evident from the sections above, delivering therapeutic concentrations of antineoplastic agents to cancer cells within the CNS is challenging. Barriers to effective delivery occur within the systemic circulation, the BBB, and the tumor itself. In order for the active agent to have access to the CNS, it must first reach adequate concentrations within the plasma and can be affected by drug interactions such as those seen in patients with brain tumors on EIAED or glucocorticoids. In addition, it is likely only free drug that will have access to the CNS; thus a highly protein-bound drug is unlikely to reach the brain tumor tissue in adequate concentrations. A series of other important factors play a role at the level of the BBB. These include relative integrity of the BBB within and around the tumor and the administered agent's molecular weight, lipid solubility, and charge, whether the agent is a substrate for one of the transporters or drug resistance mechanisms. The ability of a drug to penetrate the brain also depends on its ability to penetrate the lipid component of the endothelial cell membrane of the BBB. The octanol/water partition coefficient, a physicochemical measure of the lipophilic nature of a compound, is one measure of the permeability of a drug at the BBB [84]. The permeability and the rate of cerebral blood flow control the rate of transfer of drugs whose octanol/water partition coefficient lie between 1 and 0.1 [85]. If a drug's octanol/water partition coefficient is >1 ($\log p > 0$), then its rate of transfer across the BBB is rapid and mainly limited by the availability of the drug or the blood flow rate. On the other hand, if a drug's octanol/water partition coefficient is <0.1 ($\log p < -1$), the permeability across the BBB is low and is limited from entering the brain. At the level of the tumor, issues regarding local blood supply, tissue hypoxia, tumor cell-related transporters, and drug resistance mechanisms can also alter availability at the site of action. In addition, most brain tumors are quite resistant to chemotherapeutic agents even in tissue cultures where drug delivery is not an issue.

4.2 Routes of Drug Administration

Therapeutic agents for the treatment of brain cancer can be administered systemically via oral, intravenous, or intra-arterial routes or they can be delivered more locally using intrathecal or intraparenchymal approaches.

4.2.1 Systemic Administration

Despite decades of clinical trials using systemically administered agents, only nitrosoureas, temozolomide, and bevacizumab are currently approved for the treatment of high-grade gliomas. Until recently, BCNU was the primary chemotherapy used in the therapy of brain tumors. BCNU is a highly lipophilic drug which allows transmembrane transfer by passive diffusion rather than by active transport. However, in large phase III trials comparing radiation alone to radiation plus BCNU, no difference was seen in median survival or survival at 12 or 24 months [86, 87]. Similarly, studies using combination chemotherapy with CCNU, procarbazine, and vincristine (The Medical Research Council Brain Tumour Working Party [88]) provided no survival advantage over radiation therapy alone in glioblastoma or anaplastic astrocytoma [89, 90]. Two meta-analysis of clinical trials for high-grade gliomas subsequently demonstrated a very modest increase in 1-year survival of 6 % and in 2-year survival of 5 % from the addition of a nitrosourea [91, 92]. However, these marginal improvements were accompanied by substantial toxicities with no curative potential.

Temozolomide is well absorbed after oral administration [93]. The average volume of distribution is reported to be 17 l/m^2, and the half-life is approximately 1.8 h [93, 94]. Following oral administration, temozolomide is hydrolyzed in aqueous solution to methyl-triazeno-imidazole-carboxamide (MTIC) which is rapidly converted to the inactive 5-aminoimidazole-4-carbozamide (AIC) and to the electrophilic alkylating methyldiazonium cation that transfers a methyl group to DNA [95]. The DNA methyl adducts are responsible for cytotoxicity. Alkylation of the O^6 position of guanine accounts for only about 5 % of DNA adducts but is primarily responsible for the cytotoxic effects of temozolomide. The O^6-methylguanine (O^6-meG) lesion leads to DNA double-strand breaks and subsequent cell death via apoptosis and/or autophagy [96–98]. MGMT repairs the O^6-meG lesion such that high levels of MGMT are thought to contribute to temozolomide resistance [77, 99].

Temozolomide CSF concentrations reach 30–40 % of plasma. Microdialysis catheters were used to measure temozolomide concentrations in the brain, demonstrating a T_{max} of 2.0 ± 0.8 h.

Temozolomide is currently used as the primary chemotherapeutic agent used in patients with high-grade gliomas. It was first approved by the FDA in 1999 for patients with recurrent anaplastic astrocytomas with subsequent approval as concomitant therapy with radiotherapy and then as maintenance treatment in newly diagnosed glioblastoma [2]. A recent report demonstrates that the improvements in survival are durable with improved results lasting over 5 years for newly diagnosed glioblastoma patients [7]. Similar studies are now underway to determine if these results can be duplicated in patients with anaplastic astrocytoma, anaplastic oligodendroglioma, and low-grade gliomas.

The FDA approved the use of bevacizumab in May of 2009 for single-agent use for recurrent and progressive GBM [26, 27, 100]. This approval was based on improvements in patient outcomes and MRI scans, but without survival data. GBMs are highly vascular tumors which overexpress VEGF, and this overexpression has been linked with poor prognosis [101, 102]. VEGF was originally discovered as a factor inducing vascular permeability [103] but was subsequently found to be an important endothelial cell mitogen [104, 105]. VEGF is transcriptionally upregulated under hypoxic conditions by hypoxia inducible factor 1α (HIF1α). It is mostly expressed by tumor cells and to a lesser extent by stromal cells and vascular endothelium. VEGF increases vascular permeability allowing plasma proteins and other circulating macromolecules to cross the endothelium [106]. This subsequently leads to increased interstitial fluid pressure and increased vasogenic edema around the tumor [23]. Endothelial cells are stimulated by VEGF to proliferate and to migrate. Bevacizumab binds to VEGF and prevents the proliferation of endothelial cells and formation of new blood vessels [107]. Clinical experience with bevacizumab suggests that it repairs BBB integrity thereby reducing contrast enhancement, mass effect, and peritumoral edema on MRI scans. This reduction in peritumoral edema results in clinical improvements in patients as seen with the use of high doses of glucocorticoids. Studies with other short-acting oral anti-VEGF agents demonstrate that these rapidly repair BBB integrity, but if they are withdrawn, the BBB quickly reverts to its prior state. These data suggest that bevacizumab has its major effects on the blood vessels of the brain tumor. Recent data suggests that this agent may favor a more invasive tumor phenotype and that the potential short-term benefits on the BBB could be outweighed by further tumor dissemination. Randomized prospective trials that have been presented, but not yet published, have evaluated the use of combination of radiation, temozolomide, and bevacizumab in the front line setting in patients with newly diagnosed GBM with no improvement in overall survival observed.

High-Dose Chemotherapy

High-dose systemic chemotherapy with autologous bone marrow or peripheral stem cell rescue has been considered as a treatment paradigm for chemotherapy-sensitive brain tumors. Studies utilizing this approach used either high-dose BCNU [108, 109], high-dose BCNU with intra-arterial cisplatin [110],

or combination of BCNU, etoposide, and thiotepa [111, 112]. Most of these efforts were uncontrolled with small patient numbers and selected patient populations making it difficult to determine if survival advantages were valid. Local tumor concentrations of the drugs were not measured. Moreover, these regimens were associated with a 5–20 % toxicity and mortality rates [113, 114]. As a result, this approach has not been adopted by the medical community.

High-Dose Methotrexate

Primary CNS lymphomas were originally treated with radiation therapy alone which resulted in high initial response rates, median survivals of 1 year, and uniformly devastating neurotoxicity in the few long-term survivors. Systemic lymphoma regimens such as CHOP resulted in early responses followed by rapid progression once the integrity of the BBB was restored and no improvement in median or long-term survivors. Subsequently, high-dose methotrexate-based regimens have gradually replaced radiation-based treatment approaches. These regimens do not result in long-term neurotoxicity and have increased the median survival to over 4 years with about 30 % of patients being long-term survivors. Of note, ocular and CSF relapses with this regimen are very unusual [11, 12]. Cerebral drug penetration of methotrexate is greater in contrast-enhancing tumor than nonenhancing tissue using microdialysis techniques [115]. Methotrexate concentrations in extracellular fluid exceeded 2 μM for 20–26 h in both regions of the tumor. While high-dose methotrexate appears critical to the treatment of patients with primary CNS lymphoma, it did not result in responses in patients with recurrent glioblastoma. Ultimately, this approach could be of value if a suitably active agent was identified.

Intra-arterial Administration of Therapy

Intra-arterial delivery of chemotherapy increases a drug's systemic concentration by eliminating first pass metabolism. Intra-arterial injection also allows the tumor perfused by a specific arterial supply to receive a higher concentration of the chemotherapy. BCNU was studied extensively as an intra-arterial agent in brain tumor trials. Initial reports were positive prompting the development of a phase III, four-arm randomized study conducted by the Brain Tumor Cooperative Group [116]. This study enrolled 315 patients with malignant glioma who were randomized after surgery to receive 200 mg/m^2 BCNU intra-arterial versus IV every 8 weeks with or without the addition of 5-FU. All patients then received full-dose radiotherapy. The patients treated with intra-arterial chemotherapy developed serious toxicities (9.5 % incidence of fatal leukoencephalopathy, 15.5 % of unilateral amaurosis) and had shorter survival compared with the IV arm (11.2 vs. 14 months).

Cisplatin is a hydrophilic drug with low penetration into the brain but reaches two- to threefold higher concentration in brain tumors after intra-arterial injection. A randomized phase II study of the Brain Tumor Cooperative Group studied the survival following intra-arterial administration of 60 mg/m^2 of cisplatin with 100 mg/m^2 of the nitrosourea PCNU given IV every 8 weeks in a total of 311 patients recurring after radiotherapy [117]. Overall survival was found to be shorter in the intra-arterial arm (10 vs. 13 months). Ocular toxicity was encountered in the intra-arterial arm (7.2 %) along with nausea/vomiting and different forms of neurotoxicity. Systemic toxicities are usually modest due to lower drug exposure [118].

Other agents that have also been used for intra-arterial administration include methotrexate [119] and carboplatin [120, 121]. As this approach is invasive and associated with excess toxicities and the results have proven no better than with intravenous drug administration, it is not currently being used in patients with primary brain tumors.

Hyperosmolar Disruption of the BBB

A modification of the intra-arterial approach has been studied where the BBB is transiently opened using a hyperosmolar agent prior to the intra-arterial injection. This has been shown to increase drug CNS concentration while preserving neurocognitive function and minimizing systemic toxicity [122]. The transient disruption of the BBB is achieved by delivering pre-warmed 25 % mannitol via internal carotid artery or vertebral artery at a predetermined flow rate (3–12 ml/s) after which the intra-arterial chemotherapeutic agent is infused. These hypertonic agents cause vasodilation and shrinkage of the endothelial cells, resulting in increased diffusivity and bulk flow across the BBB [123]. Cell shrinkage, along with the contraction of the endothelial cytoskeleton, results in widening of the tight junction to approximate 20 nm, which results in a tenfold increase in permeability of some compounds. An alternative mechanism might be an alteration in Na^{+}–Ca^{2+} exchange which might play a role in the osmotic disruption of the BBB [124]. The effects of hypertonic mannitol on BBB disruption lasts for about 30 min with the BBB function remaining impaired for several hours afterwards [125].

Biochemical Disruption of the BBB

Many vasoactive compounds such as leukotrienes, histamine, 5-hydroxytryptamine (5-HT), TNF-α, and bradykinin [126] are capable of modifying the permeability of the cerebral microcirculation, but the relevant systemic effects and/or rapid catabolism contraindicate their administration to the brain. RMP-7, also known as Cereport is a synthetic bradykinin-derived nonapeptide that is resistant to angiotensin-converting enzyme. After intra-arterial or IV administration, it induces rapid and reversible dilatation and permeability of the blood–tumor barrier, leaving the BBB of the normal nervous system intact with no systemic toxicity [127]. It selectively binds to the B2 bradykinin receptor and, via the generation of the nitric oxide, it activates G-proteins to trigger downstream signal transduction pathways thereby increasing intracellular Ca^{2+} and phosphatidylinositol turnover [128]. A loosening and disengaging of tight junctions has been visualized by electron microscopy [129]. In murine brain tumor models, RMP-7 increases the uptake of hydrophilic compounds in a wide range of molecular weights. After about 30 min, the permeability effect rapidly disappears due to receptorial tachyphylaxis which does not produce interstitial edema. Therefore, RMP-7 cannot be used for prolonged infusions and its administration should follow intra-arterial or IV chemotherapy injection by approximately 10 min just when the brain endothelium is expected to be exposed to the higher concentration of the cytotoxic agent [130].

The use of RMP-7 with carboplatin has conflicting results. Whereas a 32 % response rate was reported in 45 chemo-I malignant gliomas treated with IV carboplatin concomitantly with RMP-7 [131], no responses were seen in another clinical trial of 46 patients with recurrent glioma using RMP-7 and carboplatin [132]. Major side effects with the use of RMP-7 have been transient flushing, headache, and abdominal pain. Given the variable results of most trials, RMP-7 has also not been routinely implemented into clinical practice. However, it is possible that this approach could be useful in opening the BBB and providing enhanced access of some systemically administered agents to CNS malignancies.

4.2.2 Intrathecal Therapy

Access to the cerebrospinal fluid (CSF) for the administration of chemotherapy can be via lumbar puncture or an implanted subcutaneous reservoir attached to a catheter that goes to the lateral ventricle. Humans have a total approximately 140 cm^3 of CSF with the subarachnoid space at any time. However, approximately 5–8 times as much CSF is created daily by the choroid plexus. As a result

there is a continual flow of CSF from the lateral ventricles to the third and fourth ventricles, down the spinal cord, and then up over the cortical convexities where it empties into the systemic circulation via the arachnoid granulations. When cancer cells gain access or chemotherapy is administered to the CSF, they are carried by the flow of this fluid throughout the neuroaxis. Tumor cells frequently obstruct CSF flow pathways causing increased intracranial pressure when CSF is continually produced but cannot leave the subarachnoid space normally [133]. As a result, CSF flow scans are often indicated before intrathecal chemotherapy is initiated. If there is tumor blocking CSF flow from the ventricles, in the spinal canal, and/or over the cortical convexities, radiation is usually used to open the pathways. This is important as a block to ventricular outflow would result in the brain adjacent to the ventricles receiving toxic doses of methotrexate and the spinal CSF would never receive methotrexate administered through an Ommaya reservoir.

Currently only methotrexate, thiotepa, and cytarabine are routinely used for intrathecal therapy. Patients with leptomeningeal metastases generally have very short survivals and it is not clear that the intrathecal therapy provides a survival advantage. Quantitative autoradiography studies have documented that intrathecally administered chemotherapy penetrates only a thin layer of brain adjacent to the CSF and thus this administration route is not useful for patients with intraparenchymal tumors [134].

4.3 Local Administration

4.3.1 Intratumoral and Intracavitary Therapy

Langer and Folkman [135] were the first to describe the concept of implantable polymers able to release chemotherapeutic agents directly into the CNS thus bypassing the BBB. They reported the sustained and predictable release of macromolecules from a nonbiodegradable ethylene vinyl acetate (EVAc) copolymer. A drug incorporated into the polymer would be released by diffusion through the micropores of its matrix. This polymer delivery system was limited by its permanence at the site of implantation in the body. Following international phase III trials, biodegradable polymers containing BCNU (Gliadel) were approved by the FDA for patients with recurrent high-grade gliomas in 1996 and for newly diagnosed patients with high-grade gliomas in 2003. The survival advantages of the BCNU containing polymer to standard therapy are modest but no comparison to intravenous BCNU was performed. The toxicity profile for BCNU wafer is very mild with a slight increased risk of seizures during the first 5 days after implantation and uncommon wound dehiscence, delayed wound healing, and subdural or wound effusions. The hematologic side effects from the BCNU wafers are far less than with the systemic administration of BCNU as pharmacologic studies document that plasma BCNU concentrations are minimal after the wafer implants.

4.3.2 Convection-Enhanced Delivery

Another approach to regional drug delivery is convection-enhanced delivery (CED) [136]. One or more catheters are stereotactically implanted through a burr hole into or adjacent to either the enhancing portion of a tumor or the nonenhancing infiltrative surrounding tissue. A pressure-driven flow of drug and solute is achieved via an infusion pump, and the agent is directly infused into the target tissue at a predetermined concentration, rate, and duration. The increased interstitial fluid pressure observed in brain tumor creates a pressure gradient that drives the infusate out of high-pressure areas within the tumor into relatively low-pressure areas in surrounding normal tissues. The advantages to CED include bypassing the BBB and infusing therapeutic agents with both small and large molecular weights. It also provides the targeted delivery into a specific region where the catheter is placed.

Systemic toxicity as well as neurotoxicity may be limited by the direct delivery of the chemotherapeutic agent [137]. CED within the defined infusion does not produce cerebral edema or measurable increases in intracranial pressure [138]. However, the marked heterogeneity of drug distribution within the tumor itself is a potential limiting factor in the use of this therapeutic approach [139].

Both paclitaxel and topotecan have been used as chemotherapeutic agents in CED. Lidar et al. [140] examined the efficacy of intratumoral CED of paclitaxel in 15 patients with GBM. Fifteen patients received a total of 20 cycles of intratumoral CED of paclitaxel. The patients were observed daily by performing diffusion-weighted magnetic resonance (MR) imaging to assess the convective process and routine diagnostic MR imaging to identify the tumor response. Effective convection was determined by the progression of the hyperintense signal within the tumor on diffusion-weighted MR images, which corresponded to a subsequent lytic tumor response displayed on conventional MR images. Of the 15 patients, 5 complete responses and 6 partial responses were observed, giving a response rate of 73 %. Lack of convection and a poor tumor response were associated with leakage of the convected drug into the subarachnoid space, ventricles, and cavities formed by previous resections and were seen in tumors containing widespread necrosis. Complications of the procedure included transient chemical meningitis in six patients, infectious complications in three patients, and transient neurological deterioration in four patients. Topotecan has also been studied in phase I trials using CED in intratumoral cavity; however, results of the trial have not been published as of yet.

5 Methods of Evaluating Drug Delivery to CNS

Serum pharmacokinetic and pharmacodynamic information of a systemically administered agent are usually insufficient to determine if the drug will reach the intended target in the CNS at therapeutic concentrations. As a result, efforts to definitively determine intratumoral drug concentrations of investigational agents are increasingly being recognized as an important step in early drug development. To meet this endpoint, there are different techniques being utilized. The first includes surgical resection and analyzing drug concentrations in the resected specimens. Patients can be treated with a drug prior to a planned resection, and the resected tissue can be sampled for the drug concentration and markers of drug activity. This approach gives a direct sample and visualization of the effect of a drug; however, it is clearly invasive and allows for assessment of a drug concentration at only one point in time.

Placement of a microdialysis probe is another approach for providing detailed pharmacokinetics of systemically and locally administered drugs and is discussed further in Use of Microdialysis in Preclinical and Clinical Development of Anticancer Agents Chapter. This technique has been used in preclinical trials to assess drug pharmacokinetics within a tumor and in brain cancer patients [115, 141]. Its use is limited due to the invasive nature of placing a catheter within the brain, the limited area of sampling around the catheter, and the time and expertise required to have this system work properly.

Brain imaging is another approach whereby the delivery of a drug into the CNS may be monitored by noninvasive means. Molecular imaging of targeted anticancer agents is used to measure the area of distribution and degree of target inhibition for critical tumor pathways using nuclear medicine techniques [142]. Standard magnetic resonance imaging (MRI) can also be used to measure delivery of drugs to a tumor particularly with agents known to shorten T1 [143]. Novel MRI sequences that measure cerebral blood volume are also being used to assess tumor vasculature after exposure to antiangiogenic agents [144]. Other methods of evaluation include positron emission tomography (PET) imaging using radiolabeled therapeutic agents and magnetic resonance spectroscopy (MR spectroscopy). These imaging modalities are increasingly being used in preclinical and clinical studies to evaluate drug penetration into the CNS.

6 Conclusion

Improving the treatment outcomes for patients with CNS malignancies poses a series of difficult challenges. The EIAED and glucocorticoids may make it difficult to achieve adequate plasma concentrations of systemically administered agents. The presence of the BBB requires careful consideration of each agent's molecular weight, lipid solubility, charge, and protein binding and whether it is likely a substrate for the common BBB-associated efflux pumps. Furthermore the use of agents which restore the integrity of the BBB such as dexamethasone or anti-VEGF strategies could limit drug penetration into the tumor. High-grade gliomas in particular have regions of hypoperfusion and hypoxia, have inherent drug resistance mechanisms, and are relatively chemotherapy resistant at baseline. All this leads to attempts to increase drug delivery to the brain tumor. This has been done by using high doses of systemic chemotherapy, biochemically modifying BBB integrity to allow more drugs across this barrier, or using intra-arterial or intraparenchymal delivery techniques. Delivering higher concentrations of anticancer agents to the brain is obviously associated with a higher risk for neurotoxicity.

However, the major challenge at this time is learning which approach is likely to get more drugs into the tumors. Negative clinical trials are of little value if concerns remain about whether the administered agent reached the tumor in therapeutic concentrations. As a result, considerable effort must be devoted to surgical, microdialysis, and imaging efforts to quantify the concentrations of novel therapeutic agents within brain tumors. Clinical pharmacologists have much to contribute to this effort.

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Radiation and Altering Clinical Pharmacology

DeeDee Smart and Kevin Camphausen

Abstract Ionizing radiation is a widely used therapeutic option in both curative and palliative cancer treatments, as well as in specialized benign conditions. Because the majority of cancer patients will receive radiation at some point in their treatment course, and because radiation is often given concurrently or sequentially with chemotherapy, it is critically important to understand the biology of how ionizing radiation affects tumor cells as well as normal tissue, how pharmacotherapy may alter the effectiveness of radiation, and how radiotherapy may augment standard pharmacologic interventions.

Keywords Ionizing radiation • Radiosensitizer • Radiation modifier • Clonogenic survival

1 Introduction

Radiation therapy is used as a localized treatment modality for a wide variety of malignant and benign diseases and is frequently used for both definitive therapy and palliation. Annually, approximately 1.5 million cancer cases are diagnosed [1] with more than 500,000 patients per year receiving courses of radiation treatment [2]. Additionally, 85 % of cancer patients will receive radiation therapy as a component of their treatment during their disease course. The total dose of radiation delivered and the number of treatments in which this total dose is delivered vary depending on the indication. Ionizing radiation is employed because it can be absorbed in tissues and produces disruptions in atomic structure, which, in turn, produces chemical and biological damage on the subcellular level. The mechanism of action of ionizing radiation appears to be multifold. The ability of X- and γ-rays to hydrolyze water produces breakage of chemical bonds, particularly within DNA [3]. This fixation of double-stranded DNA breaks leading to mitotic catastrophe is the most supported mechanism of radiation-induced cell death [4]. Apoptosis in response to radiation occurs less often, mostly in cell populations that have diminished repair capacities such as lymphoma and leukemias [5]. However, in addition to effects on the nucleus, there is growing evidence to suggest that oxidation of the lipid bilayer [6], changes in microvascular permeability [7] cell–cell junctional complex rearrangements [8], and mitochondrial alterations inducing additional oxidative stress [9] are also subcellular targets for ionizing radiation. Because of these effects, radiation has the capacity to alter tumor microenvironment,

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cellular architecture, permeability of tumor vasculature and permeation of drugs within the tumor, and produce biochemical alterations which allow for additive or synergistic cell killing in combination with pharmacologic agents. Chemoradiation has demonstrated particular success with cancers of the gastrointestinal tract, gliomas, and squamous cell carcinomas of the head and neck, situations in which the amount of radiation alone that would be required to destroy a tumor would greatly exceed normal tissue tolerance, otherwise rendering radiation therapy as a single modality ineffective and highly toxic [10]. The benefit of a chemoradiation approach combines systemic treatment for gross tumor and micrometastatic control of subclinical disease (potentially outside the radiation field) at the same time as augmenting the effectiveness of localized radiation therapy.

1.1 Fractionation Versus Single Dose

The ability of ionizing radiation to successfully treat a cancer is dependent on producing adequate cell killing within the target without destroying the normal tissues in the path of the ionizing radiation. The concept of fractionation involves the division of a total prescribed radiation dose over a defined period of time. In vivo, fractionation allows for multiple logs of tumor cell killing while also allowing time for normal tissue repair to occur [11]. Standard daily radiation fraction doses of 1.8–2 Gy (Gy, a standard measure of absorbed dose) are used in a wide variety of regimens where concurrent chemotherapy is delivered. Altering fractionation schemes to allow multiple radiation treatments per day within the same total treatment time is defined as hyperfractionation and may be used in specialized indications to minimize normal tissue toxicities or to overcome tumor cell repopulation between radiation fractions, as is the case with certain head and neck cancers or small-cell lung carcinoma, respectively. Alternatively, delivery of larger doses of radiation per treatment to shorten the total time required for administration of a prescribed dose is defined as hypofractionation and may be used in clinical situations to overcome increased repair capacity of a tumor, as with melanoma [12]. Large fraction sizes (>4 Gy), such as those used for stereotactic body radiation or stereotactic radiosurgery, may also have a secondary effect on the tumor vasculature leading to vascular collapse and tumor necrosis [13], as compared to standard fractionation. However, doses >4 Gy at a time are classically limited to cases where the extent of disease is easily defined and where the volume of disease is limited and in a location that can tolerate the treatment. However, regimens with nonstandard fractionation in combination with sensitizing therapies remain largely untested, principally due to concerns for increased toxicity without a defined, clear benefit. Nevertheless, this is an area of radiation therapy where pharmacodynamics and pharmacokinetics of therapeutic agents in combination with radiation remains promising for future investigation.

1.2 Oxygen and Cell Cycle Effects

Hypoxic cells require higher doses of radiation to produce the same amount of cell kill [14]. As initial radiation doses induce lethal damage within the well-oxygenated outer cell layers of the tumor, the tumor volume decreases, allowing for reoxygenation of previously hypoxic-cell populations which increases efficiency of killing with subsequent radiation fractions. Attempts to sensitize hypoxic cells to the effects of radiation have focused on delivery of compounds to the tumor that mimic the bio chemical effects of oxygen. Pharmacologic agents to improve the effectiveness of radiation in hypoxic cells in vitro and in animal models have included halogenated pyrimidines, misonidazole, quinine antibiotics, nitroaromatic compounds, and benzotriazine di-N-oxides [15]. However, these agents have so far met with limited clinical success due to limited potency or their toxicity at biologically relevant

concentrations, and despite further investigation into less toxic bioreductive agents, hypoxic-cell sensitizers with distinctive biolocalization properties, and allosteric modifiers of hemoglobin [16].

Attempts to increase oxygenation of tumor cells through the use of supplemental oxygen or hyperbaric oxygen have seen promising results in animal studies [17]. In experimental tumors, carbogen breathing together with nicotinamide can enhance oxygenation and override hypoxic-cell radioresistance, particularly in shorter than conventional radiation regimens. Phase II trials of accelerated radiotherapy, carbogen, and nicotinamide showed a high level of tumor control for advanced head and neck squamous cell carcinoma and bladder cancers, although outcomes demonstrating a clinically relevant impact on tumor control in these patients as compared to standard therapy are pending [18].

2 Radiation Sensitizer Versus Radiation Modifier

A radiation sensitizer is an agent which increases the sensitivity of cells to radiation [3]. An ideal radiosensitizer would not have any cytotoxic effects on its own, being inert to both normal and tumor cells. However, most agents have a measureable amount of cytotoxicity which is separate from their effects in altering the cellular response to radiation damage. Thus, agents that have inherent cytotoxicity which additionally produce increased sensitivity to a dose of radiation are often referred to as radiation modifiers. When using in vitro clonogenic survival assays, it is traditional that when treating with a drug alone, a radiation sensitizer has a plating efficiency (PE) >90 % and a radiation modifier has a PE between 40 and 90 % [19].

The response of solid tumors to radiation depends primarily on three factors: (1) the intrinsic radiosensitivity of the tumor cells, (2) the oxygenation of the tumor cells, and (3) the number of tumor cells undergoing division between radiation treatments. Modification or changes in any of these factors would be expected to modify the radiation response of tumors. However, only modifiers of intrinsic radiosensitivity can be assessed with the use of standard in vitro studies. Therefore, many candidate radiation modifiers, such as those that change tumor oxygenation, tumor immunogenicity, tumor stroma, or tumor vascularity, must be assayed in vivo or in carefully defined in vitro conditions (e.g., hypoxic conditions or as spheroids) [20].

Traditional "radiosensitizers", cytotoxic chemotherapeutic agents such as 5-fluorouracil, cisplatin, and taxanes, are administered on a regimented schedule to optimize the interaction between the agent and radiation [21–24] and were devised on an empirical approach where the chemotherapy is administered prior to scheduled delivery of radiotherapy. Although often highly effective in experimental models, the results obtained when these combinations are applied in a clinical setting have been generally less than expected, primarily due to concomitant increases in radiation-induced normal tissue injury. Based on an increased understanding of the molecular mechanisms of radioresponse, current efforts to develop strategies for enhancing tumor radiosensitivity have focused on the use of agents that target molecules putatively involved in regulating radiation-induced cell death. However, complicating this approach, it has also become increasingly clear that cellular radiosensitivity is the sum effect of a combination of a wide variety of signaling and effector molecules. The ability of a single molecule to affect radioresponse also varies with changes in the genetic and epigenetic background [25]. Accordingly, there are numerous examples in which targeting a selected radioresponse-associated molecule affects radiosensitivity in a cell-type-dependent manner [19, 26].

The recent explosion in targeted therapies development has made it possible to allow radiosensitization with less toxicity to normal tissues, more effective augmentation of radiation-induced tumor cell death, and more flexible administration routes and regimens compared to traditional cytotoxic chemotherapies [27]. A candidate radiation modifier should demonstrate in vivo tumor radiosensitization using fractionated radiation (2–4 Gy/fraction) with minimal radiosensitization of a normal tissue in two different tumor models. Ideally, the mechanism of action of the agents should be known, and

Table 1 Commonly used and investigational radiation modifiers

Agent	Disease site	Mode of delivery	Mechanism of action	DEF
Gemcitabine	Pancreas	IV	Impaired DNA synthesis and ribonucleotide reductase inhibition	1.30–2.00 [30]
Cisplatin	GI, head and neck ca, lung	IV	DNA cross-linking	1.10–1.80 [31]
5-FU	GI	IV (or oral capecitabine)	Folate metabolism	1.60–2.40 [32]
Taxanes	Lung	IV	Microtubule disruption	1.20–2.40 [33]
Temozolomide	Glioma	Oral	DNA methylation	1.30 [34]
Vorinostat	Mycosis fungoides, breast ca, glioma	Oral	Histone deacetylases	1.50 [35]
Cetuximab	Head and neck	IV	EGFR monoclonal antibody	1.30–1.70 [36]
Bexxar/Zevalin	Lymphoma	IV	CD20 monoclonal antibody	Direct action [37]
17-AAG/DMAG	Cervix, head, and neck	IV	HSP90	1.30–1.70 [38]

The DEF, also known as the DEF_{10}, (dose enhancement factor) is a quantitative measure of this effectiveness and is defined as the ratio of doses of radiation required to produce equivalent surviving fractions, at 10 % survival, in the presence of the drug divided by the dose of radiation required to produce an equivalent surviving fraction in the absence of the drug

the in vivo response of the tumor to a particular agent should be correlated with that mechanism of action. If an agent is intended to modify a particular molecular target, radiosensitization should be correlated with modification of the target [20].

However, given that a single molecule's influence on regulating cellular radioresponse is dependent on a variety of genetic/epigenetic circumstances, the possibility exists that the effectiveness of target-based radiation sensitizers against solid neoplasms could be significantly limited by inter- and intratumor heterogeneity. As a means of reducing the consequences of cell-type specificity, targeting more than one of the potential molecular determinants of radiosensitivity has been suggested as a strategy for increasing the probability and/or degree of radiosensitization. Overcoming such a limitation would involve identifying markers that indicate which tumors may be susceptible to a given target-based radiosensitizer [28] and then using a multipharmacologic approach to improve clinical results.

In either situation, an effective radiation modifier must show a differential effect between tumor cells and normal tissue. When the differential effect is to enhance radiation-induced tumor cell damage, the agent is identified as a radiosensitizer. When the differential effect is to reduce the damage of ionizing radiation on normal tissue, the agent is identified as a radioprotector. Effective agents in either category are developed by exploiting biological differences between normal tissue and tumor. For example, cisplatin, an empirical radiation modifier, induces cell death by cross-linking DNA, thereby taking advantage of the fact that most cancer cells have a higher proliferation rate than the surrounding normal tissue. Conversely, amifostine, a classic radiation protector, distributes preferentially in salivary gland tissue compared to tumor tissue, which makes it useful in reducing the toxicity of xerostomia in radiation treatment of head and neck cancer [29].

Table 1 lists selected radiosensitizers and radiation modifiers that are in clinical use or under clinical investigation.

2.1 In Vitro Models

The clonogenic survival assay is the gold standard for in vitro identification of radiosensitizing and radiation-modifying agents (Fig. 1) where the surviving fraction of cells in a logarithmic distribution is plotted against a linear distribution of varying doses of radiation delivered to the cells in a single

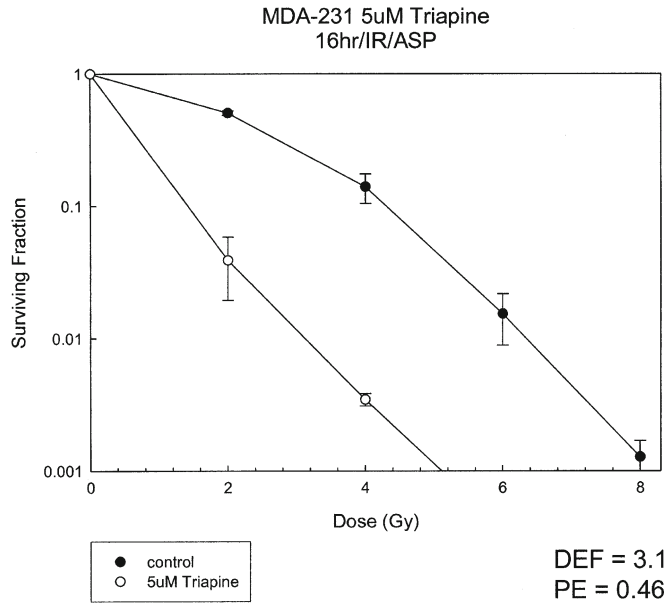

Fig. 1 In vitro clonogenic cell survival assay (original figure, submitted by Camphausen, K). The effects of Triapine on (3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP)) tumor cell radiosensitivity. Cells were exposed to 5 μM Triapine for 16 h before irradiation. Cells were fed fresh growth medium immediately after irradiation. Colony-forming efficiency was determined 10–14 days later and survival curves were generated after normalizing for the cytotoxicity induced by Triapine alone. MDA-MB-231BR cells (5 μmol/L). Points = mean; bars = SD; *DEF* dose enhancement factor, *IR* irradiation, *PE* plating efficiency

fraction, producing a log–linear curve. Radiation sensitizers, by definition, allow fewer number of surviving cells at a given dose of radiation in the presence of the agent as compared to radiation alone. The DEF_{10} (dose enhancement factor), or more simply referred to as DEF, is a quantitative measure of this effectiveness and is defined as the ratio of doses of radiation required to produce equivalent surviving fractions, at 10 % survival, in the presence of the drug divided by the dose of radiation required to produce an equivalent surviving fraction in the absence of the drug. The clonogenic survival assay is the only assay that measures total cell killing whether it occurs by mitotic catastrophe, apoptosis, terminal differentiation, or other modes of cell death [39–44]. This assay measures colony formation from single cells derived from an established tumor cell line that has a high plating efficiency. Typically, a cell line that has a plating efficiency of at least 20 % is required for use in clonogenic assays, which can be one potential limiting factor of the assay, as certain cell lines as well as primary cultures may not have a plating efficiency of greater than 20 %. A second limitation of the clonogenic survival assay is the difficulty in evaluating fractionated radiation in combination with a radiosensitizing agent.

The tetrazolium-based (MTT) and trypan blue exclusion assays are often used in the literature to evaluate the effects of radiation on cell lines in vitro. However, they are typically not appropriate for measuring overall killing of cells associated with radiation modifiers because they only measure the short-term effects of radiation on cells and can underestimate the effects of delayed radiation killing. One exception would be the use of such assays with lymphoid cells, which undergo rapid apoptosis within 4–6 h of exposure to radiation [45]. However, all potential radiation modification candidates should be confirmed by using a definitive clonogenic survival assay prior to further development.

To quantitate radiation-induced double-stranded DNA breaks and repair, standard techniques have traditionally included pulsed-field gel electrophoresis and the neutral comet assay. However, the assays are rather insensitive and usually require larger fractions of irradiation, which may not be clinically relevant. More recently, γ-H2AX expression has been established as a sensitive indicator of DNA double-stranded breaks [46, 47]. At sites of radiation-induced DNA double-stranded breaks, the

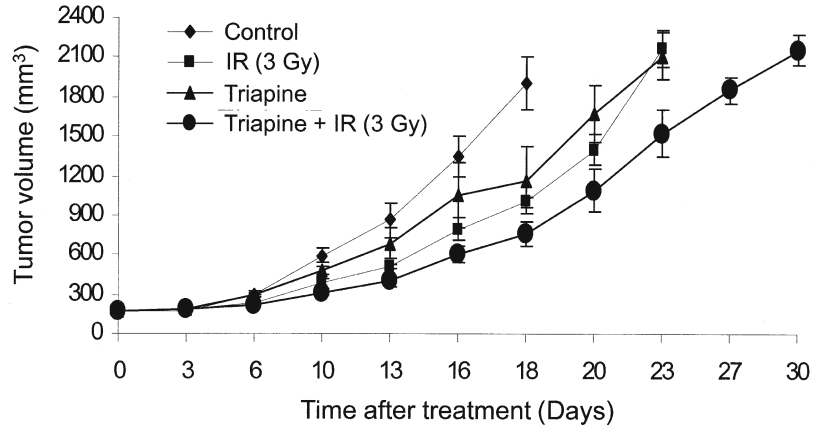

Fig. 2 In vivo tumor growth delay assay with radiosensitizer (original figure, submitted by Camphausen, K). The effects of Triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP)) on radiation-induced tumor growth delay. Tumor volume in mice after treatment with Triapine ± irradiation was plotted as a mean volume ± standard deviation (SD). When tumor reached 172 mm^3 in size, mice were randomized into four groups: vehicle, Triapine, irradiation (3Gy), or Triapine ± irradiation. A single Triapine dose was delivered as p.o (p.o. gavage) at 1 h before delivery of 3 Gy to the tumor. To obtain a tumor growth curve, perpendicular diameter measurements of each tumor were measured every 2 days with digital calipers, and volumes were calculated using formula $(L \times W \times W)/2$. Each group contained six mice except control group (10 mice). The data shown are representative of two independent experiments

histone H2AX becomes rapidly phosphorylated producing γ-H2AX which forms readily visible nuclear foci by immunohistochemistry. The expression of γ-H2AX foci in irradiated cells correlates with the repair of DNA double-stranded breaks and correlates with clonogenic survival after irradiation. Immunofluorescent staining for γ-H2AX thus allows the quantitation of nuclear foci which can be used as a surrogate measure of radiation-induced DNA damage and can be used on clinically relevant doses of radiation [48].

2.2 In Vivo Models

In vivo/in vitro tumor excision assays, tumor regrowth delay assay, and the tumor control/cure dose assay are standard assays for quantitating the response of a solid tumor to radiation treatment [49, 50]. With in vivo/in vitro tumor excision assays, animals bearing implanted tumors are treated with an anticancer therapy, and at predetermined time points, the tumors are removed from the animal, disaggregated into single cells, and assayed for clonogenic survival. Complete disaggregation of the excised tumors into single-cell suspensions prior to assay for colony formation is required for this assay to be effective and, therefore, has practical limitation since this assay can be used only with tumors for which a single-cell suspension can be made. Clonogenic evaluation can be determined in vitro, or alternatively the tumors may be disaggregated into single cells and injected intravenously into animals where the resulting lung metastases are scored.

The tumor growth delay assay measures the time required for a tumor to reach a predetermined size after treatment. The tumor size selected should allow for tumors to regrow at the same rate as the untreated tumors and is often 2–4 times the volume of the tumor before treatment [51]. The growth delay assay (see Fig. 2) may be used to evaluate both single and fractionated doses of radiation. Because the assay often requires nude or SCID mice when a human tumor xenograft is used, confounding variables such as inherent differences in immunogenicity and differences in the rates of tumor growth between immunocompetent and immunocompromised mouse strains may be present.

The tumor control/cure dose assay measures the radiation dose at which 50 % of the tumors are locally controlled (TCD50). A benefit of this assay is that it is the most similar to the biological endpoint of radiotherapy in human subjects, but it is an extremely costly assay to perform, requiring an extended time and large numbers of tumor-bearing mice to obtain one TCD50 value [52].

2.3 Interpretation of In Vivo and In Vitro Assays

Changes in radiation sensitivity are evaluated as changes in the slope of the survival curve under conditions when the agent itself produces little or no toxicity. When a pharmacologic agent has demonstrable cytotoxicity by itself, the determination of a synergistic effect between the pharmacologic agent and radiation requires isobologram analysis, which consists of a separate, full dose–response curve for each agent [53, 54]. When isobologram evaluation provides no evidence for radiosensitization by an agent, in vivo assays of antitumor effect additivity to demonstrate improved local tumor control and survival may be performed. Additional consideration must be given to drug dosing and timing, allowing for the drug to be delivered in a capacity that is appropriate for its mechanism of action. As with any experiment, controls such as postirradiation administration of an agent should be included in the assay. For some agents, extended exposure prior to radiation therapy is required, particularly if the agent has to permeate the cell and be delivered to a subcellular compartment [55].

Ideally, assays demonstrating a difference in radiation effects between tumor and normal tissue should be obtained to support the rationale for an improved therapeutic ratio for the agent which is administered concurrently with radiation. Consideration of late normal tissue endpoints may allow a quantitation of this difference. If no appropriate in vivo normal tissue toxicity models are available, the demonstration of a lack of radiosensitization using non-immortalized human cells in vitro may be used as a substitute assay [20].

2.4 Clinical Considerations of Radiation Sensitizers

In vivo and in vitro preclinical data using radiation and drug doses to model the intended clinical treatment should be obtained prior to examining the agent in a therapeutic trial. This means planning in vivo assays with fractionated treatment regimens at clinically appropriate doses (typically 2 Gy/fraction). The appropriate pharmacokinetic and pharmacodynamic data of the agent in humans should be obtained to allow for administration of the suggested radiosensitizer at doses which are clinically relevant. These considerations allow for the generation of more effective experiments which produce data that is more easily transferrable into the clinical realm. If this is not possible, a range of doses of the agent should be used in combination with fractionated irradiation to obtain evidence that the efficacy of the agent can be correlated with changes in the target or physiologic process that is being targeted. When there are no human pharmacokinetic or toxicology data for a radiation modifier, toxicology data from single-agent and combined therapy animal studies are used to choose the initial human dose, which is typically one-tenth of the dose lethal to 10 % of treated animals (LD10) of either the single-agent or combined therapy (agent plus radiation) dose, whichever is lower.

The consideration of a radiosensitizing agent should be limited to a disease site or process where an indication exists for curative or palliative radiation therapy alone as standard of care. Clinical trials with radiosensitizers typically seek to determine the dose of the modifier that is to be administered concurrently with radiotherapy, although radiation regimens may differ greatly based on the tumor type and clinical scenario. The trials are designed so that the agents are administered exclusively concurrently with radiation. When agents are administered neoadjuvantly or adjuvantly along with

concurrent chemoradiotherapy, it can be an extremely difficult task to attribute the outcome to a radiation modifier effect, particularly if the agent exhibits significant cytotoxicity. Because of this, direct anticancer properties need to be examined prior to a combination of sequential chemotherapy with a chemoradiation regimen. Endpoints such as complete response rates, local control rates, locoregional time to progression, and survival are generally preferable to overall response rates and are determined by the primary tumor being studied.

Toxicity evaluations for radiation modifiers differ based on anatomical location with radiotherapy and are divided into discrete sets of acute and late toxicities. Those effects occurring from the start of therapy and within 90 days following treatment are referred to as acute toxicities, whereas late toxicities typically develop after the 90-day mark. Although evaluation of acute toxicity within the 90 days surrounding radiation is usually the focus of toxicity evaluation, the collection and analysis of data on late toxicity effects are essential because such data can often assist with interpretation of late events seen in the initial stages of phase III trials [20]. As an added consideration, dose-limiting toxicities for clinical evaluation of radiosensitizers are often defined by the organ and site, as determined by the body areas targeted with radiotherapy, and, therefore, initial clinical investigations are often performed in a curative as opposed to a palliative setting.

Novel serum biomarkers are currently under investigation which may one day provide a reliable early marker of clinical response to radiation therapy and may provide a means to evaluate the clinical effect of radiation sensitizers as part of an individualized cancer treatment strategy. Instead of categorical radiation dosing techniques, serum proteomic tests may allow us to better quantify the biological effect of serial exposures, making it easier to avoid toxicity while maximizing therapeutic efficacy [56].

2.5 Novel Radiation Modifiers

Histone acetylation, controlled by histone acetylases and histone deacetylases (HDAC), modifies nucleosome and chromatin structures and regulates gene expression. The aberrant HDAC activity leading to transcriptional repression of tumor suppressor genes is considered to be a common event contributing to tumor formation. Accordingly, molecules that can inhibit histone deacetylases and reverse the aberrant epigenetic changes associated with various cancers are being investigated. HDAC inhibitors have been shown to induce tumor cell differentiation, apoptosis, and/or growth arrest in several in vitro and in vivo experimental models. Multiple HDAC inhibitors have also been shown to affect radiosensitivity in preclinical models [57]. One of these HDAC inhibitors, vorinostat (suberoylanilide hydroxamic acid), a novel synthetic hybrid polar compound, has been shown to inhibit HDAC activity and enhance radiosensitivity in multiple cell lines and in animal models [35]. Clinical trials are currently being conducted using these novel radiation sensitizers to determine their clinical safety and efficacy. Additionally, the use of antiangiogenic agents to augment the effectiveness of established radiosensitizers, such as temozolomide, is being investigated and represents another paradigm to affect radiosensitization [58].

3 Radiation as Immunomodulator (Abscopal Effect)

One of the more exciting possibilities is the use of radiation in stimulating immune responsiveness. Local radiation therapy that produces systemic effects on distant tumors has become known as the abscopal effect [59] and has suggested a potential use for radiation as an adjunct to tumor immunotherapy [60]. Experiments in animal models have suggested that the biological mechanisms which result in the abscopal effect may be multifactorial [61] and are most likely dependent on CD4+ and

CD8+ T cells and NK cells. The combination of radiation and IL-2 treatment results in increased antigen presentation and lymphocyte invasion in tissue at the site of irradiation, along with initiation of a systemic immunosensitization. For example, targeted radiation improves systemic responses to interleukin-2 (IL-2) and is associated with increased tumor cell surface expression of MHC class I [62]. In contrast, irradiated tumor demonstrates an influx of Mac-1^+ cells [61]. Because irradiated tumor results in changes in cell surface antigen presentation which leads to targeted immune-mediated cytotoxicity [63], the abscopal effect may present an opportunity to allow targeted radiotherapy to enhance the efficacy of immunotherapeutic agents such as sirolimus and rapamycin as well as the development of effective tumor vaccines. The combination of radiation- and vaccine-based immunotherapy has resulted in improved response rates versus radiotherapy alone in cervical cancer, localized and metastatic prostate cancer, hepatoma, and metastatic renal cell carcinoma, providing the groundwork for consideration for trials in other disease sites [64–69].

4 Summary

With current advances in molecular radiobiology, strategies for enhancing radiosensitivity now focus on targeting the molecules and processes that regulate cellular radioresponse on a localized and systemic level. A wide variety of pharmacologic agents have been shown to influence radiosensitivity affecting such fundamental processes as cell cycle checkpoints, DNA repair, gene expression, and apoptosis. However, to be clinically relevant, a molecular target must not only serve as a determinant of radiosensitivity but should also be susceptible to pharmacologic manipulation and, importantly, be selective for tumor cells over normal tissue.

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Therapeutic Cancer Vaccines: An Emerging Approach to Cancer Treatment

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Abstract In the late 1990s, monoclonal antibodies and targeted molecular inhibitors revolutionized treatment options for patients with cancer. Since their development, outcomes for patients with chronic lymphocytic leukemia, non-Hodgkin's lymphoma, and Her-2^{+} breast cancer, among others, have significantly improved. While these anticancer agents continue to evolve, therapeutic cancer vaccines could be the next major therapeutic advance for cancer patients. Immunotherapy is already an accepted treatment for some cancers. BCG is a standard treatment for localized bladder cancer, while interferon alpha and interleukin-2 (IL-2) are used to treat melanoma and renal cell cancer. These nonspecific types of immunotherapy induce a broad immunologic response that may have an antitumor effect in a minority of patients. However, therapeutic cancer vaccines that can induce a specific, targeted antitumor immune response are currently in clinical development. Therapeutic cancer vaccines in metastatic prostate cancer have demonstrated overall survival advantages relative to placebo in multiple phase II and III trials, and there are compelling data for the clinical benefit of therapeutic cancer vaccines in other cancer types. Additional strategies are being investigated that employ combinations of vaccines and standard therapeutics, including hormonal therapy, radiation, and chemotherapy, in an effort to optimize the effects of vaccines.

Keywords Therapeutic vaccine • Immunotherapy • Combination therapy • Clinical trials

1 Introduction

1.1 Rationale for Therapeutic Cancer Vaccines

Like targeted molecular inhibitors and monoclonal antibodies, therapeutic cancer vaccines are designed to target specific epitopes on malignant cells. Human cytotoxic T lymphocytes (CTLs) are able to recognize 9–14-mer antigenic peptides expressed within the major histocompatibility complex

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Table 1 Selected tumor-associated antigens as targets for vaccine

Antigen	Cancers
Carcinoembryonic antigen	Gastrointestinal (stomach, colon, rectum, pancreas), breast, cervical, and non-small cell lung
Cancer-testis antigens (NY-ESO-1 and MAGE)	Melanoma, brain, ovary, non-small cell lung, pancreas, and hepatocellular
Mucin 1	Breast, lung, prostate, kidney, and pancreas
Prostatic acid phosphatase	Prostate
Prostate-specific antigen	Prostate

(MHC) on the surface of cells. These peptides are derived from endogenously expressed proteins that are processed by proteases within cells [1, 2]. Some antigens are uniquely expressed or overexpressed by malignant cancer cells, and these tumor-associated antigens (TAAs) can be targeted by CTLs, leading to immune-mediated, tumor-specific cell lysis [3].

Unfortunately, many cancer patients develop deficient immune recognition, wherein CTLs do not effectively recognize TAAs and are unable to eliminate malignant cells. Several factors may play a role in a tumor's ability to evade immune surveillance, including decreased intracellular processing and expression of TAAs. Local immune modulators, including cytokines, may also diminish the ability to generate an effective immune response in the tumor microenvironment [4]. The ultimate goal of cancer vaccines is to generate an immune response against these TAAs through CTL stimulation.

1.2 Targeting Tumor-Associated Antigens

The ideal TAA is unique to, or overexpressed on, the surface of cancer cells. One reason that early trials of prostate cancer vaccines have shown promising results could be that there are multiple TAAs associated with prostate cancer. The most studied prostate TAA is prostate-specific antigen (PSA), a 34-kDa protein uniquely expressed in prostate cancer cells [5, 6]. Another is prostatic acid phosphatase (PAP), a 102-kDa glycoprotein overexpressed in prostate cancer cells that may play a role in disease progression [7, 8]. These antigens are also expressed on normal prostate cells; however, because the prostate is a nonessential organ, there is minimal toxicity from any cross-reactivity between CTLs targeting prostate cancer cells and normal prostate cells.

TAAs are not unique to prostate cancer (Table 1). Mucin 1 (MUC1) is a >500-kDa membrane glycoprotein of secretory epithelial cells. Although initially identified in the 1980s as a marker in breast cancer, MUC1 overexpression is also seen on cancer cells of the lung, prostate, kidney, and pancreas [9] and may play a crucial role in carcinogenesis. MUC1 has been shown to alter apoptotic pathways, transform cells by allowing anchorage-independent growth, and enhance estrogen-mediated cell growth in breast cancer [10–13]. Since MUC1 overexpression may contribute to the aberrant growth patterns that lead to tumor growth and is underglycosylated in malignant cells, this TAA differentiates tumor cells from normal epithelial cells, making MUC1 an immunologic target. Furthermore, CTLs stimulated in vitro have been shown to target and lyse cells with increased MUC1 expression [14].

Human carcinoembryonic antigen (CEA), a 180-kDa immunoglobulin-like molecule expressed on the cell surface, plays a role in cellular adhesion [15]. Malignant cells, including those from gastrointestinal tract (stomach, colon, rectum, and pancreas), breast, cervical, and non-small cell lung tumors [16, 17], often overexpress CEA, which can result in structural distortion that disrupts normal cellular differentiation and growth inhibition [18]. Furthermore, increased cellular adhesion may enhance the metastatic potential of cancer cells that break away from the main tumor [19, 20]. These characteristics make CEA an attractive target for vaccine-based therapies.

Cancer-testis antigens are a group of antigens generally limited to normal testis tissue, but they can also be expressed in the female reproductive tract [21–23]. Although their primary function is unclear, they may play a role in cellular transcription regulation, the disruption of which could lead to tumorigenesis [24]. Cancer-testis antigens are an attractive target for immunotherapy because they are overexpressed in many tumor types. The most notable and studied cancer-testis antigens include NY-ESO-1 and the MAGE family of antigens, which are overexpressed on tumors that originate in the brain, skin, ovary, lung, pancreas, and liver [24, 25]. Expression of these TAAs has been associated with aggressive tumors and poor prognosis [26–28].

1.3 Antigen Cascade

Although vaccines focus on specific TAAs, the subsequent immune response may not be limited to the targeted TAAs. In fact, the immune system is exposed to additional TAAs once cancer cells are killed in an immunologically relevant manner, creating an immune response that will likely target additional TAAs. A preclinical study demonstrated this broadening of the immune response, referred to as "antigen cascade." Tumor-bearing mice were treated with a vaccine targeting CEA, but expanded CTLs targeted other antigens also overexpressed on tumor cells, including p53 and gp70. The immune response to gp70 was even greater than the response to CEA, suggesting that while vaccine may initiate an immune response, antigen cascade serves as a vital step in the overall antitumor effect [29]. Antigen cascade has also been seen in clinical studies [30].

1.4 Developing Effective Therapeutic Cancer Vaccine Strategies

Once a suitable TAA has been identified as a vaccine target, the immune system must be activated to elicit a focused immune response that may ultimately contribute to antitumor activity. Initial efforts to develop therapeutic cancer vaccines often focused on synthetic peptides generated to mimic the 9–14-mer antigenic peptides expressed on the surface of all cells within the MHC. These strategies were largely unsuccessful, even when immunologic adjuvants were administered to enhance the immunogenicity of peptide-based vaccines [31–33]. Nevertheless, peptide vaccines are still being investigated using novel adjuvants and delivery strategies.

A second approach in vaccine development is the use of allogeneic whole tumor cell vaccines, which are generated by ex vivo expansion of cancer cell lines that are subsequently irradiated to eliminate malignant potential. These cells are then injected subcutaneously, where they cause a localized immune reaction that may be augmented by an immunologic adjuvant such as bacillus Calmette–Guerin (BCG) or granulocyte-macrophage colony-stimulating factor (GM-CSF). During this immune response, antigen-presenting cells (APCs) of the immune system recognize and process TAAs on the tumor cell surface. These APCs then present the processed TAAs in an immunologic context to CTLs. Once activated the CTLs attack tumor cells that share the same TAAs as the whole tumor cell vaccine. GVAX is a whole tumor cell prostate cancer vaccine developed from 2 prostate cancer cell lines, LNCaP and PC-3, that have been transfected with a human gene that encodes GM-CSF. After injection, GVAX secretes GM-CSF to serve as an adjuvant [34].

A third form of therapeutic cancer vaccine is vector based. In this strategy, a vector such as a genetically modified poxvirus serves as a vehicle for vaccine delivery, transporting the genetic material for TAAs into the body to trigger a targeted immune response. Once injected into subcutaneous tissue, the poxviral vectors infect APCs. The vectors then enter the cellular cytoplasm, where the transgenes for TAAs are expressed. These gene products are then processed by the APCs and

Table 2 Selected therapeutic cancer vaccines in clinical development

Vaccine	Cancer	Stage of development
Sipuleucel-T	Prostate	Two completed phase III trials showed overall survival advantage
PSA-TRICOM	Prostate	Phase II trial demonstrated overall survival benefit. Phase III trial pending
Idiotype vaccines	Follicular lymphoma	Three vaccines in late-stage development, but only one has demonstrated time-to-progression benefit. Vaccine requires further optimization and clinical investigation to confirm benefit
L-BLP25	Lung	Multiple phase III trials accruing in stage III lung cancer
gp100	Melanoma	Phase III trial demonstrated time-to-progression benefit, but survival benefit may require further follow-up
GM2 ganglioside	Melanoma	Phase III trial has completed accrual; awaiting survival data

presented within the MHC on the surface of the APCs. When the APCs interact with CTLs, the CTLs recognize the antigen within the MHC and are activated to lyse tumor cells with those antigens [35].

There are many advantages to using poxviral vectors such as vaccinia for cancer vaccines. Their foreign proteins induce an inflammatory reaction that draws immune cells to the injection site, and they are able to infect APCs at high rates. Furthermore, the poxvirus genome is large, allowing for the inclusion of multiple transgenes such as those for costimulatory molecules, resulting in enhanced antigen presentation and CTL activation. As an added benefit, vaccinia's long history of use in the widely administered smallpox vaccine provides a significant safety record [35–39]. Poxviral vaccines targeting PSA in prostate cancer and MUC1 and CEA in other human carcinomas are in clinical development [40, 41].

Cancer vaccine approaches using autologous APCs rely not on in vivo antigen stimulation but on ex vivo stimulation of APCs that are then reinjected into the same patient's bloodstream. The sipuleucel-T vaccine (Provenge®; Dendreon Corp., Seattle, WA) is developed from peripheral blood mononuclear cells exposed ex vivo to a prostate cancer antigen. Dendritic cells, T cells, B cells, and natural killer cells are selectively collected from the patient by leukapheresis. Then, in a process requiring 48 h, these APCs are activated and matured ex vivo. The activated APCs are then reinfused into the same patient with the goal of generating an antitumor response [42, 43].

Another form of autologous vaccine targets idiotypes on the cell surface of hematologic malignancies. The idiotype is a group of weakly immunogenic antigenic determinants located on the hypervariable region of antibodies that defines clonality of cells bearing such immunoglobulins [44, 45]. These idiotypes vary from patient to patient and can be used as TAA targets for autologous vaccines for patients with non-Hodgkin's lymphoma [46]. One common approach in developing an idiotype-based vaccine is to isolate the idiotype from the blood of a patient, conjugate it ex vivo to an immunogenic molecule such as keyhole limpet hemocyanin (KLH), and then administer the idiotype conjugate subcutaneously, perhaps with an immunologic adjuvant such as GM-CSF.

1.5 Selected Therapeutic Cancer Vaccines in Clinical Development

1.5.1 Prostate Cancer Vaccines

Although prostate cancer is only weakly immunogenic, the most promising clinical results involving therapeutic cancer vaccines have been in metastatic castration-resistant prostate cancer (mCRPC) (Table 2). Sipuleucel-T, an APC-based vaccine, has demonstrated an overall survival benefit in 2 phase III trials. An initial phase III trial demonstrated a >4-month improvement in overall survival compared to placebo (25.9 months vs. 21.4 months; $P=0.01$) [47]. Recently, a larger phase III trial

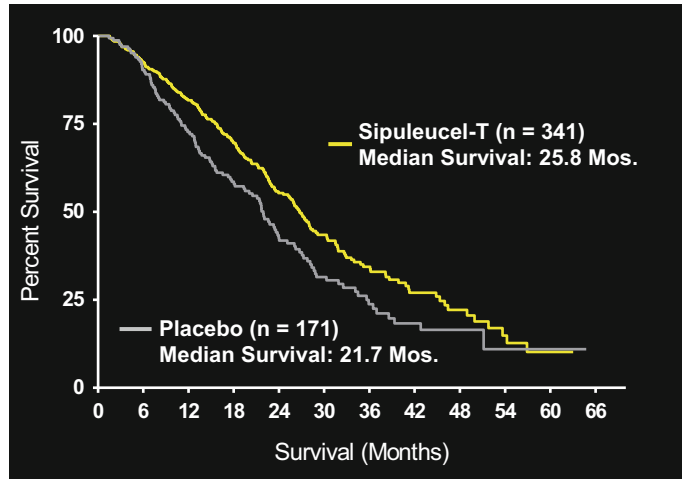

Fig. 1 Sipuleucel-T vaccine in metastatic prostate cancer. This large phase III trial was the second phase III trial with this antigen-presenting cell-based vaccine to demonstrate an overall survival advantage ($P=0.032$ (Cox model); HR = 0.775 [95 % CI, 0.614, 0.979])

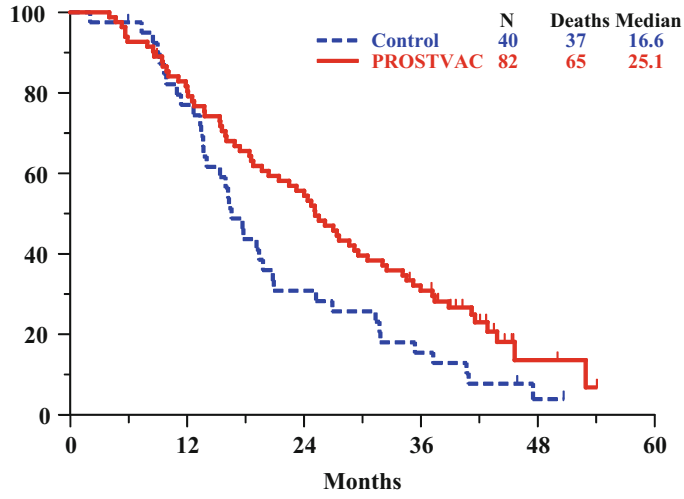

Fig. 2 Phase II PSA-TRICOM in metastatic prostate cancer. A phase II trial in metastatic castration-resistant prostate cancer treated 125 patients randomized 2:1 (PSA-TRICOM vs. placebo). The vaccine arm demonstrated an 8.5-month improvement in overall survival relative to placebo (25.1 months vs. 16.6 months; $P=0.006$)

with more than 500 patients demonstrated a similar survival benefit (25.8 months vs. 21.7 months; $P=0.032$) relative to placebo [48] (Fig. 1). In late 2009, Dendreon Corp. filed this phase III data with the US Food and Drug Administration (FDA), which could result in approval of the first therapeutic cancer vaccine in early 2010 [49].

Sipuleucel-T is not the only vaccine to have demonstrated a survival advantage in mCRPC. PSA-TRICOM, a vector-based vaccine, demonstrated an 8.5-month improvement in overall survival relative to placebo ($P=0.015$) in a multicenter randomized phase II trial [50] (Fig. 2). A smaller trial at the National Cancer Institute, also in mCRPC, demonstrated that PSA-TRICOM can generate a PSA-specific CTL response within 3 months and that these antigen-specific immune responses are associated with favorable survival outcomes [51]. Based on these findings, plans are under way for a multicenter phase III trial of PSA-TRICOM in this same patient population in 2010 [52].

Not all clinical trials employing vaccines in prostate cancer have been successful. GVAX, a whole tumor cell prostate cancer vaccine that includes cell lines genetically modified to secrete GM-CSF as an immune adjuvant [34], showed promising results in preclinical studies and phase II clinical trials. But two phase III trials investigating this vaccine platform (VITAL-1 and VITAL-2) were abruptly

terminated in 2008 [53, 54]. In retrospect, poor trial design may have limited the opportunity for success in one trial, and corporate decisions prematurely curtailed the second [55].

VITAL-2 randomized patients with symptomatic, metastatic prostate cancer to receive either docetaxel and prednisone or docetaxel and GVAX. The trial was discontinued in August 2008 after an interval analysis found 67 deaths in the vaccine arm vs. 47 deaths in the docetaxel-alone arm. Notably, a review of the data did not suggest that the vaccine itself conveyed any additional toxicity and that the majority of deaths were due to disease progression [56]. Enrollment of patients with symptomatic disease may explain the relative ineffectiveness of the addition of vaccine to docetaxel, as these patients had a predicted survival of only 13 months as measured by the Halabi nomogram [56, 57]. This is potentially significant because the NCI trial of PSA-TRICOM suggested that patients with a Halabi-predicted survival of >18 months had a substantially greater survival advantage than predicted after treatment with vaccine, while those with a predicted survival of <18 months did not [51]. Taken together, these findings suggest that vaccine therapy is best utilized in patients with earlier-stage disease or with more indolent disease characteristics. Therefore, VITAL-2 may have evaluated vaccine in a less than ideal patient population [55]. VITAL-1 compared GVAX vs. docetaxel and prednisone in patients with asymptomatic metastatic prostate cancer. Although preliminary data suggested the relative equivalency of these two treatments, with a trend toward the superiority of vaccine in patients with more indolent disease as well as substantially fewer serious adverse events, the study was not designed as a noninferiority study and, given the financial repercussions of VITAL-2, VITAL-1 was terminated in October 2008 [54, 58].

1.5.2 Non-Hodgkin's (Follicular) Lymphoma Vaccines

Three trials have evaluated different strategies of employing patient-specific, autologous, tumor-derived idiotype vaccines in follicular lymphoma (Table 2). The first two trials to report results did not meet their endpoints of improved overall survival [59, 60]. In the third trial, however, vaccine treatment demonstrated an improvement in time to disease recurrence ($P=0.045$) [61]. While some have criticized this trial for its borderline statistical significance, it differed from the first two trials in patient population [62]. All patients enrolled in the third trial had shown complete responses to previous therapy, whereas the two previous studies that did not meet their endpoints enrolled patients with measurable disease. Perhaps, similar to the prostate cancer studies, patients with lower disease volume are more likely to respond to vaccine-based therapy.

Further investigation is required to determine the optimal use of idiotype vaccines. The vaccines used in the three trials described above had subtle differences in schedule and preparation. Furthermore, a comprehensive review of the patients in these trials and their ultimate clinical responses may identify the kinds of patients who are best able to respond to idiotype vaccines. In addition, given the increased toxicity of chemotherapy compared to vaccine therapy, future trials should consider a noninferiority endpoint [62]. Perhaps greater understanding of the results of previous trials, along with definitive data from future trials, will lead to the optimal use of idiotype vaccines in follicular lymphoma.

1.5.3 Lung Cancer Vaccines

BLP25 liposome vaccine is a modified version of a peptide-based vaccine that targets the exposed core peptide of the MUC1 TAA (Table 2). L-BLP25 (Stimuvax®; EMD Merck, Serono, Darmstadt, Germany) is a lyophilized preparation consisting of BLP25 lipopeptide, the immunoadjuvant monophosphoryl lipid A, and three lipids (cholesterol, dimyristoylphosphatidylglycerol, and dipalmitoylphosphatidylcholine), forming an immunogenic liposomal product. A randomized phase IIB trial of L-BLP25 evaluated patients with stage IIIB/IV non-small cell lung cancer (NSCLC) with stable

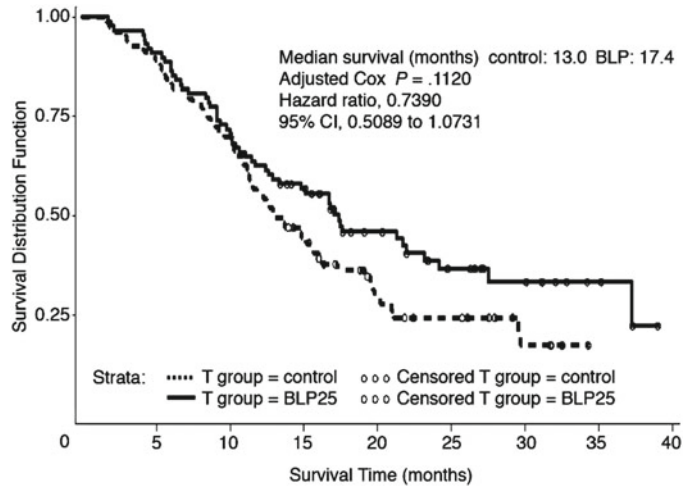

Fig. 3 Phase II trial of L-BLP25 in stage IIIB and IV lung cancer. The lipopeptide-based vaccine targeting MUC1 demonstrated an overall survival benefit in stage IIIB and IV lung cancer (17.4 months vs. 13 months for placebo)

disease or after response to primary chemotherapy. L-BLP25 was administered weekly for 8 weeks, with the option to proceed to maintenance therapy consisting of vaccination every 6 weeks starting in week 13. As part of the vaccine construct, all patients received a single infusion of cyclophosphamide 300 mg/m^2 for 3 days prior to initial vaccine administration, which has been shown to enhance response to vaccine. There were 88 patients in the vaccination arm and 83 in the best supportive care (BSC) arm. The median overall survival was 17.4 months for the vaccine arm vs. 13.0 months for the BSC arm ($P = 0.066$, unadjusted Cox) (Fig. 3). The greatest improvement in survival was observed in patients with stage IIIB locoregional disease (adjusted HR = 0.524; 95 % CI, 0.261–1.052; $P = 0.069$) [63] (Fig. 3). A subsequent study of L-BLP25 evaluated 22 patients with stage III NSCLC who had stable disease or response to chemotherapy. The median overall survival in this study was similar to the randomized phase IIB study at a median follow-up of 53 months [64].

A multicenter phase III, randomized, double-blind, placebo-controlled study of L-BLP25 in NSCLC patients with unresectable stage III disease is currently accruing. The START (Stimulating Targeted Antigenic Responses To NSCLC) trial is expected to enroll more than 1,300 patients who have had a response or stable disease after at least 2 cycles of definitive platinum-based chemoradiation [65]. A similar study will enroll over 400 Asian patients with the same stage of disease in order to explore any differential effects on that population [66]. These studies will try to improve on the current 5-year overall survival that is less than 50 % [67].

1.5.4 Melanoma Vaccines

Melanoma was once considered the best candidate for immune-stimulating cancer vaccines because of its many immunogenic TAAs. In addition, melanoma tumors can be responsive to nonspecific cytokine therapy, such as IL-2, in a minority of cases. However, after years of research, results of melanoma vaccine therapy have been disappointing [68]. For example, the whole tumor cell vaccines Canvaxin® (CancerVax Corp., Carlsbad, CA) and Melacine® (Corixa Corp., Seattle, WA) showed preliminary benefit in phase I and II trials, only to fail in randomized phase III trials [68–72].

However, recent data from a phase III trial in melanoma have suggested clinical benefit for a peptide-based vaccine (Table 2). Gp100 is a TAA that is capable of eliciting a response from CTLs within melanoma tumors [73–75]. The phase III trial randomized patients with locally advanced stage III or IV melanoma to high-dose IL-2 alone or IL-2 plus a gp100 peptide vaccine [gp100:209-217(210M)]. Both progression-free survival and overall survival favored the vaccine plus IL-2 arm

(2.9 vs. 1.6 months, $P=0.0101$, and 17.6 vs. 12.8 months, $P=0.0964$, respectively) [76]. Longer follow-up will be required to determine if this treatment provided a significant survival advantage.

Another melanoma vaccine that is further along in clinical development targets gangliosides, which are glycolipids present in cell membranes and overexpressed on the surface of melanoma cells. Similar to a peptide-based vaccine, a ganglioside vaccine is derived from a particular target ganglioside and administered with an immune adjuvant to enhance immune response [77]. A randomized trial in 1,694 stage IIB/III melanoma patients suggested that the vaccine construct (including KLH) targeting the GM2 ganglioside was more effective when administered with high-dose interferon than with a second adjuvant (QS-21) [78]. A phase III trial employing this vaccine was conducted by the European Organization for Research and Treatment of Cancer in over 1,300 patients with stage IIA melanoma. That trial is closed to accrual and final results are pending appropriate follow-up [68].

1.6 Rationale for Combining Vaccines with Standard Therapies

Although therapeutic cancer vaccines hold great promise, their ultimate utility may be in combining them with other standard therapeutic interventions. Increasing data suggest that the immune response and immune-mediated tumor killing induced by cancer vaccines can be activated and enhanced by conventional anticancer therapies. Standard treatments may upregulate MHC molecules and TAA expression or induce apoptosis by increasing the expression of death receptors such as Fas, TNF receptor, and TNF-related ligand receptors [79]. Multiple treatment modalities are being investigated in combination with vaccines, including radiation, chemotherapy, hormone therapy, and targeted molecular inhibitors.

1.6.1 Radiation

Radiation therapy has cytotoxic effects on cancer cells, but preclinical and clinical data have shown that even at low doses radiation can modulate gene expression, causing phenotypic changes in tumor cells. Following radiation, Fas, MHC class I, ICAM-1, and several other TAAs are upregulated, making them more amenable to CTL-mediated antitumor activity [80–82]. A vector-based vaccine targeting CEA and containing T-cell costimulatory molecules was combined with radiation in a murine model. The combination demonstrated a 50 % reduction in tumor mass and infiltration of T cells into the tumor. This finding was superior to either vaccine or radiation as a single modality [29]. The combination regimen used in this study upregulated Fas on tumor cells, leading to improved vaccine-mediated tumor killing, as evidenced by increased infiltration of tumor-specific $CD8^+$ T cells.

In a clinical study in men with localized prostate cancer, a vector-based vaccine plus standard radiation also generated an immune response. In this small study, 11 patients received radiation alone, and 19 patients received the combination regimen of vaccine and radiation. Of the 17 patients who completed all eight scheduled vaccinations, 13 showed increases of at least threefold in PSA-specific CTLs. These immunologic responses were superior to those induced by radiation alone ($P<0.0005$) [30].

1.6.2 Hormone Therapy

There is increasing interest in the use of therapeutic cancer vaccines in combination with hormone therapies, especially in the treatment of hormone-sensitive cancers such as breast and prostate. Increasing data suggest that androgen-deprivation therapy (ADT) in prostate cancer can augment the immune response by increasing CTL infiltration into the prostate [83]. Furthermore, ADT has been

shown to decrease immune tolerance of TAAs, increase the size of the thymus (where CTLs are produced), and enhance the CTL repertoire [84–87]. In breast cancer, the aromatase inhibitor anastrozole has been shown to increase pro-inflammatory cytokines and may enhance CTL stimulation by reducing the number of regulatory T cells (Tregs) that can hinder an immune-mediated, tumor-specific response [88].

A clinical study in men with nonmetastatic CRPC has also suggested a possible synergy between vaccines and hormone treatment. Patients were randomized to receive either vaccine or standard ADT in the form of nilutamide, an FDA-approved androgen receptor antagonist. After 6 months, patients with rising PSA but no metastasis could cross over to receive a combination of both treatments. For patients who received both therapies, the median time to treatment failure (defined by PSA rise or development of a metastatic lesion) was 13.9 months for patients who started on the vaccine arm and had nilutamide added at time of PSA progression. In contrast, patients who started on the nilutamide arm and had vaccine added at time of PSA progression had a median time to failure of 5.2 months. This study suggests that giving vaccine during early-stage disease, followed by nilutamide, may have greater clinical efficacy than nilutamide followed by vaccine [89]. A subsequent follow-up survival analysis of these patients revealed a 75 % 5-year survival rate for patients who were treated first with vaccine and then added nilutamide, compared to a 43 % 5-year survival rate for patients who received nilutamide first and added vaccine later [90].

1.6.3 Chemotherapy

Cytotoxic chemotherapy agents serve an important role in the conventional treatment of solid tumors, although most patients with metastatic disease are rarely cured with chemotherapy alone. While the primary benefit of chemotherapy derives from its cytotoxic properties, it may also alter tumors phenotypically, enhancing TAA and MHC class I expression, both of which can make cancer cells more amenable to vaccine-induced CTL activity [12, 91–94].

Chemotherapy agents have other immunomodulating properties. Doxorubicin can increase macrophage number and activity, while in vitro studies of docetaxel have demonstrated increases in pro-inflammatory cytokines [95–97]. Cyclophosphamide has been shown to reduce the number and function of Tregs that can limit immune stimulation after treatment with a vaccine [98, 99]. Therapeutic cancer vaccines can take advantage of these favorable dynamics to enhance antitumor response. Studies in murine models have indicated that vaccine in combination with docetaxel has a greater antitumor effect than either agent alone [100]. Furthermore, chemotherapy-induced cell lysis in the presence of a vaccine-activated immune response exposes the immune system to an array of additional TAAs not specifically targeted by the vaccine. Exposure and subsequent immune response to such an antigen cascade could broaden antitumor immune responses and enhance clinical benefit [30, 100].

There has long been concern that chemotherapy may limit an immune response. However, a phase II clinical trial in metastatic prostate cancer demonstrated that this was not the case. Patients were randomized to receive vaccine alone or vaccine with weekly docetaxel. The results of this study showed an equal increase in PSA-specific CTLs in both arms following 3 months of therapy. Furthermore, immune responses to other prostate cancer-associated TAAs were also detected postvaccination [101].

Administering vaccine prior to chemotherapy may take advantage of the dynamic immune response to TAAs initiated by the vaccine. Subsequent chemotherapy may yield the benefits of cytotoxicity in combination with an ongoing (and perhaps potentiated) immune response. Murine models have demonstrated that vaccine and docetaxel combined are more effective than either treatment alone but also that vaccine followed by docetaxel generates a greater vaccine-induced immune response than vaccine given after chemotherapy [100].

Anecdotal reports from several clinical trials support these findings. A phase I study treated 17 patients with a plasmid/microparticle-based vaccine targeting cytochrome P450B1, which is overexpressed in some tumors. The five patients who generated an immune response had a longer-than-anticipated clinical response to salvage chemotherapy [102]. Similarly, 29 patients with NSCLC were treated with an adenovirus-based vaccine targeting p53, resulting in a higher-than-expected (61.9 %) objective response rate to salvage chemotherapy administered after vaccine [103]. A large prostate cancer trial employing an APC-based vaccine also followed patients after treatment on a placebo-controlled study. Fifty-one patients treated with vaccine who went off study to receive chemotherapy had an overall survival of 34.5 months, compared to 25.4 months for the 31 patients treated with placebo followed by chemotherapy ($P = 0.023$) [104]. Clinical trials are currently being planned to prospectively validate the benefits of vaccine followed by chemotherapy.

1.6.4 Targeted Molecular Inhibitors

The advent of targeted molecular inhibitors has revolutionized the treatment of some cancers, and these agents may also have clinical benefit in combination with therapeutic cancer vaccines. One example of such a combination is sunitinib malate (Sutent®; Pfizer Inc., New York, NY), a tyrosine kinase inhibitor FDA approved for the treatment of metastatic renal cell cancer. Preclinical data suggest that sunitinib may selectively reduce the efficacy of Tregs in cancer patients [105, 106]. Since Tregs may limit the body's ability to generate an antitumor response, decreasing their effectiveness could enhance the immune response generated by vaccine. An ongoing clinical trial is investigating an autologous whole tumor cell vaccine in patients with renal cell cancer. Patients in this trial will also be treated with sunitinib to see if it can augment the effectiveness of the vaccine [107]. Similar clinical trials will be required to further explore this hypothesis.

1.7 Future Directions

Although there is a sound preclinical basis and emerging clinical data to support the use of therapeutic cancer vaccines alone and in combination with standard agents in the treatment of cancer, significant hurdles must still be overcome. Vaccines are different from cytoreductive therapy and may not result in immediate tumor regression, but rather may stabilize disease and delay long-term disease progression. A patient treated with chemotherapy may see an initial 30 % reduction in tumor volume, but at 6 months the tumor may have grown to twice its original size. Vaccines, on the other hand, may affect tumor growth patterns by altering the biology of the tumor microenvironment through an active antitumor immune response. Thus, a vaccine may initially produce no significant reduction in tumor size, while at 6 months the tumor may have grown by only 15 %. This could explain why several prostate cancer vaccine trials have demonstrated no significant change in disease progression as measured by standard parameters after 2–3 months, but the long-term endpoint of overall survival is improved in patients treated with vaccine [47, 48, 50]. Indeed, immunotherapy trials in melanoma have suggested that the disease may initially flare in some areas before more beneficial results are seen radiographically [108].

This concept of long-term benefit without marked initial tumor reduction makes biological sense in terms of immune response but represents a significant departure from the standard approach in cancer clinical trials of treating patients with a drug and assessing radiographic response within 2–3 months. Any increase in tumor volume greater than 20 % is considered disease progression, and the patient is removed from the study [109, 110]. However, if we are to understand the true clinical benefit of cancer vaccines, the current paradigm of drug development, which judges radiographic response but not biological response, needs to evolve [111]. In addition, the current trial design model evaluates new drugs in patients with late-stage disease who have been heavily pretreated. This may not

represent the best way to evaluate vaccines that are less effective in patients with heavy disease burdens, as demonstrated by the GVAX prostate cancer trials and the idiotype vaccine trials in follicular lymphoma. Furthermore, numerous prior chemotherapy regimens have been shown to reduce the potential of the immune system to respond to a vaccine [111, 112]. Thus, an ideal setting for future vaccine trials would involve patients with smaller disease volume and longer term trial endpoints [55]. These changes would allow investigators to accurately assess the potential clinical effectiveness of therapeutic cancer vaccines.

Although human biology dictates that vaccines will probably not mount a vigorous antitumor response, patients and practitioners cannot be expected to idly observe tumor growth while waiting for a delayed therapeutic benefit from an immune-based treatment. Thus, there is an urgent need to develop standardized biomarkers to evaluate biological response in the absence of clinical response. The ELISPOT assay is able to measure CTL response to specific TAAs in the form of gamma interferon production ex vivo, which correlates with CTLs' ability to lyse cells bearing such TAAs in vivo [113]. Although this can be an effective way of assessing immune response and has been associated with clinical benefit, it has limitations, including significant variability from institution to institution and the fact that the test may be restricted to patients with certain tissue types. Even if these shortcomings can be overcome [114], the broader complication of assessing a dynamic immune response remains. In other words, as discussed above, if a vaccine targets a TAA, and a subsequent immune response results in a vigorous attack on a separate TAA via antigen cascade, it may be difficult to know which TAA the immune system is attacking and therefore which to assess. Furthermore, various patients given the same vaccine may have significant immune responses to different TAAs not contained in the vaccine. Thus, if only the response to a specific TAA is assessed, the actual benefit of the vaccine may be underestimated. If vaccines are to move forward in clinical development and have broad application, biomarkers of response will be required.

Therapeutic cancer vaccines have been in development for several decades, initially with disappointing results. Recent trials in prostate cancer, especially, have renewed hope that the initiation of a dynamic immune response by a therapeutic cancer vaccine can have a long-term, beneficial clinical impact for cancer patients. These initial successes in prostate cancer may allow investigators to optimize vaccines for other diseases as well. The generally indolent nature of prostate cancer, and a dearth of effective systemic treatments for metastatic disease, may explain why prostate cancer vaccines have had more success than other cancer vaccines. Therefore, employing vaccines in appropriate diseases and disease states may yield greater benefit from the use of vaccines. The potential for broader application of vaccines in prostate cancer may provide an opportunity to observe many patients and develop appropriate biomarkers to assess response in other diseases. Also, the use of vaccines in combination with standard therapies may allow for greater benefit than with either treatment alone. Additional clinical trials are required to answer many of these questions, but immune-mediated antitumor treatment may one day become as common as treatment with monoclonal antibodies and targeted molecular agents, which were also relegated at one time to the realm of scientific curiosity rather than therapeutic reality. While these treatments have clearly transformed current cancer care, therapeutic cancer vaccines as a new modality with few side effects and the potential for long-term immune responses that can add clinical benefit to subsequent therapies hold the promise of revolutionizing cancer treatment.

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Recombinant Immunotoxins

Robert J. Kreitman

Abstract Recombinant immunotoxins contain a recombinant antibody and a protein toxin, capable of killing a cell after internalization and transport of the toxin to the cytosol. Growth factor fusion toxins, including the approved molecule denileukin diftitox, contain a growth factor such as interleukin-2 and truncated toxin. Recombinant immunotoxins furthest along in clinical development are BL22 (CAT-3888) and HA22 (CAT-8015 or moxetumomab pasudotox) targeting CD22 and LMB-2 targeting CD25. These agents have induced complete and partial responses in patients with chemoresistant hairy cell leukemia (HCL) and partial responses with other hematologic malignancies. Clinical development is continuing with these and other agents for different forms of cancer.

Keywords Monoclonal antibody • Fusion toxin • CD22 • CD25 • Diphtheria toxin • Pseudomonas exotoxin • Ricin • KDEL receptor • Fv • Chronic lymphocytic leukemia • Hairy cell leukemia • Adult T-cell leukemia • SS1P • CD19 • Mesothelin • CD3

1 Introduction

1.1 Protein Toxins with Catalytic Domains

Protein toxins are unique among cell-killing agents because they kill cells catalytically and therefore at extremely low concentrations. A single molecule in the cytoplasm has been shown to be sufficient for cell killing by plant toxins like ricin, abrin, and modeccin [1, 2] and by bacterial toxins like diphtheria toxin (DT) [3] and Pseudomonas exotoxin (PE). Plant toxins function as ribosomal inactivating agents by preventing the association of elongation factors 1 and 2 (EF1 and EF2) with the 60s ribosomal subunit. This occurs after removal of adenine4324 in the 28 s ribosomal RNA molecule [1, 4]. The bacterial toxins PE and DT, in contrast, directly inactivate EF2 catalytically by ADP ribosylation [5, 6]. Ricin and mistletoe viscumin [7] are holotoxins, each containing a binding and catalytic domain disulfide bonded together. Other plant toxins, including gelonin [4], saporin [8], pokeweed antiviral protein [9], BRIP [10], momordin [11], and trichosanthin [12], are hemitoxins, containing catalytic domains without known binding domains. Bacteria as prokaryotes are unable to produce multichain

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toxins joined by disulfide bonds and instead produced single-chain toxins [13]. In DT, the catalytic domain is at the amino terminus, while the binding domain is at the carboxyl terminus. In PE the orientation is reversed. Recombinant immunotoxins are produced by replacing the normal binding domain of the toxin with a recombinant antibody. Immunotoxins originally were produced as chemical conjugates of antibody with toxin. Recombinant immunotoxins have advantages over these earlier molecules, including smaller size, homogenous toxin-ligand junction, and more efficient production from bacteria.

2 Mechanisms of Cell Death

2.1 Mechanism of Cell Death by PE

The PE protein is composed of several domains [14, 15], including domain Ia at the amino terminus for binding and domain III at the carboxyl terminus for catalytic ADP ribosylation of elongation factor 2 (EF2). A current model for intoxication includes (1) proteolysis by carboxypeptidase of the carboxyl terminal lysine residue at position 613 [16]; (2) binding of domain Ia (amino acids 1–252) to the alpha-2 macroglobulin receptor present on animal cells [17]; (3) internalization and unfolding of the toxin at low pH; (4) proteolytic processing by furin of domain II (amino acids 253–364) between Arg279 and Gly280 [18–20]; (5) reduction of the disulfide bond linking the two toxin fragments [21]; (6) trafficking of the carboxyl terminal fragment of the toxin (amino acids 280–613) from the transreticular Golgi to the endoplasmic reticulum through binding of the REDL carboxyl terminus to the KDEL receptor; (7) translocation of the toxin fragment to the cytosol [22, 23]; (8) once in the cytosol, catalytic ADP ribosylation of the diphthamide residue [24] in EF2 by PE amino acids 400–602 [5], requiring residues His440 and Glu553 [5, 25–27]; and finally (9) apoptotic cell death [28–31].

2.2 Mechanism of Cell Death by DT

The DT protein is 535 amino acids, composed of a catalytic A domain (amino acids 1–193) and binding B domain (amino acids 482–535) [32–34], separated by the transmembrane translocating (T) domain [35]. DT is thought to intoxicate cells by (1) proteolytic processing by furin between Arg193 and Ser194, generally prior to binding [36], leaving a disulfide bridge at Cys186 and Cys201; (2) binding via amino acids 482–535 to CD9 plus heparin-binding EGF-like growth factor precursor on the cell surface [32, 37]; (3) internalization and unfolding at low pH; (4) insertion of a hairpin from the TH8 (amino acids 326–347) and TH9 (amino acids 358–376) domains into the endocytic vesicle, allowing translocation of the catalytic domain to the cytosol [38–43]; (5) binding of NAD to DT amino acids Glu148, Tyr65, Tyr54, and His21; (6) transfer of the ADP ribose of NAD to EF2 [24, 44–46]; and, finally, (7) apoptosis resulting from ADP ribosylation, leading to cell death [28, 47].

3 Construction of Recombinant Immunotoxins

PE40 was the earliest truncated form of PE used to selectively target cells, composed of PE amino acids 253–613 [14, 48]. Amino acids 365–380, containing an unnecessary disulfide bond, were removed, resulting in PE38, without compromising cytotoxicity or ease of production [49, 50]. DT-based recombinant immunotoxins originally contained methionine followed by amino acids 1–485, called DAB_{486}, which was missing domain 5 [51]. To improve cytotoxicity and toxicity to

normal tissues, a disulfide bond within the T domain was removed, leaving the first 388–389 amino acids of DT (DT388 or DAB_{389} or DT390) for making recombinant immunotoxins [52, 53]. Single-chain Fv fragments, containing variable heavy (VH) and light (VL) domains fused via a flexible $(G_4S)_3$ linker, were first fused to truncated bacterial toxins to make recombinant immunotoxins [50, 54, 55]. To increase stability, cysteine residues were engineered into the variable domains so that they would be ~5 Å apart and join VH and VL, resulting in disulfide-stabilized recombinant immunotoxins [56–58]. Typically, VH rather than VL is fused directly to the toxin, since VH is less stable than VL as a single domain, and free VH-toxin is less likely to contaminate the recombinant immunotoxin than free VL-toxin. The disulfide-stabilized immunotoxins are considered recombinant since chemical conjugation is not needed for production.

3.1 Production of Recombinant Immunotoxin

The DNA fragment encoding the recombinant immunotoxin, or the single domain of a disulfide-stabilized recombinant immunotoxin, is ligated into a T7 promoter-containing plasmid [59], which is then introduced into *Escherichia coli* containing a repressed RNA polymerase. During fermentation, T7 RNA polymerase is induced by adding isopropyl-*b*-D-thiogalactopyranoside (IPTG), a lactose analog, which leads to plasmid expression and production of the recombinant immunotoxin. The protein may stay soluble in the periplasm [60] or cytoplasm [61] of the *E. coli* cell, but the highest yields of recombinant immunotoxin are possible when the protein is stored in insoluble inclusion bodies [62–65]. The insoluble inclusion body protein is then homogenized, detergent washed to removed endotoxin, dissolved in 7 M guanidine or 8 M urea, denatured, and reduced. The reduced-denatured protein is then renatured after dilution into redox buffer, permitting pairing of disulfide bonds to the native structure. Dialysis, ultrafiltration, or dilution can then be used to decrease ionic strength prior to ion-exchange chromatography. Ultrafiltration is often used in high-yield production. After anion exchange, the pure protein is obtained by sizing chromatography. By this method, 5–20 % of total renatured recombinant inclusion body protein can generally be obtained pure, although industrial methods may improve significantly on this percentage. Recombinant immunotoxins are also produced from eukaryotic *Pichia pastoris* cells containing mutant EF2 to prevent cell death [66, 67].

3.2 Denileukin Diftitox and Other Growth Factor Fusion Toxins

Recombinant fusions of growth factors and bacterial toxins are often considered as types of recombinant immunotoxins, since the ligand, although not an antibody, is of immunologic interest. The one example of this class which has been approved is denileukin diftitox, indicated for relapsed and persistent cutaneous T-cell lymphoma (CTCL) [68]. Denileukin diftitox, earlier called Ontak or DAB_{389}IL-2, also has reported activity in non-Hodgkin's lymphoma (NHL) and chronic lymphocytic leukemia (CLL) [69, 70]. Several recombinant fusion toxins containing truncated PE were tested clinically. TP40, targeting the epidermal growth factor receptor (EGFR) in bladder cancer, contains TGF alpha and a form of PE40 [71]. IL-4(38-37)-PE38KDEL, containing circularly permuted interleukin-4 toxin and a variant of PE38 with KDEL replacing REDLK at the carboxyl terminus, targeted glioblastoma multiforme (GBM) and other solid tumors [72–75]. Interleukin-13-PE38KDEL, containing human interleukin-13 fused to the same truncated toxin, targeted GBM [76–78]. CD4-PE40 was tested against HIV-infected cells in AIDS patients [79]. Other growth factor fusion toxins besides denileukin diftitox which contain truncated DT have been tested in different types of tumors. Examples include DT388-GM-CSF (DTGM) for acute myelogenous leukemia (AML) [80–82], DT388-interleukin-3 for AML [83–85], and DAB_{389}EGF for EGFR-expressing solid tumors [86]. The remaining chapter will review recombinant immunotoxins tested clinically, each of which contains a cell-binding Fv fragment fused to truncated bacterial toxin.

4 Immunotoxins in Clinical Testing

4.1 Recombinant Immunotoxins Targeting IL-2-Receptor Alpha (CD25)

An early target expressed preferentially on malignant cells is the interleukin-2 receptor (IL-2R) [87–91]. This receptor is composed of subunits CD25 (alpha), CD122 (beta), and CD132 (gamma), which if all present have high affinity ($K_d \sim 10^{-11}$ M) for IL-2. CD25, if alone, has low affinity ($K_d = 10^{-8}$ M) for IL-2. The complex of CD122 with CD132 binds IL-2 with intermediate affinity ($K_d = 10^{-9}$ M) [92, 93]. CD25 is usually the most prominently expressed IL-2R subunit [87, 88]. To target CD25 with a high affinity, an Fv fragment was produced from the mAb anti-Tac, which binds to CD25 with high affinity ($K_d \sim 10^{-10}$ M), and fused to PE40 [54]. A PE38-containing version containing the deletion of amino acids 365–380 was created, called anti-Tac(Fv)-PE38 (Fig. 1) or LMB-2 [50], and was cytotoxic toward CD25+ cell lines, activated T-cells, and freshly obtained leukemia cells [50, 94, 95]. LMB-2 showed antitumor activity against human xenografts in mice which expressed human CD25 [96]. LMB-2 in biodistribution studies in mice was found to concentrate into CD25+ tumors and CD25-negative normal tissues, with highest concentrations being in the kidney [97, 98]. Cynomolgus monkeys, which were required for toxicity studies since only primate CD25 binds LMB-2, showed reversible toxicity at 300, 750, and 1,000 μg/kg every other day for 3 days (QOD × 3). The most common toxicity was transaminase elevations [94]. In pharmacokinetic studies, performed in mice and monkeys, biphasic disappearance in the plasma was observed with alpha 58 min and beta 170 min.

4.2 Clinical Activity of LMB-2

A phase I trial was performed in 35 patients with leukemia, lymphoma, and Hodgkin's disease (HD) using 2–63 μg/kg QOD × 3 of LMB-2. Dose-limiting toxicity (DLT) was reversible and at the highest level (50–63 μg/kg QOD × 3) consisted of transaminase elevations and cardiomyopathy [99]. All of four patients with hairy cell leukemia (HCL) responded with one complete remission (CR) and three partial responses (PRs) [100]. One patient each with CLL, ATL, CTCL, and HD also responded with PR. The most common toxicities were transaminase elevations associated with fever, possibly representing evidence of cytokine release [101, 102]. Six (17 %) out of 35 patients had immunogenicity after the first cycle which prevented retreatment. Phase II trials were begun in CD25+ HCL, CLL, ATL, and CTCL. In targeting ATL, two limiting factors are immunogenicity and progression between cycles because of incomplete response, the latter possibly caused by high tumor interstitial soluble CD25 (sCD25), which can block LMB-2 distribution to tumor cells [103]. A trial is ongoing to block immunogenicity and decrease intratumoral sCD25, by treating with fludarabine and cyclophosphamide (FC) prior to LMB-2. Since about 20 % of chemoresistant HCL patients have the CD25-negative HCL variant (HCLv), HCL patients on immunotoxin trials are usually receiving anti-CD22 recombinant immunotoxin, reviewed next.

4.3 Recombinant Immunotoxin BL22 Targeting CD22

The siglec family member CD22 [104] contains three immunoreceptor tyrosine-based inhibitory motifs (ITIMs), associates with the B-cell receptor, and inhibits activation by recruiting SHP-1, an inhibitory tyrosine phosphatase. Earlier nonrecombinant chemical conjugates, which contained ricin A chain connected to RFB4 or truncated PE connected to LL2, targeted CD22 on B-cell malignancies [105–111]. A recombinant anti-CD22 immunotoxin was produced using the Fv of RFB4 [111] and

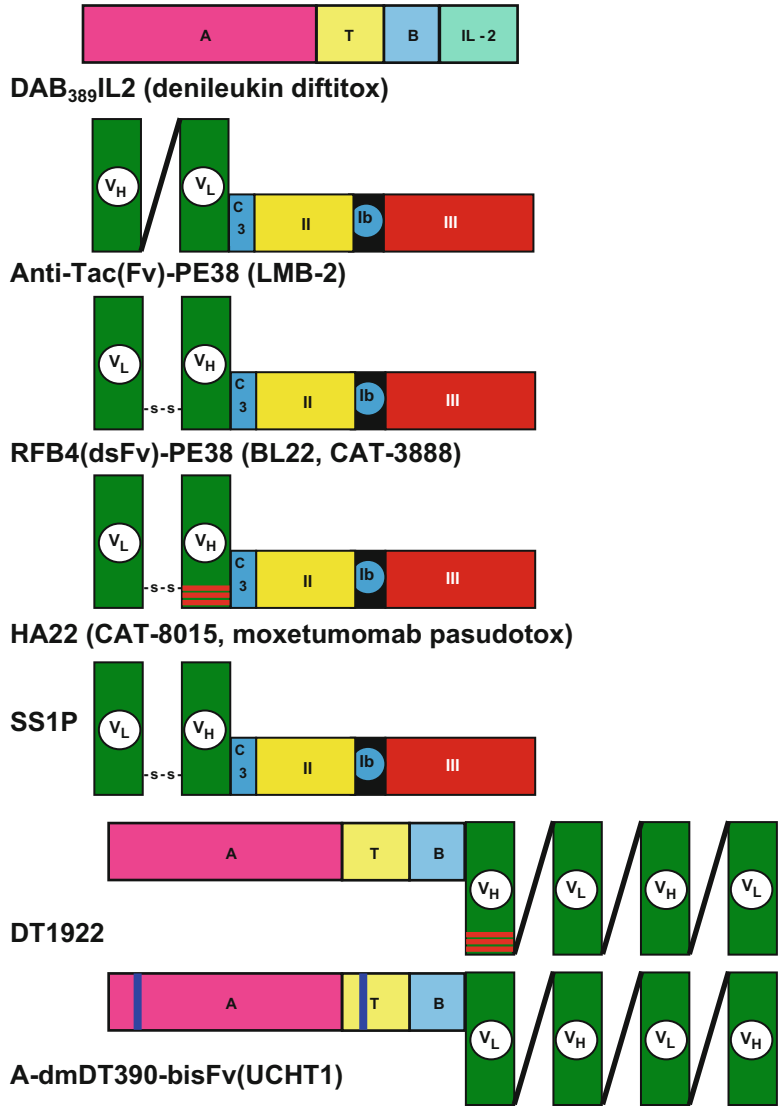

Fig. 1 Recombinant immunotoxins in use or under development. Denileukin diftitox (DAB_{389}IL-2 or DT388-IL-2) contains DT amino acids 1–388, which are composed of the activity (A) chain, the transmembrane (T) domain, and part of the binding domain (B). The ligand is interleukin-2 (IL-2). DT-containing recombinant immunotoxins include DT1922, containing sFvs against CD22 and CD19, and A-dmDT390-bisFv(UCHT1), containing two anti-CD3 sFvs in tandem. LMB-2 is composed of anti-Tac mAb VH and VL joined by the peptide linker $(G_4S)_3$ and with VL joined via the C3 connector ASGGPE to PE amino acids 253–364 and 381–613. In BL22 and HA22, VL and VH are disulfide bonded together using engineered cysteine residues replacing Arg44 of VH and Gly100 of VL. HA22 is a higher affinity mutant of BL22 containing THW replacing SSY at positions 100, 100a, and 100b of VH. The same point mutations are present in the RFB4(VH) domain of DT2219. SS1P contains an engineered disulfide bond permitted by VH R44C and VL S105C mutations. S18A and N235A are point mutations in A-dmDT390-bisFv(UCHT1) which prevent glycosylation during its eukaryotic expression

was converted to the disulfide-stabilized recombinant immunotoxin RFB4(dsFv)-PE38, also called BL22 (Fig. 1). The dsFv contained mutations R44C in VH and G100C in VL which allowed VH and VL to become disulfide bonded [112]. BL22 induced CRs in CD22+ lymphoma xenograft-bearing mice. Mice with durable tumor regression had plasma levels similar to those achieved in cynomolgus

monkeys [113], and monkeys were required for toxicity analysis since only primate CD22 binds RFB4. BL22 was also cytotoxic toward leukemic cells directly from patients with CLL and NHL [114]. Thus, BL22 could kill cells expressing orders of magnitude less than CD22 compared to cell lines.

4.3.1 BL22 in Phase I Testing

A total of 46 patients, including 31 with HCL, 4 with B-NHL, and 11 with B-CLL, received 265 cycles of BL22 at 3–50 μg/kg QOD × 3 [115, 116]. Patients required treatment because of either cytopenias (neutrophils <1,000/mm^3, hemoglobin <10 g/dl, platelets <100,000/mm^3) or lymphocytosis (circulating HCL cells >20,000/mm^3) or tumor-related symptoms like painful splenomegaly or frequent infections. A total of 19 (61 %) CRs and 6 (19 %) PRs were observed in the 31 HCL patients, an overall response rate (ORR) of 81 % in HCL. Three of 31 HCL patients had the poor-prognosis variant HCLv, which is poorly responsive to even first-line cladribine [117], and all three of these HCLv patients had CR with BL22. Of 19 CRs, 11 were achieved after 1 cycle, and the other CRs required 2–14 cycles of BL22. By definition [118], CR in HCL required regression of disease visible by light microscopy using non-immunologic methods, and 1 of the 19 CRs had evidence of minimal residual disease in the bone marrow biopsy by immunohistochemistry [116]. Because CD22 is so highly expressed in HCL (median 44,000 sites/cell), plasma levels were low at first and increased with repeated cycles after patients responded. DLT in one patient consisted of a cytokine release syndrome with fever, bone pain, hypotension, and weight gain (VLS) but no pulmonary edema, lasting 3 days. A completely reversible hemolytic uremic syndrome (HUS) was observed in four patients on phase I, confirmed by renal biopsy. HUS presented with hematuria and hemoglobinuria by day 8 of cycles 2–3, was treated with 6–10 days of plasmapheresis without dialysis and completely resolved. Of the four patients with HUS, three achieved CR, suggesting benefit even in patients with DLT. The cycle 1 MTD of BL22 was 40 μg/kg QOD × 3, which was well tolerated in 12 of 12 patients. Thus, in phase I testing of chemoresistant HCL, BL22 achieved CRs in 61 % of patients, usually after 1 cycle, and with retreatment a completely reversible form of HUS was observed in 13 % of patients.

4.3.2 BL22 in Phase II Testing of HCL

To minimize the rate of HUS while maintaining activity, our goal was to determine the effect of 1 cycle and retreat only those patients who did not achieve resolution of cytopenias after 1 cycle. Resolution of cytopenias was defined as the normal blood counts needed for CR, namely, neutrophils ≥1,500/mm^3, Hgb ≥11 dl, and platelets ≥100,000/mm^3. Patients not achieving these normal blood counts could still qualify for PR with ≥50 % improvements in normal blood counts. Patients who achieved resolution of cytopenias but not CR due to residual disease in the bone marrow were classified as hematologic remission (HR) and like the CRs would not be retreated after 1 cycle. HCL patients on the phase II trial received the phase I-determined MTD (40 μg/kg QOD × 3) for cycle 1 and were retreated only if not achieving HR by 8 weeks after cycle 1. Thus, patients achieving HR after cycle 1 would undergo bone marrow biopsy to document CR but regardless of the result would be observed without retreatment. Patients with PR (not achieving HR) or stable disease after 1 cycle would begin retreatment at 30 μg/kg QOD × 3 every 4 weeks, until 2 cycles past CR. Retreatment also required absence of high levels of neutralizing antibodies. After 1 cycle of BL22 in 36 HCL patients, there were 9 (25 %) CRs, 3 (8 %) HRs, and 6 (17 %) PRs, an overall response rate (ORR) of 50 % [119]. After retreating 56 % of the 36 patients, the final response rates included 17 (47 %) CRs, 5 (14 %) HRs, and 4 (11 %) PRs, an ORR of 72 % [119]. Of the 17 CRs, MRD was positive in 3 (18 %) patients by BMBx IHC. Patients with CR after retreatment with BL22 had no MRD. Disease-free survival (DFS, CR duration) for phase II had not been reached at a median of 32 (range 4–62) months,

with 12 (71 %) of 17 CRs still ongoing. Patients with spleens <200 mm had higher CR rate after 1 cycle (41 % vs. 0 %, $p=0.006$) or ≥1 cycle (64 % vs. 21 %, $p=0.019$) compared to either patients with spleens >200 mm or patients having had prior splenectomy. The ORR was also higher after 1 cycle (73 % vs. 14 %, $p=0.002$) or ≥1 cycle (95 % vs. 36 %, $p=0.0002$) than in patients with smaller spleens. This observation may have been due to more advanced disease in patients with larger spleens or splenectomy, or possibly more limited tumor penetration of BL22 into large spleens or densely packed marrow disease which may occur after splenectomy. Therefore, BL22 experimental therapy may be most appropriate before patients have either massive splenomegaly or splenectomy.

4.3.3 Conclusions from Phases I and II Testing of BL22

In comparing phase II with phase I testing of BL22, the average dose/cycle was similar (33 vs. 29 μg/kg × 3), but with selective retreatment, the average cycle/patient was 3.6 vs. 8.6 ($p<0.0001$). There was a significant decrease in immunogenicity (11 % vs. 39 %, $p=0.002$). The rate of dose-limiting HUS on protocol for phase II was <50 % that of phase I (2 of 36 vs. 4 of 31), which was not statistically significant due to the low number of events on each trial. Response rates for phase II were not significantly lower compared to phase I (CR 47 % vs. 61 %, $p=0.3$, ORR 72 % vs. 81 %, $p=0.8$, respectively). Thus, BL22 achieved CR rates of 47–61 % in relapsed/refractory HCL and had a safety profile that supports continued development.

4.4 Targeting CLL with Anti-CD22 Recombinant Immunotoxins

CLL was less responsive than HCL to BL22 [116], attributed to the lower CD22 expression, with a median of 1,250 sites/cell on CLL vs. 44,000 sites/cell on HCL cells. The relatively high off-rate of BL22 indicates that a high percentage of molecules binding disassociate before internalizing. To increase the percentage of bound molecules which internalize, the off-rate of BL22 was decreased by mutagenesis of the CDR3 domain within "hot spots" for somatic mutations. The mutation of residues 100, 100a, and 100b of VH from SSY to THW resulted in a mutant with 15-fold improved binding affinity [120], and 8-fold improved cytotoxicity. The higher affinity version of BL22, called HA22 (or CAT-8015 or moxetumomab pasudotox; Fig. 1), is now undergoing multicenter testing in phase I trials in CLL, HCL, and NHL. So far, moxetumomab pasudotox in HCL has resulted in only two cases of non-dose-limiting HUS. Additional improvements have been made by removing a large section of domain II of PE containing lysosomal protease sites, which also contains immunogenic epitopes. The resulting molecule HA22-LR was much more cytotoxic to CLL samples than HA22, probably by avoiding destruction of the toxin in lysosomes while it is trafficked to the ER [121]. More improvements are being made to mutate and remove mutagenic epitopes to avoid the immunogenicity problem [122, 123].

4.4.1 Targeting Both CD22 and CD19

A bispecific single-chain immunotoxin was engineered containing methionine followed by the first 389 amino acids of DT and then followed by two Fv fragments in tandem, one directed to CD22 and the other to CD19 [124]. DT2219 (Fig. 1) was cytotoxic to CD22+/CD19+ cell lines like Daudi with an IC50 of 2 nM (~200 ng/ml). The affinity was improved using the SSY → THW mutation at position 100 of VH, first used for BL22 [120], lowering the IC50 to 0.3 nM (~30 ng/ml). DT2219 showed both tumor regression and increased survival in murine xenograft studies [124]. DT2219 subsequently began clinical testing in patients with CD22+/CD19+ B-cell tumors.

4.4.2 Targeting CD3 with Recombinant Immunotoxin

The anti-CD3 mAb UCHT1 [125] was previously chemically conjugated to toxin and shown to kill CD3+ T-cells ex vivo and prevent graft-versus-host disease in patients [126]. To determine if a recombinant anti-CD3 immunotoxin would be better or equivalent, the recombinant immunotoxin DT390-UCHT1-sFv was constructed [127]. Due to a 22-fold loss of binding affinity compared to the free sFv, a divalent recombinant immunotoxin (A-dmDT390-bisFv) was engineered (Fig. 1). This molecule is composed of an N-terminal alanine, DT amino acids 1–389, the ASAGGS connector, and the tandem Fv UCHT1 VL-VH-VL-VH with three $(G_4S)_3$ linkers placed between four variable domains [128]. Two mutations in DT, S18A and N235A, were used to prevent glycosylation during expression and production in CHO cells [128]. The protein expressed by *Pichia pastoris [*66], containing mutant EF2, was harvested through 0.1 μm hollow-fiber micro-filtration. The pure protein was obtained by purification on a hydrophobic affinity column (Butyl 650 M) and was then purified by Borate Poros 50 HQ anion exchange chromatography, removing glycoproteins, and then finally purified by Poros 50 HQ anion exchange chromatography [67]. A 69 % yield of pure protein was obtained from culture supernatant [67], and the plasma lifetime was 18 min when given at 56.25 μg/kg twice daily for 4 days. Preclinical toxicities included transient transaminase elevations, lethargy, and weight loss, but no decrease in organ function [129]. A-dmDT390-bisFv(UCHT1) subsequently began phase I/II testing in relapsed/refractory T-cell malignancies.

4.5 Older Trials of Recombinant Immunotoxins for Solid Tumors

Because of more difficult tumor penetration and higher rates of immunogenicity, targeting solid tumors with recombinant immunotoxins has been more challenging than targeting hematologic malignancies. An early target was the epidermal growth factor receptor (EGFR), targeted by either EGF or transforming growth factor alpha (TGF alpha) connected to truncated PE or DT [130–134]. Clinically, the TGF alpha-toxin TP40 was tested for intravesical treatment of bladder cancer [71], TP-38 was tested as intracerebral injection of glioblastoma multiforme [135], and $DAB_{389}EGF$ was tested systemically in patients with various solid tumors [136]. Chemical conjugates and recombinant immunotoxins were made to target LeY on solid tumors, and responses were reported in phase I trials [137, 138]. Erb-38, a recombinant immunotoxin targeting erbB2, had excessive liver toxicity when administered systemically, attributed to low expression on liver [139], but direct injection of scFv(FRP5)-ETA, also targeting erbB2, into breast and colon tumors, avoided this systemic toxicity [140, 141]. Direct injection of the circularly permuted interleukin-4 (IL-4)-toxin IL-4(38-37)-PE38KDEL was used to target glioblastoma multiforme [73–75, 142, 143], and it was also tested systemically [72]. The interleukin-13 toxin IL-13-PE38QQR was also tested by direct injection of glioblastoma multiforme [76–78, 144]. These trials demonstrated modest clinical activity with limitations including impaired distribution of toxin to tumor cells and immunogenicity during systemic delivery.

4.6 Targeting the Mesothelin Antigen on Solid Tumors

Mesothelin is a 40 kDa membrane glycoprotein which allows binding of CA-125, which then mediates malignant invasion [145–147]. Mesothelin is expressed on a variety of solid tumors, including mesotheliomas [148–152]. To target mesothelin, both chemical conjugates and recombinant immunotoxins were constructed [153–155]. The recombinant immunotoxin was produced by immunizing mice with DNA encoding mesothelin and screening phage Fv expression libraries [155].

Table 1 Recombinant immunotoxins under clinical development

Recombinant immunotoxin	Antigen	Toxin	Target disease(s) tested	Type of DLT	References
BL22	CD22	PE	HCL, CLL, NHL	HUS VLS	[115, 116, 119]
HA22	CD22	PE	HCL, CLL, NHL	TBD	[30, 120]
LMB-2	CD25	PE	HD, NHL, HCL, PTCL, CTCL, ATL	LFTs Heart	[99]
A-dmDT390-bisFv(UCHT1)	CD22	DT	T-cell malignancies	TBD	[67, 129]
DT2219	CD22, CD19	DT	B-NHL	TBD	[124]
SS1P	Mesothelin	PE	Mesothelioma, lung CA	VLS	[160, 161]

Dose-limiting toxicity (DLT) included liver function test abnormalities (LFTs), cardiomyopathy (heart), hemolytic uremic syndrome (HUS), vascular leak syndrome (VLS), and to be determined (TBD)

The resulting recombinant immunotoxin was engineered into a high-affinity mutant called SS1P (Fig. 1) by hot spot mutagenesis [156–159]. SS1P was tested in two phase I trials, one by bolus (30 min infusions) to 34 patients [160] and one by continuous infusion for 10 days to 24 patients [161]. Clinical activity was observed on these trials, along with frequent immunogenicity. Clinical development of SS1P is continuing in combination with chemotherapy, based on the synergy observed in vivo using animal models [103, 162, 163] (Table 1).

5 Conclusions and Future Directions

Two major advantages of recombinant immunotoxins over other types of targeted cancer therapy are extreme potency and alternative mechanism of action with less susceptibility to drug resistance. The ability to kill a cell with a single molecule in the cytosol is unique in nature and may be the only way to selectively target cells with limited numbers of sites/cell. Antibody-based toxins using a non-immunogenic small molecule may be able to kill cells in tissue culture or as xenografts which express a high number of sites/cells, such as established cell lines, but may not always be able to kill malignant cells found in patients, because receptor expression may be orders of magnitude less. The use of recombinant immunotoxins may obviate drug resistance in a way that targeted chemotherapy cannot. The trade-off for these unique potential advantages is the challenge of immunogenicity. While immunogenicity is more problematic for solid tumors than for hematologic malignancies, it remains a significant problem even in hematologic malignancies [99, 116, 119]. The approaches mentioned above to avoid immunogenicity involve mutating immunogenic epitopes without losing toxin efficacy [122, 164–166] and removing large sections of toxin which contain immunogenic epitopes [121]. Alternatively, it might be possible to safely decrease the immune system in the patient by pretreating with immunosuppressive chemotherapy. Since patients with CLL never made high levels of neutralizing antibodies to LMB-2 or BL22 in published studies [99, 116, 119], if one could decrease immune function to the level of a CLL patient, at least temporarily, immunogenicity toward recombinant immunotoxins might be lessened. As mentioned above, a pilot trial is now underway at NCI pretreating ATL patients with fludarabine and cyclophosphamide prior to LMB-2. An additional goal of this approach is that chemotherapy in mice appears to decrease the concentration of soluble receptor in tumors, which can be 10–100 times higher than the level of soluble receptor in blood [103]. These approaches may improve the targeting of both solid and hematologic tumors with recombinant immunotoxins.

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Monoclonal Antibodies

Shuang Bai, Rong Deng, Hong Xiang, Manish Gupta, Luna Musib, Banmeet Anand, and Bert Lum

Abstract Since the approval of rituximab in 1997 for the treatment of non-Hodgkin's lymphoma, over 11 monoclonal antibodies (mAbs) have been approved for the treatment of cancer and hundreds are under development. Characterizing pharmacokinetics (PK) and pharmacodynamics (PD) of an antibody is a fundamental aspect for selecting the right drug and right regimen for the right patients. As mAbs have complex pharmacology and the PK/PD depends on their structures and target antigens, understanding the biological characteristics of mAbs and their mechanisms of action is essential.

To fully understand the complex nature of this class of agents, we provide a brief overview of the biology and mechanisms of absorption, distribution, metabolism, and excretion of mAbs at the beginning of this chapter. Differences between mAbs and small molecules are highlighted. In the second portion of the chapter, a detailed review of selected therapeutic agents is provided, primarily focusing on the clinical PK/PD of approved mAbs for solid tumor and hematological malignancies. Since mAb clinical development is highly dependent on identifying target drug exposure using preclinical models, these data are also summarized for each specific molecule, where such data are available.

The unique PK/PD behavior of mAbs provides great opportunities and challenges during all phases of drug development. The discovery of new targets/epitopes, advancement of antibody engineering, and identification of new PD and pharmacogenetic markers, coupled with the necessity for the understanding of key drivers of PK/PD relationships, will inevitably further expand therapeutic potential of antibodies to bring exciting new treatment options to improve the lives of patients with cancer.

Keywords Monoclonal antibody • Pharmacokinetics and pharmacodynamics • Target-mediated disposition (clearance) • Immunogenicity

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1 Introduction

Therapeutic biologics are a class of agents that include proteins, protein conjugates, proteoglycans, and polypeptides. Monoclonal antibodies (mAbs) are a subclass of the protein biologics that broadly include cytokines, enzymes, growth factors, vaccines, and protein receptors.

In contrast to small molecules, mAbs have unique features that make the study of the pharmacokinetics and pharmacodynamics (PK/PD) complex. The target-mediated drug disposition of these agents may lead to nonlinear distribution and elimination. In addition, other factors such as the development of host immune responses to the mAb may further complicate the assessment of these drugs. For the reader to fully understand the complete nature of this class of agents, we provide a brief overview of the biology and the mechanisms of absorption, distribution, metabolism, and excretion and provide a detailed review of selected therapeutic agents.

In this chapter we primarily focus on the clinical PK/PD of approved mAbs for solid tumor and hematological malignancies. Because mAb clinical development in the oncology setting is highly dependent on identifying target drug exposure using preclinical models, these data are summarized for each specific molecule, where such data are available.

2 Biology of Monoclonal Antibody

Since the first US Food and Drug Administration (FDA) approval of rituximab in 1997 for the treatment of non-Hodgkin's lymphoma (NHL), at least eight unconjugated and three conjugated mAbs have been approved for the treatment of cancer and hundreds are undergoing preclinical and clinical development [1]. Characterizing PK and PD of an antibody is a fundamental aspect for selection of the right clinical candidate and the right regimen for a target indication. As mAbs have complex pharmacology and the PK/PD depends on mAb structure and target antigen, understanding the biological characteristics of mAbs and their mechanisms of action is essential.

2.1 IgG Structure

Antibodies (also known as immunoglobulins, abbreviated Ig) are a group of glycoproteins present in blood or other body fluids of vertebrates as part of the immune system to identify and neutralize foreign objects. Among the five isotypes (IgG, IgE, IgA, IgM, and IgD), IgGs have the most therapeutic potential as they are more permeable to extravascular spaces and have longer half-life through binding with the neonatal Fc receptor (FcRn). They also exhibit a range of biological effector functions through their abilities to interact with complement and various Fc receptors [1–3]. The longer half-life offers the benefit of less-frequent dosing and continuous interaction with the target.

IgG is a symmetrical "Y"-shaped molecule that consists of four polypeptide chains: two identical heavy chains (~50 kDa each) and two identical light chains (~25 kDa each) with the molecular weight of approximately 150 kDa (Fig. 1). The heavy chain has one variable domain (V_H) and three constant domains (C_H1, C_H2, C_H3), whereas the light chain has one variable domain (V_L) and one constant domain (C_L). IgG when treated with papain yields two Fab fragments (fragment of antigen-binding), which is composed of one constant and one variable domains from each heavy and light chains [4, 5]. Each variable domain contains three short stretches of peptide known as the complementarity-determining regions (CDRs). The CDRs are the major determinants of antigen-binding affinity and specificity. The Fc region of the antibody is associated with effector functions through the interaction with Fcγ receptors and disposition through the interaction with the neonatal Fc receptor (FcRn). The binding characteristics of the antibody to the antigen and Fc receptors affect PK/PD behavior of the antibody.

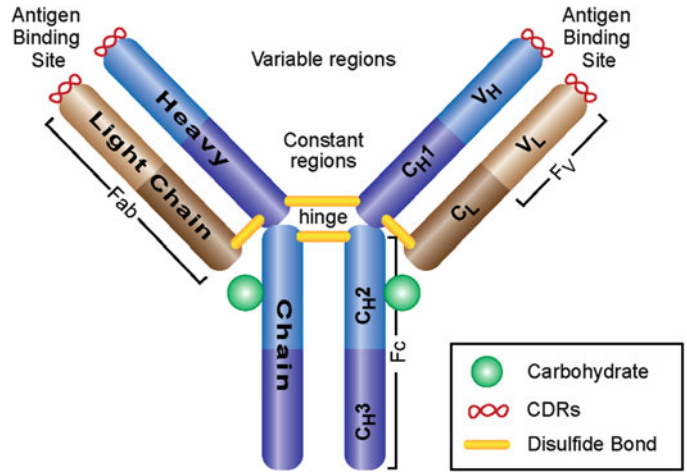

Fig. 1 IgG1 antibody structure. IgG is a symmetrical "Y"-shaped molecule that consists of four polypeptide chains: two identical heavy (H) chains and two identical light (L) chains. The heavy chain has one variable domain (V_H) and three constant domains (C_H1, C_H2, C_H3), whereas the light chain has one variable domain (V_L) and one constant domain (C_L). The hinge region is a segment between C_H1 and C_H2 domains. IgG treated with papain yields two Fab fragments (fragment of antigen-binding), which is composed of one constant and one variable domain from each heavy and light chain. The variable domains on Fab are referred as F_V region and there are complementarity-determining regions (CDRs) in this region. The antibody-binding sites (epitopes) on the CDRs are the major determinants of antigen-binding affinity and specificity. Fc fragment (fragment crystallizable) formed by C_H2 and C_H3, on the other hand, is associated with effector functions

2.2 Antibody Engineering

Since Koehler and Milstein invented the hybridoma technique in 1975 to generate mAbs [6], therapeutic antibodies have evolved from murine, chimeric, and humanized to human mAbs and differ in the degree of similarity to human antibody. Murine mAbs (named as -omab) have the lowest homology with human mAbs. Chimeric mAbs (named as -ximab) share 60–70 % similarity with human mAbs through combination of the human constant region with the intact mouse variable regions [7, 8]. Humanized mAbs (named as -zumab) contain only the CDRs of the mouse variable region grafted onto the human variable region framework with 90–95 % homology with human mAbs [9, 10]. Human mAbs (named as -umab) are generated through phage display library or genetically engineered mice [11–13]. Both humanized and human mAbs have been widely developed as therapeutic candidates to reduce the incidence and severity of immune responses to the drug and to improve PK/PD and safety profiles.

2.3 Antibody Mechanism of Action

Monoclonal antibodies target-specific antigens through the Fab region and the target could be soluble or membrane-bound antigens [2, 14]. The binding of antibody to the soluble antigen (e.g., bevacizumab to vascular endothelial growth factor; VEGF) neutralizes and reduces the concentration of the circulating antigen to achieve its activity. The binding of antibody to membrane-bound antigen overly expressed or differentially expressed on tumor cells or supporting cells exerts its therapeutic effect through modulation of receptor density by internalization, shedding, and downregulation or through

the block of ligand binding. Antagonistic antibodies (e.g., cetuximab) prevent ligand binding to provide antitumor activity, while agonistic antibodies (e.g., anti-DR4 and anti-DR5 antibodies) mimic a natural ligand's function to enhance its antitumor activity (i.e., apoptosis) [15, 16]. In addition, some antibodies (e.g., rituximab) eliminate receptor-positive cells through the effector function, such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) [2].

As the knowledge of mAbs grew during the past decades, innovative extensions for the use of these agents emerged, such as the development of antibody–drug conjugate (ADC) or immunoconjugates, whereby mAbs have been linked to potent cytotoxic chemical entities or radioisotopes [15]. In addition, another area of brisk and exciting development is bispecific antibodies, which are engineered to bind to two distinct antigens in hopes that the simultaneous blockade of several targets may yield better therapeutic efficacy than inhibition of a single target [15, 17]. These agents are early in clinical development and will not be extensively discussed in this chapter.

3 Overview of Pharmacokinetics and Pharmacodynamics of Monoclonal Antibodies

Monoclonal antibodies have several unique and complex PK/PD characteristics that differ from the small molecule drug (SMD). Typical metabolic enzymes and transporter proteins such as cytochrome P450 and multidrug resistance (MDR) efflux pumps that may be critical for small molecule drugs are not involved in the disposition of mAbs. In contrast, mAbs are mainly eliminated via a large-capacity nonspecific IgG elimination pathway and a specific target-mediated drug disposition pathway. Protein binding is common for small molecules but not for mAbs. Further discussion of the absorption, distribution, and clearance is provided below. The PK/PD characteristics of mAbs have been extensively reviewed recently [1, 18–20] and are summarized in Table 1.

Table 1 Pharmacokinetics and pharmacodynamics of small molecule drugs versus monoclonal antibodies

Characteristics	Small molecule drug	Monoclonal antibody
PK	Nonspecific binding (can affect multiple enzymes)	Specific binding to target
	PK linearity dependent on enzymatic capacity	PK linearity dependent on target level
	Therapeutics doses: typically linear PK	Low doses: nonlinear PK
	High doses: nonlinear PK (saturation of metabolic enzymes)	High doses: linear PK (saturation of target-mediated clearance)
	Fast clearance and short half-life (in hours)	Slow clearance and long half-life (in days)
	Oral dosing preferred	No oral dosing
	Parenteral routes possible	Common routes: intravenous, subcutaneous, or intramuscular injection
	Significant serum protein binding	No serum protein binding
	Binding to tissues, high volume of distribution	Distribution limited to blood and extracellular space
	Metabolism by cytochrome P450s or other enzymes	Metabolism by specific and nonspecific clearance mechanisms
		No P450s involved
	Significant renal clearance	No renal clearance of intact antibody
PD	Intra- and extracellular targets	Extracellular targets with high affinity and specificity
	Polypharmacology exists	
	Off-target toxicity common	Limited polypharmacology
		Limited off-target toxicity
PK/PD relationship	PK and PD are independent	PK and PD are interdependent

3.1 Pharmacokinetics

3.1.1 Absorption

Monoclonal antibodies are administrated parenterally because of their limited gastrointestinal stability, poor lipophilicity, and molecular size. Intravenous administration is still the most common route of administration, which allows for immediate systemic delivery of large volume of drug product and provides complete systemic availability. Currently marketed anticancer antibody therapeutics are most commonly administrated via intravenous infusion. Subcutaneous or intramuscular administration has been used, but mainly in immunology therapeutic areas. The pulmonary route of administration is under investigation and holds promises for the treatment of lung cancers [21].

Absorption following subcutaneous or intramuscular injection is facilitated by the lymphatic system with bioavailability generally in the range from 50 to 80 % [20]. The incomplete bioavailability is due to the proteolytic degradation of mAbs in the interstitial fluid or lymphatic system. The absorption rate is also slow, and it typically takes a few days to reach the peak serum concentration after subcutaneous or intramuscular injection.

3.1.2 Distribution

The antibody distribution is far more complex than small molecules and involves multiple processes including convection, diffusion, endocytosis, and transcytosis [20]. Convection is considered as the primary mechanism for movement of antibodies from the vasculature to the interstitial spaces. Other potential factors affecting antibody distribution are the binding affinity, kinetics of antigen–antibody complex, and receptor expression levels (i.e., antigen sink) for specific or nonspecific binding [20, 22, 23].

Monoclonal antibodies are typically designed to bind their target antigen in tissue sites with high affinity, and consequently a large apparent volume of distribution might be expected. However, the large molecular weight and hydrophilicity of mAbs limited their ability to distribute from the blood compartment to peripheral tissue. This results in slow distribution and low volume of distribution in most of cases. Generally the volume of distribution in the central compartment (V_c) and the steady-state volume (V_{ss}) in humans are in the range of 2–3 L and 3.5–7 L, respectively, suggesting that mAbs are largely confined to the vascular and interstitial spaces [18].

Antibodies hold high therapeutic promises with their ability to bind specifically to antigens and selectively target the disease. Bound antibodies can destroy tumor cells by recruiting immune effectors, blocking proliferative signaling, or delivering cytotoxic agents. However, heterogeneous distribution of systemically administered antibodies in tumor has been a recognized issue for immunotherapy for over 20 years [24]. The heterogeneous distribution can significantly impact the therapeutic response by leaving a fraction of cells untargeted, which may also lead to drug resistance. It is believed that the heterogeneous distribution is due to a complex process, which involves plasma clearance, extravasation across tumor capillaries, blood flow through tumor, convection, diffusion and binding within tumor interstitium, and internalization and catabolism in the tumor cells [25]. Multiple factors may influence this process, including dose, diffusivity, permeability, affinity, and antigen density [25]. Understanding the causes of incomplete antibody distribution into tumors is important to make rational dosing regimen selection and improve clinical efficacy.

3.1.3 Clearance

Owing to their large molecular size, mAbs do not undergo renal elimination/excretion or cytochrome P450-mediated hepatic metabolism of parent drugs. Antibodies are primarily cleared by proteolytic catabolism and broken down into peptide fragments and amino acids that can be recycled as energy

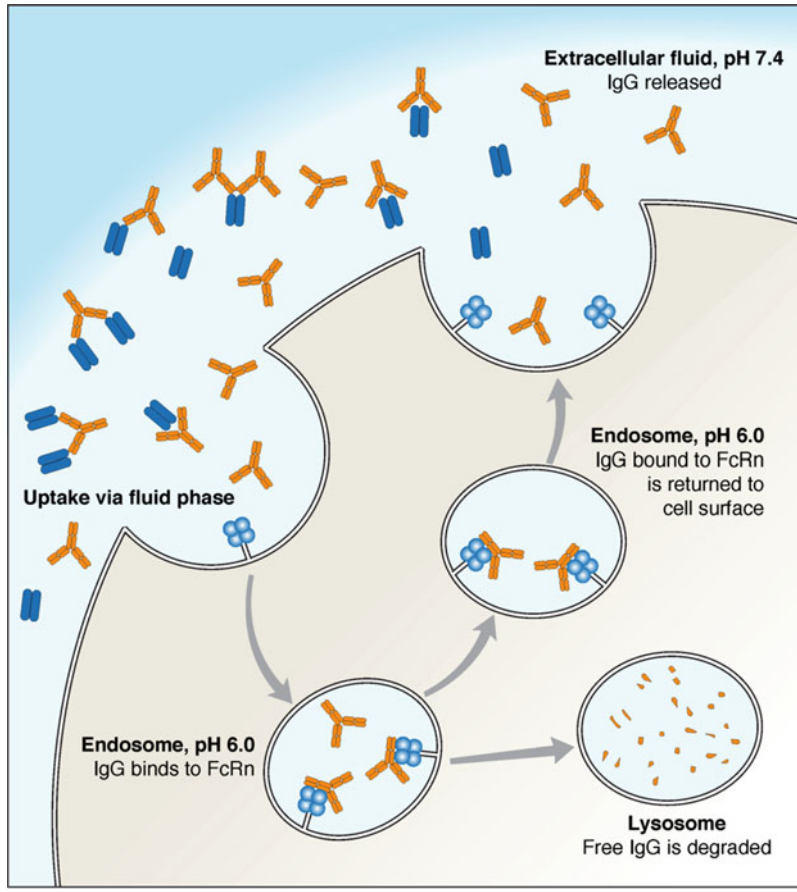

Fig. 2 Schematic regulation of IgG catabolism by FcRn. Schematic regulation of IgG catabolism by FcRn: (1) IgG in the extracellular fluid is uptaken by fluid-phase endocytosis and delivered to endosome; (2) IgG binds to FcRn at acidic pH (6.0) at endosome; (3) IgG bound to FcRn is transported and released back to the extracellular fluid, where dissociation occurs at a neutral pH; (4) IgG that is not bound to FcRn in the endosome undergoes transport to and proteolysis in the lysosomes

supply or for new protein synthesis. Therapeutic mAbs often exhibit two distinct catabolic pathways: (1) a nonspecific, linear clearance pathway mediated by interaction of the Fc region of a mAb with Fc receptors (i.e., FcRn and Fcγ receptors) and (2) a specific, nonlinear (target-mediated) clearance pathway mediated by the specific interaction of the CDR in the Fab region of a mAb with its pharmacological target [1, 18–20].

IgG catabolism seems to be widely distributed in the liver, skin, muscle, spleen, intestine, and tumor. The liver and peripheral tissues (e.g., skin and muscle) are the major sites of IgG catabolism, each accounting for 25–50 % of antibody degradation, whereas the spleen, intestine, and tumor sites are shown to be minor degradation sites [26]. The nonspecific clearance pathway is independent of specific interaction between a mAb and its pharmacological target and is a common pathway for both endogenous IgGs and therapeutic IgG mAbs. This pathway has large capacity, and the clearance is generally low and constant across the therapeutic dose range. The low clearance of mAbs is believed to be a result of the protection mechanism of the FcRn receptor [27–29]. FcRn is a heterodimer comprising of a β2-microglobulin (β_2m) light chain and a MHC-class-I-like heavy chain [30] and is ubiquitously expressed in cells and tissues [31]. Following the cellular uptake of IgG, it binds to FcRn where bound IgG is redirected to cell surface and then released to the extracellular fluid, while unbound IgG is delivered to lysosome for degradation (Fig. 2). This salvage mechanism helps to

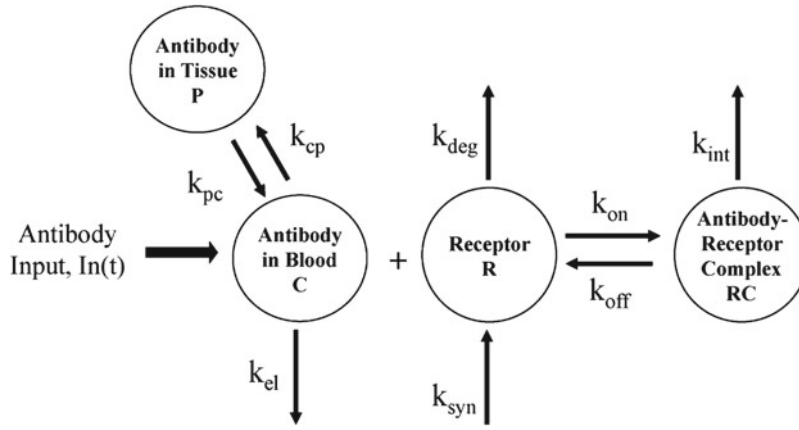

Fig. 3 Target-mediated drug disposition model. In(t) is the input rate of the antibody. Once the antibody is administered, it distributes from the central compartment (blood) to the peripheral (tissue) compartment. This process can be described by a two-compartment PK model. The antibody binds to the target receptor to form the antibody–receptor complex (PD effect). C and P are free antibody concentrations in the blood and tissue. R and RC are concentrations of the free receptor and antibody–receptor complex. k_{el} is the nonspecific elimination rate constant of the antibody. k_{pc} and k_{cp} are distribution rate constants of antibody from blood to tissue and tissue to blood. k_{deg} and k_{syn} are rate constants for degeneration and production of the receptor. k_{on} and k_{off} are association and dissociation rate constants of the antibody–receptor complex. k_{int} is the internalization (elimination) rate constant of the antibody–receptor complex

explain the much longer elimination half-life of IgG (~21 days in humans) compared with other immunoglobulin classes (IgA ~6 days, IgE ~2.5 days, IgM ~5 days, IgD ~3 days). Within the IgG class, IgG1, IgG2, and IgG4 subclasses exhibit long half-life around 21 days and clearance about 3–5 mL/day/kg, with the exception of IgG3 (half-life of 7 days). The difference in elimination half-life of IgGs has been attributed to differences in the binding of FcRn [32].

Although with large capacity, FcRn salvage can be saturated at high IgG concentrations. This is supported by the findings that decreased IgG elimination half-life was observed in patients with elevated serum IgG concentrations following administration of 1–2 g/kg intravenous immunoglobulin (IVIG) therapy [33, 34]. However, the doses of therapeutic mAbs less likely saturate the FcRn receptors and result in alteration of the nonspecific clearance.

For the specific clearance pathway, also known as the target-mediated clearance pathway, binding of a mAb to its target antigen (typically cell-bound receptor) can result in the internalization and subsequent intracellular degradation of the mAb in lysosome (Fig. 3). As the number of receptors within the distribution space of the mAb is limited, they may become saturated at therapeutic doses of the mAb. Thus, nonlinear clearance can be observed with faster clearance at low doses and slower clearance at higher doses. The nonlinear PK may also reflect on time-dependent changes if the receptor density or tumor burden is altered by mAb activity (e.g., CD20+ B-cell depletion by rituximab treatment) [35, 36]. mAbs binding to a soluble ligand can also experience target-mediated clearance, particularly at low doses of the therapeutic mAb or in instances of high ligand levels. In addition to the receptor density, the binding affinity of mAbs to the target and target turnover rate could affect the target-mediated clearance. In general, mAbs with high affinity appear to exhibit faster clearance [37]. Figure 4 presents typical linear and nonlinear PK profiles of mAbs.

In addition to the specific and nonspecific clearance pathways, several other factors are important in determining the disposition of mAbs. These include the immunogenicity of antibody (Sect. 3.2), the degree and the nature of antibody glycosylation, effector function, the susceptibility of antibody to proteolysis, and potential drug–drug interaction between mAbs and SMDs (Sect. 3.3) [20, 32, 38].

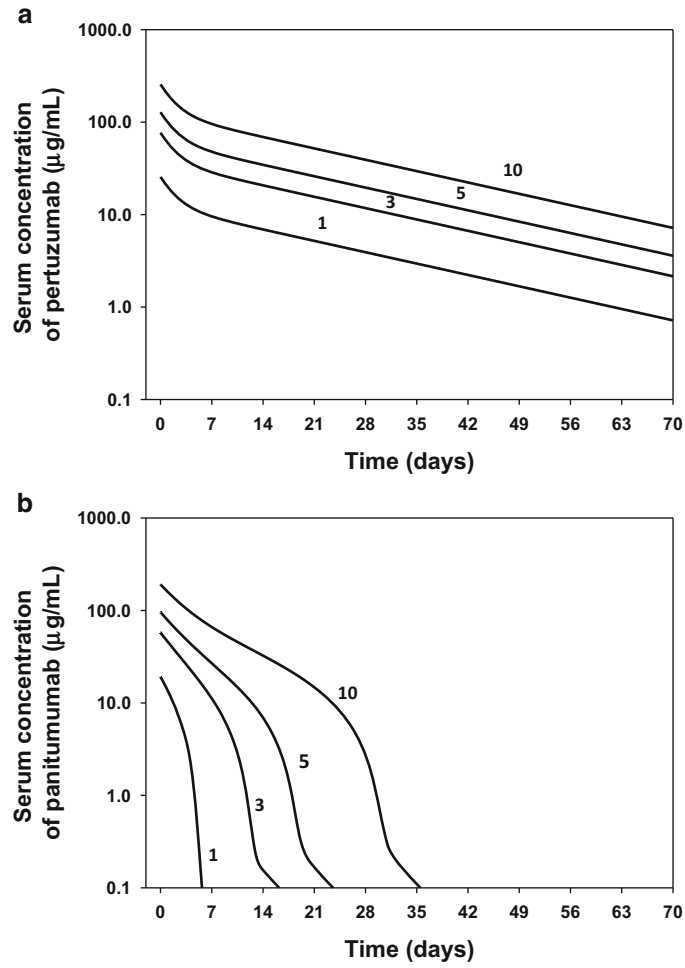

Fig. 4 Linear versus nonlinear pharmacokinetic profiles for monoclonal antibodies. Representative serum concentration–time profiles after intravenous doses (1, 3, 5, and 10 mg/kg) of pertuzumab (**a**, linear PK) and panitumumab (**b**, nonlinear PK). The data are simulated based on published population PK parameters for pertuzumab [226] and panitumumab [128], respectively

3.2 Impact of Immunogenicity on Pharmacokinetics

Immunogenicity is a property of biologically derived proteins to induce humoral and cellular immune responses. It can be a significant problem in the therapeutic use of mAbs containing xenogenic protein sequences. The immunogenic potential of therapeutic biologics is governed by product-intrinsic factors (e.g., species-specific epitopes, degree of foreignness, glycosylation status, extent of aggregation or denaturation, impurities, and formulation), product-extrinsic factors (e.g., route of administration, acute or chronic dosing, and existence of endogenous equivalents), and patient-specific factors (e.g., autoimmune disease, immunosuppression, and replacement therapy) [39]. Although evolution in generation of mAbs from the use of murine to humanized/human antibodies has been crucial in reducing the immunogenicity rates, the immune response to therapeutic antibodies still has clinical relevance. All currently marketed mAbs have exhibited some level of immunogenicity [40].

The consequences of an immune reaction to a therapeutic protein range from transient appearance of antibodies without any clinical significance to severe life-threatening events. Immune complex formation in serum has been shown to accelerate clearance of mAbs by the reticuloendothelial system (RES) and/or to neutralize the antigen-binding domain, which may result in the loss of efficacy and hinder therapeutic effects following repeated mAb administration [41, 42]. Immunogenic responses

to infliximab and muromonab-CD3, two drugs approved in the immunology therapeutic area, have been shown to impact the duration and the extent of the response in patients. Serious adverse events and safety risks, such as hypersensitivity reactions, infusion reactions, anaphylactoid reactions, and induction of autoimmunity (including antibodies to the endogenous form of the protein), have also been associated with immunogenicity of mAb therapeutics [40, 43–46].

Evaluation of the immunogenicity of therapeutic mAbs therefore remains an important part of antibody–drug development. A risk-based assessment strategy has been well applied across the biopharmaceutical industry [40, 47–51]. The premise of this strategy is to consider the severity of an immunogenicity response to a protein therapeutic if one is induced. The critical factors to be considered are related to the biological function of the product or its endogenous counterpart(s), the target of the product, the mode of administration, and the health status of the subject, concomitant medications. The identification of the risk level will affect the immunogenicity testing scheme in terms of timing and frequency of sampling; neutralizing activity assessment; qualitative, semiquantitative, or quantitative measurement; as well as characterization of a positive response.

Immunogenicity results are highly dependent on the sensitivity and specificity of the test method. Two essential tests recommended are the screening and confirmation assay by drug inhibition and/or immunoglobulin depletion [47, 52]. Due to competition for product-specific antibodies between the drug and a capture reagent in the assay system, it is desired for samples to be collected after a suitable drug washout period in order to minimize the drug interference in the assay. Furthermore after positive screening, in high-risk cases, measurement of neutralizing antibody (NAb) activity should be carried out using functional cell-based assays as the most preferable methodology. Evaluation of the PK/PD or biomarker data may be helpful as an indirect assessment of the neutralizing antibody activity.

Taken together, the immunogenicity of therapeutic proteins is a concern for clinicians, manufacturers, and regulatory agencies and often requires an assessment of correlation with any pharmacological and/or toxicological observations.

3.3 Pharmacokinetic Drug–Drug Interaction

As mAbs are an integral component of combination drug therapy regimens, drug–drug interactions (DDI) have become an ongoing assessment in clinical pharmacology of mAbs. PK DDI between therapeutic mAbs and conventional SMDs are usually not expected, since they have different clearance mechanisms. Nevertheless, many clinical PK drug interaction studies have been conducted between mAbs and SMDs, mostly through cross study or substudy comparison. However, dedicated DDI studies, i.e., with formal designs, have not been commonly performed.

It was reported that mAb-induced changes in cytokine levels could result in downregulation of mRNA and decreased CYP expression and therefore potentially alter PK of SMDs [53–56]. On the other hand, effect of SMDs on mAb PK may theoretically occur through their effect on proteolytic catabolism, salvage by FcRn, receptor-mediated disposition, and the expression of Fcγ receptors [32, 57]. Some comedications have been implicated in the development of immunogenicity of therapeutic mAbs [58]. In addition to the PK interaction, PD interaction may also occur that leads to additive, synergistic, or antagonistic effect of a drug [59].

Even though some of these DDIs for mAbs have been reported to be statistically significant, they are generally moderate compared to DDIs between SMDs. Typically the exposure change as a result of a DDI for mAbs is less than two-fold, and a need for dose adjustment has rarely been reported given the favorable therapeutic window of mAbs [60].

Unlike a SMD, investigating the DDI potential of a mAb poses scientific and operational challenges due to the nature of mAb-specific clearance mechanism, difference in target expression in patient

populations versus healthy volunteers, and the long elimination half-life [60]. As an alternative, population PK modeling has been shown to be a useful tool to assess the effect of concomitant medications on mAb PK in the covariate analysis.

3.4 Pharmacodynamics

Antibodies may act by a wide variety of pathways to elicit pharmacological effects. The target specificity offers advantages for studying the mechanism of action or toxicity, as well as the overall PD behavior of therapeutic mAbs. The PD endpoints can be biomarkers or clinical endpoints. A biomarker is a characteristic that can be objectively measured and evaluated as an indicator of normal and disease processes or pharmacological responses to a therapeutic intervention. Biomarkers can be specific cells, molecules, genes, gene products, enzymes, or hormones. Based on their application, biomarkers can be classified as diagnostic biomarkers, staging of disease biomarkers, disease prognostic biomarkers, and PD biomarkers for monitoring the clinical response to an intervention. Diagnostic and prognostic biomarkers are most well studied in the oncology therapeutic areas. The joint approval of trastuzumab and the immunohistochemical assay for determination of HER2 protein overexpression in metastatic breast cancer patients was the first example applying a diagnostic biomarker in specific therapy [61]. Based on the observation that the tumor *KRAS* mutation is a major predictive marker of resistance to the anti-epidermal growth factor receptor, panitumumab was approved to treat metastatic colorectal cancer with wild-type rather than *KRAS* mutation [62]. In addition, it has been observed that the polymorphism in FcγRIIIA was associated with favorable clinical response following rituximab administration in NHL patients [63, 64]. Further discussion of the impact of pharmacogenomics in cancer drug development is included in other chapters of this book.

3.5 Pharmacokinetics/Pharmacodynamics Relationship

As discussed previously, mAbs are target-specific, and the target-mediated clearance pathway plays an important role in antibody elimination. The effect on the targets can result in changes in the PK and subsequently the PD effects of the mAb. Therefore, unlike small molecules, antibody PK and PD are interdependent.

A target-mediated drug disposition model, which accounts for the drug PK, target dynamics, and their interaction, was first described by Mager et al. [65]. This model has been used to describe PK/PD relationships for many mAbs, e.g., anti-EFGR antibody 2F8 in monkeys [66] and anti-$\alpha_5\beta_1$ integrin mAb (volociximab) in cancer patients [67].

PD biomarkers are markers of a certain pharmacological response, which are of special interest in dose optimization studies. PD biomarkers sometimes directly or indirectly relate to clinical endpoints and typically exhibit a time course after drug treatment. The PD time course is often directly related to the time course of plasma drug concentrations, possibly with a measurable delay. For this reason, exposure (PK) and response (PD) relationships can help establish proof of activity in phase I trials and guide dose selection through each sequential phase of clinical development. It has been shown that the higher peak and trough concentrations of rituximab correlated significantly with better clinical responses in patients with low-grade NHL [35]. Similarly, the achievement of better clinical response and minimal residual disease for alemtuzumab, a humanized anti-CD52 antibody for chronic lymphocytic leukemia (CLL), is correlated with higher blood concentrations of the drug [68]. Therefore, through all phases of clinical development, the best “effect” measures, such as objective tumor

response, progression-free survival (PFS), and overall survival, should be identified. Understanding the relationship between drug exposure and these effect measures is very important to allow prediction of treatment efficacy and aid in dose optimization.

3.6 Dosing Regimens

Monoclonal antibodies are administered parenterally and the intravenous infusion is the common route of administration in the oncology therapeutic area. Most mAbs exhibit linear PK within the therapeutic dose range, indicating target saturation. Within the linear PK range, IgG mAbs typically have a long elimination half-life (~2–3 weeks). This enables a less-frequent dosing schedule (usually dosed every 1–3 weeks) compared with SMDs [19]. Given the long elimination half-life, loading doses may be beneficial to achieve steady-state concentrations more rapidly. However, the utility of loading dosed also depends on the PK/PD relationship and toxicological profile of a particular molecule.

For some mAbs (e.g., cetuximab), the clinical efficacious doses are still in the nonlinear PK range, suggesting the target-mediated clearance pathway plays a significant role in the drug disposition [69]. The exposure of the mAb could be largely influenced by the tumor burden and disease status. Thus, the selection of doses and dosing frequency should be carefully evaluated, and dose modification may be considered in different patient populations to maximize the therapeutic benefit. In some particular cases, mAbs (e.g., rituximab) also exhibit time-dependent target-mediated decreases in clearance, as a result of a change in antigen expression/tumor burden following initial doses and therapeutic response [70]. The mAb dose should then be adjusted at later treatment cycles without compromising patient exposure and response (see Sect. 4.3, below).

Historically, the development of oncology mAbs used weight- or body surface area (BSA)-based dosing, with the perception that this technique leads to a reduction in interindividual variability of exposure of mAbs. Recently, a comparison of the body-size-based and fixed dosing was conducted through modeling and simulation and case studies [71, 72]. The results showed the two dosing approaches performed similarly in terms of PK/exposure/PD variability. It is suggested that the dosing paradigm for mAbs should be assessed in the context of unique PK/PD behavior of mAbs. Given the high specificity of mAb target, off-target toxicity is often limited. In general, antibodies tend to have larger therapeutic window, when compared to SMDs. The PK variability has been shown to be smaller when compared with SMDs. These PK/PD properties of mAbs potentially allow flexibility in dosing strategy and convenient integration with other therapeutically active agents. For the same reasons, fixed dosing (i.e., dose independent of body size) should be evaluated during the clinical development, as this may minimize the risk for dosing errors and offers convenience and benefits in terms of cost of manufacturing.

4 Clinical Applications of Monoclonal Antibodies

4.1 Anti-angiogenesis

Major progress in the last few decades have led to a greater understanding of tumor biology, clearly demonstrating the fact that angiogenesis can be a viable target in the treatment of cancer. It has been shown that tumor requires a vascular blood supply from neighboring host to grow beyond 1–2 mm^3 [73]. Tumors that do not establish a neovascular supply may remain dormant for many years, whereas increased tumor growth and metastatic potential are believed to be stimulated by increased expression of proangiogenic factors (e.g., vascular endothelial growth factor (VEGF), fibroblast growth factor

(FGF), platelet-derived growth factor (PDGF), and transforming growth factor-β) and by a decrease in anti-angiogenic factors (e.g., IFN-α or thrombospondin-1) [73, 74].

VEGF and its receptors have emerged as the most potent and specific positive regulators of angiogenesis, and many studies have shown that VEGF is overexpressed in most human tumors [74–77]. They play a pivotal role in normal and pathological angiogenesis, including endothelial cell mitogenic activity, vascular permeability-enhancing activity, and angiogenic properties, allowing the tumor to expand rapidly, invade surrounding tissues, and metastasize. Therapeutic strategies developed to target VEGF include mAbs directed against VEGF, or its receptors VEGFR-1 and VEGFR-2, and small molecule tyrosine kinase inhibitors that act intracellularly to prevent autophosphorylation and activation of downstream growth-promoting signaling cascades [76, 77].

4.1.1 Bevacizumab

Bevacizumab was the first humanized IgG mAb that binds to all isoforms of VEGF and inhibits angiogenesis and tumor growth [78] to enter clinical trials and was US FDA approved in 2004. It is currently approved in metastatic colorectal cancer, non-squamous non-small cell lung cancer (NSCLC), glioblastoma, and metastatic renal cell carcinoma. Several studies are ongoing in other tumor types, including pancreatic cancer, ovarian cancer, hepatic cancer, prostate cancer, and soft-tissue sarcomas (STS).

Bevacizumab is produced through recombinant biotechnology from a Chinese hamster ovary cell line and has a molecular weight of ~149 kDa. It is a humanized mAb that has 93 % human and 7 % murine protein sequence. It maintains the high specificity and affinity of the parental antibody (murine antihuman VEGF mAb A4.6.1) for VEGF, with reduced immunogenicity and longer biological half-life [73, 79].

Preclinical Pharmacokinetics and Pharmacodynamics

The efficacy of bevacizumab against various cancer types has been demonstrated in in vitro and in several in vivo preclinical studies [73]. Both bevacizumab and A4.6.1 block tumor angiogenesis and growth with almost identical potency and efficacy in human cells and tissues. Bevacizumab neutralizes all isoforms of human VEGF with a dissociation constant (K_d) of 1.1 nM. It has lower affinity to rabbit VEGF [73, 80] and does not bind to rodent VEGF [37]. It inhibits VEGF-induced proliferation of endothelial cells with ED_{50} of 50 ± 5 ng/mL. Numerous tumor xenograft studies in nude mice with bevacizumab and/or A4.6.1 showed that anti-VEGF treatment results in 25–95 % tumor growth inhibition compared with control mice across different tumor types. In most of these studies, a dose-dependent tumor growth inhibition was observed.

Bevacizumab shows linear PK, and the elimination half-life of bevacizumab is about 6–12 days in mice, rats, and monkeys following intravenous administration of 10 mg/kg [81]. Based on the concentration–response relationship demonstrated in the human tumor xenograft mice model, serum concentration of bevacizumab of >10–30 μg/mL was predicted to be necessary for satisfactory tumor suppression [82]. It was assumed that the same serum concentration of bevacizumab in humans would be associated with clinical efficacy, because the murine and human forms of the antibody have similar efficacy (IC_{50}) and binding affinity (K_d) [82].

Clinical Pharmacokinetics and Pharmacodynamics

The PK of bevacizumab has been characterized in clinical studies, with doses of 1–20 mg/kg administered at a frequency ranging from weekly to every 3 weeks [83, 84]. Bevacizumab demonstrates

linear PK over a dosage range of 3–20 mg/kg. This is consistent with other mAbs that bind soluble (serum) antigen, where specific clearance is often saturated at lower concentrations. Thus, nonlinear, target-mediated clearance does not play a significant role in the overall disposition of bevacizumab at the clinical dose range.

A population PK analysis was conducted with combined data from 491 patients across 8 trials, and the concentration–time profiles were well described using a two-compartment linear model with first-order elimination from the central compartment [83]. Consistent with other IgG1 antibodies, the PK of bevacizumab were characterized by slow clearance of 0.207 L/day and long terminal half-life of 20 days (range: 11–50 days), with steady-state concentrations achieved in approximately 100 days. The exact route of bevacizumab metabolism and elimination has not been described, but it is likely to be cleared via the typical IgG pathways described earlier in this chapter.

Of all the baseline demographic and pathophysiological covariates tested, body weight and sex accounted for the highest interindividual variability in the bevacizumab PK [83]. Bevacizumab clearance and volume of the central compartment (V_c) increased with increasing body weight. After adjusting for body weight, bevacizumab clearance was 26 % faster and V_c was 22 % larger in men than in women. In patients with low serum albumin (<2.9 g/dL) and high alkaline phosphatase (>484 U/L), both markers of disease severity, bevacizumab clearance was approximately 20 % faster than patients with median laboratory values. In addition, clearance was similar across all studies and tumor types [83]. Despite small PK differences seen with these covariates, response rates and toxic effects do not differ appreciably, and no dosage modifications are necessary.

In phase I studies with bevacizumab, it was shown that bevacizumab doses ≥ 0.3 mg/kg produced complete suppression of free serum VEGF and doses > 1 mg/kg produced serum levels of bevacizumab in the target range of ≥10 μg/mL for at least 14 days [85]. There is limited PD data published with bevacizumab. A consistent exposure–response relationship was not observed, which may be because the clinical studies in approved indications were conducted within a limited dose range. In metastatic NSCLC and metastatic renal cell carcinoma, a traditional dose–response relationship was observed; higher doses of bevacizumab appeared to be more efficacious than lower doses of the drug. Bevacizumab at 15 mg/kg appeared to be more effective than bevacizumab at 7.5 mg/kg when added to carboplatin/paclitaxel chemotherapy in metastatic NSCLC [86], and 10 mg/kg bevacizumab was more effective as a single agent than 3 mg/kg bevacizumab in metastatic renal cell carcinoma [86, 87]. In contrast, the dose–response relationship for bevacizumab was unclear in the phase II breast cancer and colorectal cancer clinical trials [86, 88, 89]. Somewhat paradoxically, treatment with 5 mg/kg bevacizumab appeared to be more effective than the 10 mg/kg dose in patients with colorectal cancer. This may have been due to the small sample size or to imbalances in patient characteristics between arms. Therefore, it is likely that optimal dosing regimen of bevacizumab may depend on the tumor type and chemotherapy combination. Bevacizumab is generally well tolerated at approved doses.

Dose and Administration

Dosage of bevacizumab varies according to indication [90]. In colorectal cancer, the manufacturer-recommended dose is 5 or 10 mg/kg via IV infusion every 2 weeks, in combination with fluorouracil-based chemotherapy [84, 90]. In non-small cell lung cancer, the approved dose is 10 mg/kg every 2 weeks or 15 mg/kg every 3 weeks with paclitaxel or carboplatin/paclitaxel, respectively. The approved dose for glioblastoma and renal cell carcinoma is 10 mg/kg every 2 weeks.

The first infusion should be given over a minimum of 90 min, and patients should be monitored for infusion-related reactions, such as fever and chills. If administration over 90 min is tolerated, the subsequent infusions can be administered over 30–60 min.

Drug–Drug Interaction

Due to reasons described in Sect. 3.3, bevacizumab presents a low theoretical DDI potential in combination with SMDs or mAbs. DDI assessments have been conducted, and as expected, no PK interactions have been demonstrated between bevacizumab and any of the following anticancer agents [73, 83, 90]: irinotecan, carboplatin, paclitaxel, interferon alfa-2a capecitabine, cisplatin, 5-fluorouracil, oxaliplatin, and trastuzumab. However, three of the eight patients receiving bevacizumab plus paclitaxel/carboplatin had substantially lower paclitaxel exposure after four cycles of treatment (at day 63) than those at day 0, compared with patients receiving paclitaxel/carboplatin alone. This analysis was limited by few patients to draw firm conclusions regarding a DDI. Overall, there is a low potential for bevacizumab to alter the PK of other drugs. Since bevacizumab exhibits a moderate to large therapeutic index, the PD impact of any concomitant medications that would alter bevacizumab drug exposure is diluted.

Immunogenicity

As with all therapeutic proteins, there is a potential for an immune response to bevacizumab. Patients in two adjuvant phase III colon carcinoma studies were tested for antibodies to bevacizumab by immunoassay. The overall incidence of antibodies to bevacizumab from the two studies was <1 % (0.79 and 0.54 %) with three antibody-positive patients developing neutralizing antibodies.

While the clinical significance of an immune response to bevacizumab is unknown, none of the adverse events observed in patients who developed antibodies to bevacizumab were considered to be associated with type I hypersensitivity or type III immune complex-mediated reactions.

Special Populations

Bevacizumab has been evaluated in the pediatric population in a phase I trial which included 21 patients. Drug disposition in pediatric patients was similar to that observed in adults. Acceptable safety profile was reported when bevacizumab was administered at doses of 5, 10, or 15 mg/kg every 2 weeks [91]. Additional studies of bevacizumab in combination with chemotherapy in children are ongoing. In the population PK analysis described previously, serum creatinine was found to not have a significant effect on bevacizumab PK. No formal studies have been conducted in patients with impaired hepatic and renal function.

4.1.2 Other Anti-angiogenesis Agents in Development

Several pathways have been identified that play critical roles in the formation of new tumor-associated blood vessels. Generally these pathways involve VEGF family, PDGF family, TGF-β family, FGF superfamily, angiopoietin (ANG) and Tie signaling, and Notch and Wnt signaling [92]. Tremendous efforts have been devoted to target these pathways to inhibit blood vessel growth in tumors. Two frontrunners, are aflibercept and ramucimab. Aflibercept is a Fc-fusion protein specifically designed to bind all forms of VEGF; ramucirumab is a fully human IgG1 mAb against VEGFR2 [93, 94].

Many new angiogenesis targets are discovered in the past few years, which include integrin α5β1, integrin avb3, angiopoietin, activin receptor-like kinase 1 (ALK1), delta-like ligand-4 (DLL4), VEGFC, VEGFR3, Bv8, placental growth factor (PlGF), Ang 1, Ang2, endoglin (CD105), bFGF, neuropilin 1 (NRP1), Tie2, PDGF-BB, PDGFR-beta, and epidermal growth factor-like domain 7 (EGFL7) [92, 95, 96]. The antibodies against these targets are being tested in early phase of clinical

development. The development of new anti-angiogenesis therapies as a single agent or in combination with chemotherapy continues growing at a fast pace, and the accumulation of the knowledge in anti-angiogenesis area might open a new door for future cancer therapy.

4.2 EGFR Pathway

The EGFR family of receptors consists of four closely related members: EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4 [97, 98]. These receptors are involved in regulating cell growth, survival, and differentiation through interlinked signal transduction involving activation of the phosphatidylinositol 3 kinase (PI3K)/Akt and the Ras/Raf/MEK/MAPK pathways [99].

All of the EGFR family receptors have an extracellular domain (ECD) responsible for ligand binding, a helical transmembrane segment and an intracellular protein tyrosine kinase (TK) domain. The binding of ligands induces dimerization of two identical (homodimer) or different (heterodimer) receptors. The dimerization partner has an important impact on the type and number of downstream effectors activated and also on the downregulation mechanism of the ligand-bound receptors. Signaling through HER2 and HER3 requires heterodimerization since HER2 has no known ligand and HER3 lacks TK activity. Importantly, HER2 is the preferred dimerization and signaling partner for all other HER receptors [100].

EGFR (ErbB-1; HER1) is constitutively expressed in many normal epithelial cells and plays an important role in tumor biology. It promotes proliferation, metastasization, angiogenesis, and inhibition of apoptosis [101, 102]. The most common EGFR alteration in tumor cells is its overexpression that may lead to ligand-independent receptor dimerization. EGFR is frequently overexpressed in human tumors including breast cancer, lung cancer, glioblastoma, bladder carcinoma, head and neck cancer, ovarian carcinoma, colorectal cancer, and prostate cancer [103]. Overexpression correlates with disease progression, poor outcome and low response to therapy, and resistance to cytotoxic treatments [104, 105].

4.2.1 Cetuximab

Cetuximab (Erbitux®) is a recombinant, human/mouse chimeric MAb that binds specifically to the extracellular domain of the human EGFR on both normal and tumor cells [106–108] and competitively inhibits the binding of EGF as well as other ligands, such as TGF-α. Cetuximab is produced in mammalian (murine myeloma) cell culture and is composed of the Fv regions of a murine anti-EGFR antibody with human IgG1 heavy and kappa light chain constant regions and has an approximate molecular weight of 152 kDa [109]. Cetuximab binds to EGFR with higher affinity (K_d = 0.1–0.2 nM) compared to EGFR natural ligands, EGF or TGF-alpha, and is able to block ligand-inducing activation of EGFR [110]. The selective action of cetuximab accounts for less toxicity compared with other EGFR inhibitors, which act on the intracellular domain of EGFR and on a vast number of tyrosine kinases.

Cetuximab is approved by the FDA for use in the treatment of head and neck squamous cell carcinoma (HNSCC) and EGFR-expressing metastatic colorectal cancer (mCRC), as monotherapy or in combination with chemotherapy. Use of cetuximab is not recommended for the treatment of colorectal cancer with *KRAS* mutations, as this biomarker has been associated with a lack of therapeutic benefit.

Preclinical Pharmacokinetics and Pharmacodynamics

Cetuximab PK has been studied in mice and monkeys. Since cetuximab does not bind to mouse EGFR, it shows linear PK with the elimination half-life approximately 37.8–42.2 h [111]. In rhesus

monkeys, cetuximab clearance decreased and terminal half-life increased with increasing dose, indicating saturation of target-mediated clearance at higher doses, a likely effect of cetuximab binding to monkey EGFR [112].

A dose response was observed in an in vivo efficacy study in nude mice bearing Geo human colon tumor xenografts [113]. At 0.04 mg, the maximum inhibition of tumoral phospho-EGFR was 53 % at 24 h and reduced to 37 % by 72 h, whereas at 0.25 mg, tumoral phospho-EGFR was maximally inhibited by 91 % at 24 h and reduced to 72 % by 72 h. In general, the time course of tumoral phospho-EGFR inhibition and recovery seemed to correlate with plasma and tumoral levels of cetuximab at these doses. It was determined that dose levels of 0.25 mg or higher was optimal for the antitumor activity in mice. The PK and PD data from mice were modeled using an inhibitory Emax model with EC_{90} of 67.5 μg/mL, which can be considered as the active plasma concentration of cetuximab that results in near complete inhibition of phospho-EGFR. The average steady-state plasma concentration ($C_{ss,avg}$) in mice at 0.25 mg was estimated to be 73.1 μg/mL [111]. The C_{ss} of cetuximab in cancer patients is in the range of 56–100 μg/mL when administered the clinical dosing regimen and is comparable to the EC_{90} in the mice model.

Clinical Pharmacokinetics and Pharmacodynamics

The PK of cetuximab was initially evaluated in three consecutive phase I clinical trials in which cetuximab was administered with three different dosing regimens including (1) a single i.v. infusion, (2) weekly infusion for 4 weeks, and (3) weekly infusion in combination with cisplatin [114]. All these studies were open-label, dose-escalation trials with doses of 5, 20, 50, and 100 mg/m^2. Cetuximab was additionally escalated to 200 and 400 mg/m^2 in the combination study with cisplatin. The maximum tolerated dose (MTD) was not reached in any of these studies. Noncompartmental PK analysis for dose levels of 20, 50, and 100 mg/m^2 showed that the systemic clearance decreased with increasing dose. In another phase I study, patients received a single dose of cetuximab at 50, 100, 250, 400, or 500 mg/m^2 for evaluation of PK and PD (EGFR expression in skin and tumor biopsies) followed by weekly 250 mg/m^2 cetuximab dosing from day 22 [115]. Clearance decreased and half-life increased with increasing dose. At doses of 50, 100, 250, and 400 mg/m^2, mean clearance values were 1.16, 0.811, 0.433, and 0.374 mL/h/kg, respectively. The mean half-life values were 26.3, 67.6, and 97.5 h at 50, 250, and 400 mg/m^2, respectively. The volume of distribution at steady state remained relatively constant and approximately equivalent to the plasma volume. These data suggest that cetuximab exhibits a nonlinear PK, with faster clearance at doses $\leq$ 100 mg/m^2 and potential saturation of EGFR binding at doses of 250 mg/m^2 as evidenced by clearance becoming linear at that dose [115].

The population PK of cetuximab has been described in two studies. In the first, cetuximab in patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck enrolled in two phase I/II studies using nonlinear mixed-effects modeling. In this analysis, a total of 912 concentrations from 143 patients were used to find cetuximab PK was best described by a two-compartment model with Michaelis–Menten-type saturable elimination. Population estimates (between-subject variability, percent coefficient of variation) of the pharmacokinetic parameters were V_{max} 4.38 mg/h (15.4 %), K_m 74 μg/mL, central compartment volume (V_1) 2.83 L (18.6 %), peripheral compartment volume 2.43 L (56.4 %), and inter-compartment clearance 0.103 L/h (97.2 %). Ideal body weight and white blood cell count were identified as predictors of V_{max} and total body weight as a predictor of V_1. Clinical dose adjustments beyond the approved body surface area-based dosing of cetuximab may be warranted in patients with extreme deviations of their actual body weight from ideal body weight. Agreement between simulated and measured concentrations monitored for up to 43 weeks of therapy indicates that cetuximab PK parameters remained constant during prolonged therapy [69].

In the second population PK analysis performed in a phase II study in metastatic colorectal cancer patients treated with cetuximab in combination with irinotecan and 5-fluorouracil, 96 patients received cetuximab as an infusion loading dose of 400 mg/m^2 followed by weekly infusions of 250 mg/m^2. Compartmental PK parameters were estimated by a population approach and were best described using a two-compartment model with both first-order and saturable (zero-order) elimination. Estimated PK parameters (% standard error) were as follows: central volume of distribution V_1 2.96 L (4 %), peripheral volume of distribution V_2 4.65 L (6 %), elimination clearance CL 0.497 L/day (4 %), distribution clearance Q 0.836 L/day (8 %), and zero-order elimination rate k_0 8.71 mg/day (10 %). Patient covariates including body weight and serum albumin were found to influence volume of distribution and clearance, respectively [116].

A clear exposure–response relationship has been observed in two phase I and II studies [115, 116]. The patients with partial response or stable disease revealed a higher grade rash (even at first appearance) and higher cetuximab trough concentrations (~60 μg/mL) than those with progressive disease (~33 μg/mL). Similarly, the progression-free survival (PFS) was significantly influenced by drug exposure (i.e., dose-normalized AUC). Time to progression of patients with dose-normalized AUC above the median value was 8.48 months, as compared with 3.25 months for other patients. The *FCGR3A*-V158F polymorphism and tumor *KRAS* status has no influence on PFS. However, the influence of dose-normalized AUC on median time to progression was significant in the wild-type *KRAS* group, not in the mutated group.

It was also observed that EGFR protein expression in skin biopsies decreased in a dose- and time-dependent manner. A significant decrease in EGFR staining intensity in skin was observed at doses of 250 and 400 mg/m^2. The study supports minimal efficacious dose of 250 mg/m^2.

On the basis of likely saturation of the EGFR and target-mediated clearance at doses $\geq$ 250 mg/m^2 and exposure–response relationship, the recommended dosing regimen for subsequent phase II/III trials was a 400 mg/m^2 loading dose, followed by 250 mg/m^2 weekly maintenance dose [115]. The weekly dose regimen is supported by the PK data (a half-life of about 4 days, no relevant accumulation) and PD analyses.

Dosage and Administration

As a monotherapy or in combination with irinotecan, the recommended initial dose of cetuximab is 400 mg/m^2 as 120-min intravenous infusion. The recommended maintenance dose is 250 mg/m^2 over 60-min infusion weekly until disease progression or unacceptable toxicity.

Drug–Drug Interaction

A drug interaction study was performed in which cetuximab was administered in combination with irinotecan. There was no evidence of any PK interactions between cetuximab and irinotecan. Population PK analysis did not reveal any meaningful effect of coadministration of chemotherapeutic agents (irinotecan, gemcitabine, fluorouracil/folinic acid, and oxaliplatin) and radiotherapy on cetuximab PK [69, 114, 117, 118]. No formal drug–drug interaction studies have been conducted.

Immunogenicity

Several clinical studies of cetuximab have obtained sampling for human anti-cetuximab antibodies. Pooled data indicate that very few patients (4 %) have a positive antibody response to cetuximab [119].

Special Populations

Formal PK studies of cetuximab have not been conducted in patients with renal or hepatic impairment patients. Based on the population analysis, cetuximab PK are not influenced by race, age, gender, renal, or hepatic status based on integrated analysis [69].

Cetuximab PK has been studied in combination with irinotecan for treatment of pediatric and adolescent patients with refractory solid tumors [120]. This phase I study enrolled patients with ages from 1 to 18 years with advanced refractory solid tumors, who received escalating weekly doses of cetuximab (75, 150, 250 mg/m^2) plus irinotecan (16 or 20 mg/m^2/day) for 5 days for 2 consecutive weeks every 21 days. Cetuximab 250 mg/m^2 weekly plus irinotecan 16 mg/m^2/day (pediatric) or 20 mg/m^2/day (adolescent) was established as the maximum tolerated dose/recommended phase II dose. Cetuximab demonstrated dose-dependent clearance in both children and adolescents, similar to that observed in adults. Clearance decreased from 0.057 to 0.015 L/h m^2 as cetuximab dose increased from 75 to 250 mg/m^2. Clearance was similar to that of the adolescent group and, when adjusted for body size differences, similar to previous values in adults. This study showed that the cetuximab/irinotecan combination could be given safely to children and adolescents with cancer, particularly in CNS tumors.

4.2.2 Panitumumab

Panitumumab is a fully human anti-EGFR IgG2 mAb and is expected to be much less immunogenic than the chimeric cetuximab. Panitumumab binds to the extracellular domain of the EGFR with high affinity (K_d 5×10^{-11} M) blocking binding of EGF and TGF-α to the receptor and leading to internalization of the receptor–antibody complex [121]. This prevents ligand-induced EGFR-tyrosine autophosphorylation and subsequent activation of key downstream signaling molecules involved in tumorigenesis. The potency of panitumumab appears to be greater than cetuximab in preclinical in vitro cell lines and in vivo in animal models [121]. Panitumumab is efficacious as a monotherapy in chemotherapy-refractory patients and in different combinations against metastatic colorectal cancer. The clinical response is restricted to tumors with wild-type *KRAS*; therefore, the *KRAS* status should be checked before treatment.

Panitumumab is indicated as monotherapy for the treatment of patients with EGFR-expressing metastatic colorectal carcinoma with wild-type *KRAS* after failure of fluoropyrimidine-, oxaliplatin-, and irinotecan-containing chemotherapy regimens.

Preclinical Pharmacokinetics and Pharmacodynamics

Pharmacological studies of panitumumab in mice and nonhuman primates have been used to simulate the PK profiles in humans and predict effective dosing regimens. Clearance of panitumumab in mice, in which the mAb is not cross-reactive, was slow and linear. In contrast, in cynomolgus monkeys, in which there is expected to be a degree of receptor cross-reactivity, clearance decreased from 20 mL/kg/day at a dose of 0.6 mg/kg down to approximately 8 mL/kg/day at a dose of 6 mg/kg and only decreased to approximately 5 mL/kg/day when the dose was further increased by a magnitude of 10. On the basis of the rapid clearance seen in the monkeys at non-saturating doses, human clearance was similarly anticipated to be nonlinear owing to the role of the EGFR itself as a mode of mAb clearance [122].

Clinical Pharmacokinetics and Pharmacodynamics

Panitumumab demonstrated nonlinear PK similar to that seen with cetuximab, which is attributable to EGFR-mediated clearance. Panitumumab exhibits predictable PK, with low intra- and interindividual

variability [123]. Doses ranging from 1 to 2.5 mg/kg/week showed serum concentrations increased nonproportionally with increasing dosage, presumably reflecting progressive saturation of EGFR. Saturation of this pathway appeared to be reached with dosages > 2 mg/kg/week [124]. A 2.5 mg/kg once-weekly dosage evaluated in early clinical studies was associated with a 100 % incidence of skin rash. Exploration of less-frequent dosing regimens revealed that trough serum levels were similar for the following three dosing regimens: 2.5 mg/kg every week, 6 mg/kg every 2 weeks, and 9 mg/kg every 3 weeks [123]. Considering patient convenience, the 6 mg/kg regimen, administered as a 60-min intravenous infusion once every 2 weeks, was chosen for further development. This regimen achieves steady-state serum concentrations after the third infusion, with peak and trough concentrations of 213 ± 59 and 39 ± 14 μg/mL, respectively. The clearance and serum elimination half-life of panitumumab were 4.9 ± 1.4 mL/kg/day and ~7.5 days (range 3.6–10.9 days), respectively [125].

A nonlinear mixed-effects modeling approach has been used to develop a comprehensive population PK model of panitumumab. This analysis included 1,200 patients compiled across 14 clinical studies in advanced solid tumors. The PK of panitumumab was best described with a two-compartment model with parallel linear and nonlinear (Michaelis–Menten) elimination pathways. For a typical male patient with colorectal cancer (80 kg, 60 years old), the estimates for the linear clearance (CL), the maximum nonlinear clearance (V_{max}/K_m), the central volume of distribution (V_1), the peripheral volume of distribution (V_2), and the Michaelis–Menten constant (K_m) are 0.273 L/day, 28.4 L/day, 3.95 L, 2.59 L, and 0.426 μg/mL, respectively. Body weight was found to be the most influential covariate on PK parameters, affecting CL, V_{max}, and V_1. The intensity of baseline tumor EGFR expression did not alter the PK of panitumumab. Overall, 18 of 530 patients developed anti-panitumumab antibodies (3.4 % rate), and this did not appear to affect panitumumab exposure substantially, as evidenced by an 8 % lower AUC in the antibody-positive patient group.

Dose and Administration

The recommended initial dose of panitumumab is 6 mg/kg administered over 60 min as an intravenous infusion every 14 days. If safety or tolerability concerns arise, dosage may be reduced by 50 %. Doses higher than 1,000 mg should be infused over 90 min.

Drug–Drug Interaction

A formal drug–drug interaction study was conducted, and the preliminary data indicated that administration of panitumumab had no impact on the PK of irinotecan [126]. Population PK analysis with data from two phase II studies indicated that paclitaxel/carboplatin- and irinotecan-containing regimens had no meaningful effect on panitumumab PK [127].

Immunogenicity

Eighteen out of the 530 patients (3.4 %) tested positive for anti-panitumumab antibodies. No difference in panitumumab exposures was seen when comparing exposures in patients who developed anti-panitumumab antibodies and in patients who did not develop these antibodies, which may be attributable to the small sample size in the anti-panitumumab-positive group [128].

Special Populations

Formal PK studies have not been conducted in patients with renal or hepatic impairment patients. Based on the population analysis, panitumumab PK are not influenced by age, gender, ethnicity,

mild-to-moderate renal or hepatic impairment, or EGFR membrane-staining intensity in tumor cells [128]. In the population PK analysis described above, when Japanese patients were compared to other racial groups, there were no significant differences in model-predicted steady-state panitumumab AUC, C_{max}, or C_{min} after accounting for the effect of body weight group [128].

4.2.3 Trastuzumab

Trastuzumab was identified initially by screening mAbs that targeted the HER2 extracellular domain (ECD) [129, 130]. The establishment of the unique role of HER2 in malignant transformation in preclinical models, coupled with the biological significance of HER2 overexpression and the preclinical demonstration of the antitumor activity of mAbs directed against HER2, encouraged the development of trastuzumab. HER2 is overexpressed in 20–25 % of breast tumors and is associated with a poor prognosis.

Trastuzumab is a humanized IgG1 antibody, composed of an antigen-binding component (from the murine mAb 4D5) combined with human IgG, and has an in vitro binding affinity for the HER2 ECD threefold that of 4D5, the precursor to trastuzumab (K_d=0.1 nM) [131]. Trastuzumab has been approved for treatment of HER2-overexpressing breast cancer and metastatic gastric cancer.

Preclinical Pharmacokinetics and Pharmacodynamics

As a humanized mAb, trastuzumab binds the target in monkeys but not in mice, and a significant species difference in PK was observed. The terminal half-life in mice (11–39 days) was considerably longer than that in rhesus monkeys (6 days at 0.5 mg/kg dose) due to the target-mediated clearance in monkeys. Nonlinear kinetics was observed at doses lower than 2 mg/kg in monkeys, while dose proportional kinetics was observed above this dose, suggesting the saturation of the target [132].

In mouse xenograft models, a significant inverse relationship was found between trastuzumab and tumor burden. A target concentration of 10–20 μg/mL was identified for clinical development [133]. This antibody concentration is associated with maximal antiproliferative effect in vitro [134].

Clinical Pharmacokinetics and Pharmacodynamics

Trastuzumab was evaluated as a single agent or in combination with chemotherapy in phase II and phase III studies with a loading dose of 4 mg/kg followed by 2 mg/kg/week. In phase I studies, trastuzumab showed nonlinear PK in metastatic breast cancer patients. Based on a one-compartment model, the half-life estimate averaged 5.8 days (range 1–32 days). The dosage regimen was designed to achieve and maintain serum trough concentrations greater than 20 μg/mL. With repeated administration, the trough levels tended to increase through weeks 16–32 and then reached steady state with a mean trough concentrations of 79 μg/mL and peak concentrations of 123 μg/mL. PK analyses of data from trials using weekly trastuzumab dosing indicated the half-life of trastuzumab is approximately 28.5 days and supported the use of a longer dosing schedule. Subsequent population PK analysis confirmed the findings concerning the approximately 28.5 day half-life [135].

Subsequently, a study using an alternative schedule of trastuzumab monotherapy (8 mg/kg loading dose followed by maintenance doses of 6 mg/kg every 3 weeks) showed that larger doses of trastuzumab do not result in undue toxicity and trough concentrations were attained, similar to that observed in the pivotal trials using weekly dosing [136]. Another clinical study demonstrated that administration of trastuzumab with the same dosing regimen in combination of paclitaxel produced favorable

response rates, with no new or unexpected toxicity, and produced serum trastuzumab trough levels similar to those achieved with the standard weekly trastuzumab regimen [137]. A more intensive loading schedule was designed using pharmacokinetic simulation to obtain the established steady-state concentration earlier in treatment for improving response to therapy. The regimen comprised of a loading dose of 6 mg/kg given weekly for 3 weeks followed by a maintenance dose of 6 mg/kg every 3 weeks until disease progression. Higher steady-state serum concentration of trastuzumab was achieved without compromising safety and efficacy [138].

Nonclinical studies have suggested that the complex formed between trastuzumab and the shed extracellular domain (ECD) of HER2 has a greater clearance than free trastuzumab. Baseline covariates (number of metastatic sites, level of shed ECD of the HER2 receptor, and patient weight) were found to be significant variables influencing either clearance or central volume of distribution, or both ($P < 0.005$). However, these covariate effects on trastuzumab exposure were viewed to be modest and not clinically important in comparison with the large inter-patient variability of clearance (43 %) [135]. Baseline shed antigen (the circulating ECD of HER2) could be detected in approximately 64 % patients with median levels of 11 ng/mL.

In a subset of patients in a clinical trial of trastuzumab administered as a single agent on a weekly schedule, mean trough levels at weeks 7 and 8 were found to be higher in complete responders (70.3 μg/mL) and partial responders (58.4 μg/mL) than nonresponders (44.3 μg/mL) [139]. The data suggest the relationship between trastuzumab exposure and the clinical response to single-agent trastuzumab in metastatic breast cancer patients.

In gastric cancer patients it has been observed that mean serum trastuzumab trough concentrations at steady state are 24–63 % lower in the gastric cancer patients, as compared to the concentrations observed in patients with breast cancer, although the mechanism for these observations is unclear [140]. A concentration versus survival analysis showed that patients in the lowest quartile of exposure (trough concentration at day 21) had substantially shorter overall survival than those with higher exposure [141]. As a result of these observations, alternative trastuzumab doses are being evaluated in the post-marketing clinical trials in gastric cancer patients.

Dose and Administration

Trastuzumab is administered as a loading dose of 4 mg/kg, followed by a maintenance dose of 2 mg/kg on a weekly schedule, or as an 8 mg/kg loading dose followed by a maintenance dose of 6 mg/kg on an every 3-week schedule. In metastatic or early breast cancer, trastuzumab should only be administered to patients whose tumors have HER2 overexpression in tumor samples. The drug should be infused intravenously over 90 min for the initial dose and 30–90 min for subsequent doses.

Drug–Drug Interaction

PK data from the phase II/III studies of trastuzumab showed that concurrent administration of the anthracyclines doxorubicin or epirubicin plus cyclophosphamide (AC), or cisplatin did not alter clearance, half-life, or exposure of trastuzumab compared to the administration of trastuzumab as a single agent. However, patients receiving paclitaxel had an average about 30 % higher exposure to trastuzumab than those receiving trastuzumab in combination with doxorubicin and cyclophosphamide [132]. It is unlikely that this difference would have clinical consequences, and therefore no dose adjustment was deemed necessary. In a phase II study evaluating the PK and safety of trastuzumab and paclitaxel given every 3 weeks to patients with HER2-overexpressing metastatic breast cancer, no apparent drug interactions were observed between trastuzumab and paclitaxel [137].

Immunogenicity

Trastuzumab has low potential for immunogenicity and resultant production of anti-trastuzumab antibodies as it is a humanized mAb. Of the 903 patients who were evaluated, human antihuman antibody (HAHA) to trastuzumab was detected in one patient, who had no allergic manifestations [132].

Special Populations

Formal PK studies of trastuzumab have not been conducted in patients with renal or hepatic impairment patients. The population PK analysis showed that trastuzumab disposition was not influenced by age or renal impairment [135].

4.2.4 Other Anti-EGFR Agents in Development

Targeting HER2 with mAbs is a current treatment approach for breast cancer; however, the critical role of other HER receptors in HER-mediated pathways of tumorigenesis is increasingly being recognized. In particular, HER3 seems to be the preferred dimerization partner when signaling occurs through the PI3K pathway and, as such, is emerging as a key target for inhibition of HER signaling. In a recent computational model of the HER signaling network, HER3 has been identified as a key node. This model predicts that HER3 antagonist would inhibit combinatorial, ligand-induced activation of the HER3-PI3K network more potently than do current marketed therapeutics [142]. Combinatorial therapies including HER3 targeting may ameliorate tumor responses by limiting escape mechanisms and resistance [143–145]. Currently therapies (e.g., U3-1287, MM-121) targeting HER3 are being designed and in early development.

Pertuzumab is a humanized IgG1 mAb that binds to the subdomain II dimerization arm and inhibits ligand-induced HER2/HER3 heterodimerization [146]. It is the first in a new class of targeted anticancer agents and has a unique mechanism of action compared with other HER-targeted therapies. In contrast to trastuzumab, pertuzumab binds to the extracellular dimerization (receptor pairing) domain of HER2. By blocking heterodimerization of HER2 and HER3, pertuzumab inhibits the key HER signaling pathways that mediate cancer cell proliferation and survival. Exploratory data from two phase II clinical trials of pertuzumab in ovarian cancer suggest that HER3 mRNA levels may predict clinical benefit from pertuzumab [99].

Trastuzumab-DM1 (TDM1), an antibody–drug conjugate (ADC), delivers a thioether-linked antimitotic maytansine derivative to HER2+ breast tumors. The potential advantage of the ADC is that the antibody (trastuzumab) delivers the cytotoxic agent DM1 (a derivative of the antimicrotubule chemotherapy maytansine) specifically to tumor cells that overexpress HER2 antigens, where the cytotoxic will be released intracellularly. This design is intended to reduce the toxicity of DM1. In addition, the antibody trastuzumab has its own anticancer activity. T-DM1 has demonstrated robust and impressive activity in phase II trials in patients with advanced HER2-positive breast cancer [147–149].

4.3 B-Cell-Directed Therapy

B cells are a fundamental component of the body's immune system. However, like most cells in the body, B cells can become cancerous—leading to diseases such as NHL and CLL. B-cell-directed therapy is targeting surface antigens on those cancerous B cells with mAbs to induce B-cell lysis through apoptosis, ADCC, or CDC. There are several B-cell surface antigens, and among them, CD20

is considered an ideal target for mAb because it is highly expressed on malignancies of B-cell precursors and mature B cells, but not in hematological stem cells, pro-B cells, normal plasma cells, or other normal tissues. And it typically is not internalized, downregulated, or shed. Consequently, treatment with anti-CD20 antibodies will lead to destruction of both malignant and normal mature B cells but allows for the recovery of B cells from normal naive pro-B cells [150, 151].

Currently two full-length mAbs (rituximab and ofatumumab) and one radioimmunoconjugate (ibritumomab tiuxetan) have been approved in the therapeutic areas of NHL, CLL, and multiple myeloma. CD52 is another surface antigen being targeted for the treatment of CLL through ADCC. The successful application of mAbs in the treatment of B-cell malignance stimulates the continue interests to search for other surface antigens as druggable targets.

4.3.1 Rituximab

Rituximab was the first mAb approved by the FDA for the treatment of relapsed or refractory, low-grade or follicular, CD20 antigen-positive B-cell NHL, either as a single agent or in combination with other therapies [152, 153]. Rituximab is an engineered chimeric mAb containing a human IgG1 Fc kappa region and murine variable region reactive with human CD20 antigen. It has an approximate molecular weight of 145 kDa and binding affinity of approximately 8.0 nM.

Preclinical Pharmacokinetics and Pharmacodynamics

A significant dose–concentration–response relationship of rituximab was observed in a murine model of human CD20+ lymphoma [154]. Tumor burden influenced both rituximab exposure (i.e., dose–concentration relationship) and rituximab efficacy (i.e., concentration–response relationship). Rituximab concentrations were inversely correlated with baseline tumor burden, where non-tumor-bearing animals have higher concentrations than tumor-bearing animals—animals with lower tumor burden having higher concentrations than those with intermediate or high tumor burden. The results can be explained that high tumor burden "captures" more antibodies, which is then quickly eliminated.

Rituximab did not improve survival at 6 mg/kg, whereas mice treated with 12, 20, or 40 mg/kg of rituximab had a significantly longer survival with median survivals of 28, 32, and 43 days, respectively. Furthermore, higher concentrations (likely in mice with lower tumor burden) were associated with a higher rate of complete tumor response, and concentrations higher than 1.5 μg/mL were associated with a longer survival. More interestingly, mice that displayed linear PK (indicating saturation of target) had complete response, whereas mice with accelerated elimination of rituximab had partial response with resumption of tumor growth 13 days after the rituximab injection. The study clearly demonstrated the relationship of tumor burden, rituximab exposure, and efficacy and offers support of dose adjustment of rituximab to tumor burden.

Clinical Pharmacokinetics and Pharmacodynamics

PK behavior of rituximab in patients is affected by different disease types and a clear PK/PD relationship exists. In a study with four groups of patients with different diseases (i.e., follicular and mantle cell lymphoma, autoimmune disorder, amyloidosis) with low tumor burdens [155], rituximab PK was characterized by a two-compartment linear model. At the standard rituximab weekly or monthly IV dose of 375 mg/m^2, the total systemic clearance ranged between 3.1 and 11.9 mL/h/m^2, which corresponded to a long elimination half-life of approximately 3 weeks (range, 10–36 days). The long elimination half-life results in significant (2.6–2.7-fold) drug accumulation following weekly dosing and is

consistent with the observation that steady-state plasma concentrations of rituximab were not reached until after 6–8 weekly infusions.

In contrast, rituximab displays nonlinear PK in patients with NHL, indicating target-mediated disposition plays a significant role in the overall drug clearance. In a multicenter phase III study involving 166 patients with recurrent or refractory low-grade NHL using 375 mg/m^2 for 4 weekly doses, the rituximab elimination half-life increased significantly between the first (half-life 76.3 h) and fourth (half-life 205.8 h) infusion [35]. The increase in half-life is most likely associated with the elimination of circulating CD20+ B cells, which results in the saturation of target-mediated drug disposition following initial infusions. Subsequently this subpopulation of cells is replenished in most patients by 9–12 months after therapy because CD20 is not expressed on hematopoietic stem cells. The recovery of B cells is considered to be a PD marker for rituximab.

Consistent with preclinical results, rituximab concentrations inversely correlate with tumor bulk and the number of circulating B cells at baseline and even with histologic subtype of the patients [35]. Rituximab levels were significantly lower for patients with International Working Formulation (IWF) Histologic Type A, presumably due to higher tumor/antigen burden. It was also observed that higher concentrations of circulating drug correlated with better clinical response. At 3 months posttreatment, median serum concentration in responders was 25.4 μg/mL compared with 5.9 μg/mL in nonresponders. The association of tumor burden, rituximab concentration, and response suggests that higher doses of rituximab may be necessary to induce responses in some subsets of patients with bulky disease.

More recently, a population PK analysis was conducted to characterize the unique PK profile of rituximab, using data from 298 NHL patients who received rituximab once weekly or every 3 weeks [36, 70]. A two-compartment model with time-varying clearance best described the concentration-time data, as the result of rapid and sustained depletion of circulating and tissue-based B cells following rituximab treatment. In this model, total clearance was comprised of a nonspecific clearance and a specific clearance that decreased with a first-order rate constant following multiple infusions. Covariate analysis revealed that patients with higher CD19 counts or sum of perpendicular diameters (SPD) of tumor burden at baseline had a higher rituximab specific clearance, whereas the central volume of distribution varied by BSA and CHOP (i.e., cyclophosphamide, doxorubicin, vincristine, and prednisone) chemotherapy. While this model serves to explain the changes in rituximab behavior as the result of changing disease parameters, the results did not identify covariates that could reduce PK variability to the extent that alterations in dose would be warranted.

Further effects of target-mediated drug disposition of rituximab are observed in patients with CLL, where the number of circulating malignant cells is expected to be higher than in NHL. Consequently, the degree of target-mediated (specific) clearance effects is expected to be greater. In a rituximab dose escalation study conducted in 40 CLL patients, a first dose of 375 mg/m^2 was administered to all patients, followed by weekly doses ranging from 500 to 2,250 mg/m^2 from second through fourth doses to different patients. Significant clinical activity and a clear dose–response relationship were observed in this trial [156]. Further acknowledgement of the potential need of higher doses to accommodate the larger tumor burden in CLL is exemplified in a study of where an alternate dose regimen was used. Rituximab was administered at the standard dose of 375 mg/m^2 but was given three times a week, rather than the weekly dose, for 4 weeks. Both studies suggest that higher doses are more effective against CLL. Pivotal trials in CLL were performed using the dose that resulted in the approved dose in CLL of 375 mg/m^2 (i.e., standard dose) with 500 mg/m^2 after the first treatment cycle [157]. Because most toxicity was observed with the initial infusion, subsequent doses but not the first dose were increased to 500 mg/m^2.

Studies from both preclinical and clinical settings consistently suggest a strong correlation of rituximab exposure, tumor burden, and efficacy. The PK/PD relationship provides valuable insights into the clinical dose selections in different indications.

Dosage and Administration

The recommended dose of rituximab is 375 mg/m^2 via IV infusion at different schedules according to therapeutic intent. In relapsed or refractory, low-grade or follicular, CD20-positive B-cell NHL, 375 mg/m^2 is administered once weekly for 4 or 8 weeks followed by retreatment for an additional 4 weeks. In untreated, follicular, CD20-positive B-cell NHL, 375 mg/m^2 is administered on day 1 of cyclophosphamide, vincristine, and prednisolone (CVP) therapy. In nonprogressing, low-grade, CD20-positive B-cell NHL after CVP therapy, dose of 375 mg/m^2 weekly for 4 weeks is administered following completion of 6–8 cycles of CVP. In diffuse large B-cell NHL, 375 mg/m^2 is administered on day 1 of chemotherapy for a maximum of eight infusions. In B-cell CLL, dose of 375 mg/m^2 is administered prior to fludarabine and cyclophosphamide chemotherapy followed by 500 mg/m^2 on day 1 of cycles 2–6.

Due to potential infusion reaction, the infusion of rituximab should start at a rate of 50 mg/h for the first infusion or 100 mg/h for subsequent infusions. In the absence of infusion toxicity, the infusion rate can be increased to 50–100 mg/h every 30 min to a maximum of 400 mg/h.

Drug–Drug Interaction

While there have been no dedicated drug–drug interaction studies of rituximab with chemotherapeutic agents conducted in target populations, effect of coadministered and concomitant medications on rituximab PK has been evaluated in the aforementioned population PK analysis. In this analysis it was observed that the CHOP therapy led to a small increase (19 %) on the central volume of distribution, based on data from approximately 40 patients. This magnitude of effect was not considered to be clinically relevant to warrant a dose adjustment [36].

Immunogenicity

Since rituximab is a chimeric mAb, it has a potential for immunogenicity. Using an enzyme-linked immunosorbent assay (ELISA), human anti-chimeric antibodies (HACA) were detected in 4 of 356 (1.1 %) patients with low-grade or follicular NHL receiving single-agent rituximab. In these four HACA-positive patients, three demonstrated an objective clinical response, indicating that the development of antidrug antibodies did not affect the efficacy. Furthermore, there was no apparent correlation between immunogenicity and safety events such as infusion-related reactions.

4.3.2 Ofatumumab

Ofatumumab is an IgG1 kappa human mAb with a molecular weight of approximately 149 kDa. In vitro studies indicate that ofatumumab targets an epitope on CD20 different from rituximab and most other CD20-directed antibodies [158]. It binds to both the small and large loops of the CD20 molecule on B cells and appears to inhibit early-stage B-lymphocyte activation. Ofatumumab is approved in the United States and Europe for treating CLL that is refractory to fludarabine and alemtuzumab and has also shown potential in treating follicular NHL and diffuse large B-cell lymphoma [159, 160].

Preclinical Pharmacokinetics and Pharmacodynamics

The PK/PD relationship of ofatumumab was evaluated in preclinical models to help understand the dose requirements for sustained in vivo activity of ofatumumab [161]. A series of in vitro experiments

demonstrated the cytotoxic effect of ofatumumab in relation with the target occupancy. ADCC induction reached the maximum level (51 % cell lysis) at a mAb concentration of about 0.1 μg/mL, at which a half of maximum target occupancy was achieved. In contrast, full target saturation at concentration of about 5 μg/mL was required for obtaining maximal CDC (68 % cell lysis). No further increase in ADCC or CDC was observed by increasing the ofatumumab concentration above the level of target saturation. In a mouse tumor xenograft model, ofatumumab inhibited B-cell tumor development at a peak plasma concentration of 5 μg/mL, at which concentration it was expected to result in saturation of CD20 binding; tumor development resumed when ofatumumab plasma concentrations were below half-maximal target saturation. In vivo, ofatumumab displayed time- and concentration-dependent PK, which was directly correlated with tumor burden. In monkeys, initial depletion of circulating and tissue-residing B cells required relatively high-dose levels, and ofatumumab concentrations greater than 50 μg/mL were shown to be sufficient for complete B-cell depletion. Once saturation of CD20 throughout the body has been reached by initial doses, subsequently lower plasma concentrations (i.e., 5–10 μg/mL) may be sufficient to saturate CD20 epitopes and to maintain depletion of B cells in peripheral blood. These provide a rationale for establishing clinical dosing schedules.

Clinical Pharmacokinetics and Pharmacodynamics

Consistent with preclinical findings, ofatumumab in patients showed time- and concentration-dependent PK as a result of both target-mediated and nonspecific elimination pathways [162, 163]. The concentration–time profile was best described by a two-compartment model with varying clearance over the course of treatment. This is in line with an observed rapid and sustained B-cell depletion. In the dose range of 100–2,000 mg, the C_{max} and AUC values increased greater than expected from linear accumulation of the drug, suggesting concentration-dependent kinetics. The low volume of distribution at steady state (1.7–5.1 L) suggests limited distribution of ofatumumab outside the circulatory compartment, typical of mAbs. Clearance was dose-dependent at doses of 100–2,000 mg but declined substantially after the first infusion, presumably because of depletion of B cells. Between the 4th and 12th infusions, the mean clearance was 0.01 L/h and the mean elimination half-life was 14 days. Body weight, age, gender, and creatinine clearance (range: 33–287 mL/min) were not associated with clinically important effects on measured ofatumumab PK parameters.

Ofatumumab PK are influenced by the severity of CLL at baseline. The PK analysis showed that SPD, a surrogate for tumor burden, was a significant predictor for clearance. High SPD at baseline was associated with faster clearance both at first and fourth infusion [162]. However, SPD only partially explained the associations between clearance and exposure. Other surrogates for tumor burden, such as lymphocyte counts or Rai and Binet stages, were not related to clearance. Positive correlations between clinical response and exposure were observed with higher exposure associated with higher probability of overall clinical response and longer progression-free survival. The understanding of PK/PD relationship is useful in designing dose regimens of ofatumumab in future clinical trials.

Dose and Administration

The dosing of ofatumumab in patients with refractory CLL is an initial IV infusion of 300 mg followed by a 2,000 mg weekly IV infusion for 7 weeks, then monthly for 4 months. Due to potential infusion reactions, premedication such as acetaminophen, antihistamine, or corticosteroid is recommended 30 min to 2 h prior to each dose. The infusion rate should be slow at the beginning of each infusion and may be increased every 30 min thereafter in the absence of infusion reaction [164].

Drug–Drug Interaction

No formal DDI studies of ofatumumab with other drugs have been conducted.

Immunogenicity

As with other mAbs, there is a potential for immunogenicity for ofatumumab. Serum samples from patients with CLL were tested for anti-ofatumumab antibodies during and after the 24-week treatment period. No antidrug antibodies were detected in 46 patients evaluated after the 8th infusion or in 33 patients evaluated after the 12th infusion [165].

4.3.3 Alemtuzumab

Alemtuzumab is a humanized IgG1 antibody binding to CD52, an antigen abundantly expressed on the surface of T- and B-cell lymphocytes, monocytes, macrophages, and eosinophils, but not on hematopoietic stem cells. Alemtuzumab is indicated for the treatment of B-cell CLL in patients who have been treated with alkylating agents and who have failed fludarabine therapy. Alemtuzumab, administered alone or in combination, was shown to induce minimal residual disease (MRD)-negative responses [166, 167].

Preclinical Pharmacokinetics and Pharmacodynamics

Limited preclinical PK and PD data have been published for alemtuzumab. In vitro studies showed that the concentrations of alemtuzumab required for CDC and apoptosis are approximately 1–10 μg/mL, whereas that for ADCC is 0.01 μg/mL [168]. To date, no in vivo data are available to define alemtuzumab concentration required for clinical activity.

Clinical Pharmacokinetics and Pharmacodynamics

A two-compartment model with nonlinear elimination best describes alemtuzumab PK. The clearance of the drug is both time- and concentration-dependent [169]. White blood cell (WBC) counts were identified as the only important covariate on maximum rate of elimination [$V_{max} = 1{,}020 \times \text{WBC count}/(10 \times 10^9/\text{L})^{0.194}$]. Following alemtuzumab administration, WBC counts generally declined rapidly. Other factors that may affect exposure to alemtuzumab include soluble CD52, tumor burden, level of CD52 expression, and concurrent chemotherapy [170]. When the baseline antigen level is high, clearance is faster because the drug binds to its epitope and is subsequently rapidly cleared from the blood. As the antigen is depleted following repeated dose administration, clearance will decrease to reach a plateau and half-life will increase. The half-life is 11 h after the first dose, but increases to 6 days (range 1–14 days) when WBCs are at their nadir after the repeated 30 mg dose.

In the PD analysis, a stimulatory indirect response model in which WBCs were rapidly depleted with repeated dosing best described the effect of alemtuzumab on WBCs [169]. A direct relationship between maximum trough concentrations and clinical response was observed, with increasing alemtuzumab exposure resulting in a greater probability of positive tumor response. In a study with 14 patients who received the alemtuzumab in combination with fludarabine (FluCam regimen), the maximum alemtuzumab concentrations were 3.35, 0.98, and 0.32 μg/mL in patients with completed response, partial response, and progressive disease, respectively [171]. Montillo et al. reported that in

alemtuzumab consolidation therapy, all 12 patients having AUC_{0-12} > 5 h μg/mL achieved a complete response [172].

A large interindividual variability was observed, both in PK and PD of alemtuzumab, which was probably reflective of broad differences in tumor burden among patients. In the population analysis, typical values (% coefficient of variation) for V_{max}, K_m, V_1, and V_2 were 1,020 μg/h (32 %), 338 μg/L (145 %), 11.3 L (84 %), and 41.5 L (179 %), respectively [169]. In a study including 30 patients with relapsed CLL with received IV alemtuzumab 30 mg three times a week for up to 12 weeks, the maximum trough concentrations of alemtuzumab ranged from 0.5 to 18.3 μg/mL with the average of 5.4 μg/mL. The cumulative dose of alemtuzumab needed to reach a trough concentration of 1.0 μg/mL was 90 mg on average with the range from 13 to 316 mg [68].

Alemtuzumab dosing regimen was developed in the absence of robust PK data and does not take into consideration of variations in patient characteristics. A PK-guided dosing schedule has been suggested to ensure sufficient exposure of alemtuzumab achieved in any given patient [170].

Dose and Administration

Alemtuzumab is administered as a 2-h intravenous infusion under a dose escalation scheme, wherein patients receive 3 mg daily until infusion reactions are grade 2 or less, followed by escalation to 10 mg daily until tolerated, and then 30 mg three times weekly for a maximum of 12 weeks [173]. The dose escalation is typically achieved in 3–7 days.

The subcutaneous route, at the same IV administration dose, has been studied in an attempt to reduce side effects and make the treatment more manageable [174].

Drug–Drug Interaction

Several studies have been conducted to evaluate the combination of alemtuzumab with fludarabine or rituximab in the treatment of CLL. No alterations in PK have been reported.

Immunogenicity

Four (1.9 %) of 211 patients evaluated for development of an immune response were found to have antibodies to alemtuzumab [175].

4.3.4 Other B-Cell-Directed Agents Approved or in Development

The successful application of engineered antibodies in the treatment of B-cell-directed therapies has contributed to the development of a variety of antibodies against hematological malignancies. Besides rituximab and ofatumumab, two radioimmunoconjugates ^{90}Y Ibritumomab tiuxetan and ^{131}I Tositumomab that target CD20 have been approved in the treatment of relapsed/refractory follicular and transformed NHL [176]. The rationale for using a targeted radiolabeled isotope is to decrease toxicity by achieving more target-specific radiotherapy using the exquisite target specificity of mAbs as a drug delivery system. GA101 is a humanized and glycoengineered anti-CD20 mAb currently in late-stage clinical development. In preclinical studies, GA101 exhibits significantly greater ADCC and direct cell death induction than rituximab, which possibly results in greater efficacy especially in patients who are carriers of the FcγRIIIa low-affinity receptor polymorphism [177]. Brentuximab vedotin (SGN-35) was recently approved for the treatment of patients with relapsed or refractory

Hodgkin lymphoma [178]. This antibody–drug conjugate is composed of anti-CD30 chimeric mAb cAC10 and the potent antimicrotubule drug monomethylauristatin E connected by a protease-cleavable linker. Treatment with single-agent brentuximab vedotin resulted in unprecedented objective response rates and complete response rates [179]. Other B-cell and T-cell targets have been identified in treating hematological malignancies, which include but not limited to CD22, CD 23, CD80, HLA-DR, and CD33 [180], and numerous target-specific antibodies are being developed. mAbs have provided powerful and relatively safe tools to specifically target cells and become indispensable in the treatment of lymphoma and leukemia.

4.4 Immunomodulatory Therapy

Over the past decades, considerable knowledge has been gained on the components that are relevant in antitumor immune responses and immune escape mechanisms. Although vaccination can lead to the induction of T-cell response, a more direct approach is to use mAbs to directly activate antitumor T-cell response. Studies in a variety of systems have shown that engagement of the T-cell receptor (TCR) is not sufficient to activate T cells to full effector function. Further signals by a wide variety of costimulatory molecules are required to amplify and/or modify the TCR signal. Several mAbs have been developed that can bind to coreceptors, either inducing or inhibiting their signal. Potential therapeutic targets under development include targeting CD28 and cytotoxic T-lymphocyte antigen 4 (CTLA-4), programmed death-1 (PD-1), PD ligand-1 (PDL01), CD40, OX40, and 4-1BB [181, 182].

Ipilimumab is a fully human mAb directed against CTLA-4 that is recently approved by the FDA for the treatment of melanoma. It inhibits the binding of CTLA-4 to B7 receptors and causes unrestrained T-lymphocyte proliferation and IL-2 production. This reaction leads to amplification of immune response to immunogenic malignancies and subsequent tumor regression [183, 184].

Based on data from a double-blind, dose-ranging phase II study, a population PK analysis found ipilimumab. Ipilimumab was characterized by a two-compartment linear model [185]. Analysis of covariate effects indicates the clearance and central volume of distribution correlate with body weight, with clearance increasing with increasing of serum concentration of lactate dehydrogenase (LDH). Model-based simulations suggest that target trough concentration of 20 μg/mL before the fourth dose in the induction phase would be achieved for over 95 % patients given 10 mg/kg of ipilimumab once every 3 weeks, but only 30 % and 0 % at the dose of 3 mg/kg and 0.3 mg/kg, respectively. The study demonstrated dose–response relationship with the best overall response rate (BORR) of 0 %, 4.2 %, and 11.1 % and the 24-week PFS rates of 2.7 %, 12.9 %, and 18.9 % in the 0.3, 3, and 10 mg/kg dose groups, respectively. The study, along with other phase II studies, showed superior BORR with ipilimumab 10 mg/kg [186]. However, in the phase III pivotal, a survival benefit was demonstrated at a dose of 3 mg/kg every 3 weeks for a total of four doses, which served as the basis for approval by the US FDA [186]. An ongoing trial is evaluating the efficacy of 10 mg/kg regimen when ipilimumab is used in combination with dacarbazine in the first-line setting [187].

Following on the success of ipilimumab, more mAbs targeting T-cell costimulatory antigens will enter the clinical development. Immunotherapy can result in enhanced or reduced immune activation and strong antitumor responses but also can cause profound toxicity or autoimmunity. This remains a challenge in the development of immunotherapy. A dose-dependent increase in immune-related adverse events (irAEs) of any grade was observed [188], and grade 3–4 irAEs affected a quarter of patients in the 10 mg/kg group reported in a phase II monotherapy study of ipilimumab [185]. The frequency and severity of irAEs have shown to be related to tumor response as well as prolonged time to relapse [189, 190]. As with many other types of treatments, further identification of patient subtypes who respond best to the therapy and section of dose regimens remains a challenge in future clinical development.

4.5 Met/HGF Pathway

Hepatocyte growth factor (HGF, ligand) and Met (receptor) expression have been observed in tumor biopsies of most solid tumors [191, 192]. Results of several clinical studies indicate that Met expression is associated with poor prognosis and the development of acquired resistance to chemotherapies [191, 193–195]. This evidence led to the development of a variety of Met pathway antagonists with potential clinical applications. Several mAbs were developed to direct against HGF ligand (e.g., rilotumumab, AV-299) or Met receptor (e.g., onartuzumab) with high specificity, and preliminary clinical results are encouraging.

Rilotumumab is a fully human IgG2 antibody being evaluated in phases I/II trials in prostate, colon, lung, gastric, esophageal, renal, and glioma cancers alone or in combination with others agents [196, 197]. Phase I study of rilotumumab showed acceptable safety profile as a monotherapy. Rilotumumab exhibited linear PK across all dose levels tested, with the mean elimination half-life of 18 days. A preliminary population PK model suggested that dosing regimens of ≥10 mg/kg every 2 weeks, 15 mg/kg every 3 weeks, or 20 mg/kg every 4 weeks should maintain serum rilotumumab trough concentrations above the IC_{90} value in the human umbilical vein cell and U-87 MG cell proliferation assays in >90 % of patients [198]. Therefore, these regimens were chosen for ongoing phase II studies. A dose-dependent increase in the levels of plasma HGF was observed upon treatment with rilotumumab, suggesting that HGF levels may serve as a biomarker for the inhibition of the HGF/Met pathway and potentially a patient selection marker for clinical studies [196].

Different from rilotumumab, which targets the HGF ligand, onartuzumab (also known as MetMAb) is a humanized aglycosylated monovalent antibody that acts as an antagonist of Met by binding the receptor, thereby blocking HGF/Met binding [199]. This monovalent antibody is composed of a full-length heavy chain, a light chain, and a truncated heavy chain that consists only of the C_H2 and C_H3 domains of the IgG protein. Onartuzumab is produced in *E. coli* and has a molecular mass of approximately 99 kDa. The unique monovalent one arm design of onartuzumab eliminates the potential for Met activation (i.e., agonistic activity) via receptor dimerization, observed with some bivalent antibodies [200]. This also makes unique PK characteristics. Since onartuzumab retains the intact Fc, it binds to FcRn with similar affinity to bivalent, glycosylated IgG1 antibodies. The binding to FcRn is expected to improve the half-life of onartuzumab in circulation when compared to a Fab fragment [201]. Results from a phase I dose escalation study indicate that onartuzumab is safe and well tolerated as a single agent at doses up to 30 mg/kg [202]. Onartuzumab PK are linear in the dose range of 4–30 mg/kg with a clearance of approximately 7.5 mL/day/kg, approximately twofold faster than a traditional bivalent antibody. A terminal half-life of approximately 10 days is observed. Results from a phase II NSCLC study suggest Met expression in tumors may be a diagnostic marker. The improvement in progression-free survival and overall survival is correlated with Met expression in patients received onartuzumab [203]. Preclinical in vivo studies have identified a target concentration approximately 15 μg/mL. PK analyses in a phase I trial showed that a dose of 15 mg/kg once every 3 weeks or 10 mg/kg every 2 weeks would achieve steady-state concentrations above the 15 μg/mL target for a majority of patients [204].

4.6 Apoptosis Pathway

Apoptosis (type 1 programmed cell death) has evolved in multicellular organisms as a means of eliminating abnormal cells. Defects in apoptosis can prolong cellular life span and contribute to carcinogenesis. Apoptosis occurs through two distinct pathways: the intrinsic and extrinsic pathways. The intrinsic pathway results in signals from the mitochondria and is activated intracellularly by a variety of stimuli, such as chemotherapeutic drugs, radiotherapy, hypoxia, and starvation. The extrinsic pathway is

characterized by activation of death receptors (DR) [205]. Numerous antibody-based anticancer agents have advanced from bench to clinic during the last two decades that target the extrinsic apoptosis pathway. The target specificity of mAbs and the relative lack of overlapping toxicities with existing treatments make them attractive therapeutic candidates [206–208]. Currently, one chimeric (LBY135) and five human or humanized mAbs (drozitumab, mapatumumab, lexatumumab, conatumumab, and tigatuzumab) and one TNF-related agonistic ligand (TRAIL), which target the DR4 and DR5 receptors or both (TRAIL), are in phase I and II clinical trials [209]. These are under development either as single agents or combination with chemotherapeutic agents or other mAbs to enhance the antitumor activity of this class of agents through cross talk between the intrinsic and extrinsic pathways. These agents have been well tolerated at the doses tested in patient populations. In clinical studies, the PK of these mAbs are linear in the therapeutic dose range with elimination half-lives of approximately 2–3 weeks in patients. With dosing frequencies of once every 1–3 weeks, steady-state drug concentrations achieved with the apoptotic mAbs are consistent with those that predict for antitumor activity in preclinical models. No significant PK drug–drug interaction has been reported.

Despite remarkable selectivity of DR expression on cancer cell surface, development of resistance to TRAIL-induced apoptosis remains a major challenge. In fact, an increasing number of publications show TRAIL resistance in primary human tumor cells, and sensitization to TRAIL-induced apoptosis is likely required for efficacy. Therefore, development of suitable diagnostic tests to identify right patient population may be essential for the success of this class of molecules [210–212]. In review of the published data, there is no PK/PD relationship has been established for the TRAIL agonistic mAbs.

4.7 IGF Pathway

The insulin-like growth factor (IGF) pathway is a fundamental mechanism of cell survival. IGF binds to its receptors IGF1R to stimulate cell growth, proliferation, differentiation, and survival through ERK/MEK and PI3K/Akt pathways. Increased expression of IGF1R or enhanced activation has been reported in many cancer types including breast, colon, melanoma, and prostate cancers [213–220]. Moreover, the resistance to EGFR therapy is associated with upregulation of IGF1R levels in tumors. These data clearly indicate that IGF1R signaling is crucial for tumor transformation and survival of malignant cells.

A number of antibodies that bind to extracellular domain of IGF1R have been developed for the potential treatment of cancer. These antibodies function through blocking ligand activation of the IGF system and simulating receptor downregulation by receptor internalization and degradation. These molecules demonstrate dose-dependent tumor growth inhibition in xenograft models, which was also associated with dose-dependent downregulation of IGF1R expression and inhibition of phosphorylation of Akt. Clinical results have been reported for several mAbs targeting IGF1R, such as figitumumab, cixutumumab, AMG 479, SCH717454, R1507, MK-0646, and BIIB022. These molecules are in various stages of clinical development. Overall these agents have been well tolerated at pharmacologically relevant doses. Figitumumab appears to have a longer elimination half-life of approximately 20 days, when compared to others in this therapeutic class, which have much shorter half-lives (varying from 4 to 14 days). The long half-life for figitumumab is explained in part by the use of a fully human IgG2 backbone [221]. The dose-dependent downregulation of IGF1R on circulating leukocytes and sustained serum elevation of IGF-1 and IGF-binding protein-3 (IGFBP-3) levels indicated that there may be potential markers for predicting clinical response during treatment with IGFR1 targeting agents [221–225]. Although favorable PK, PD, and safety properties have been demonstrated with these agents, a significant level of clinical response remains to be established.

5 Conclusion

Monoclonal antibodies over past decades have come to represent a significant and growing component of the cancer treatment armamentarium for both solid and hematological malignancies. The strategies and challenges in clinical development of these agents differ markedly from those of small molecule drugs. The unique PK/PD behavior of mAbs provides unique opportunities and challenges during all phases of drug development. The discovery and validation of new functional targets and epitopes on existing targets, increased application of antibody engineering, discovery of new PD and pharmacogenetic markers, increased knowledge of key drivers of PK/PD relationships, and the ability to conjugate potent toxins and radionuclides to these proteins will inevitably further expand therapeutic potential of these proteins to bring exciting new treatment options to improve the lives of patients with cancer.

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Clinical Pharmacology in Pediatrics

Michael Tagen and Clinton F. Stewart

Abstract Pediatric cancer patients differ from adults both in the spectra of their malignancies and in their response to drugs. Our knowledge of the developmental pharmacology of anticancer drugs has increased, and yet we often do not have sufficient understanding to optimally treat pediatric cancer patients, particularly infants and young children. Well-designed clinical pharmacology studies in this group of patients are necessary for the development of novel dosing strategies tailored to children of different ages. The first section of this chapter reviews the basic principles of drug absorption, distribution, metabolism, and elimination in infants, children, and adolescents. Subsequent sections address the appropriate methods to select drug dosages in children, practical issues associated with clinical pharmacokinetic studies in children with cancer, and the pharmacokinetics of specific anticancer drugs in children. We then review the pharmacokinetics of selected anticancer drugs used in children with cancer.

Keywords Pediatrics • Cancer • Pharmacology • Developmental pharmacology • Pharmacokinetics

1 Introduction

Pediatric cancer patients differ from adults both in the spectra of their malignancies and in their response to drugs. Since the publication of the previous edition of this book, our knowledge of the developmental pharmacology of anticancer drugs has increased, and yet many reports have shown that we do not have sufficient understanding to optimally treat pediatric cancer patients, particularly infants and young children. Recent reviews have highlighted accomplishments, including taking steps towards model-based pediatric dose selection and studies of drug-metabolizing enzyme and transporter ontogeny [1, 2]. We have furthered our knowledge of the pharmacokinetics of specific anticancer drugs.

Children, particularly infants and young children, are still at risk from inappropriate dosing of drugs. It was recently reported that children less than 4 years of age have the highest incidence of serious adverse drug events [3]. Higher rates of serious toxicities [4] and treatment-related deaths [5, 6] have been seen in treatment of infants with malignancies. Well-designed clinical pharmacology

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studies in this group of patients will be necessary for the development of novel dosing strategies tailored to young children. This process can be improved by the use of population pharmacokinetic–pharmacodynamic modeling, to take full advantage of the data generated by these studies [7]. Certainly, we must move beyond simple BSA- and weight-based dosing [8].

Even accounting for maturational changes that occur as a child ages, there is still likely to be considerable interindividual variability in the pharmacokinetics of anticancer drugs in children. This interindividual variability has significant clinical implications. For example, patients with low clearance of a drug will have increased systemic exposure, which can lead to toxicity. Conversely, those children with more rapid clearance may not have toxicity, but they may have a lack of antitumor activity [9]. Differences in drug disposition among children can be attributed not only to developmental changes in physiology but also to variation in genetic composition of individuals [10]. However, the genotype–phenotype correlation may be age dependent; a polymorphism in a gene will not likely have an effect if the gene is not yet expressed [11]. Evaluation of the relation between systemic exposure and drug effect (e.g., toxicity and efficacy) has become an important aspect of new drug development in children since many clinical pharmacology studies have reported a relationship between systemic exposure and response. Understanding the pharmacokinetics of anticancer drugs in children is essential to develop new drugs and new combination regimens and to define rational dosing schedules for these drugs.

The first section of this chapter will review the basic principles of drug absorption, distribution, metabolism, and elimination in infants, children, and adolescents. Subsequent sections will address the appropriate method to select drug dosages in children, practical issues associated with clinical pharmacokinetic studies in children with cancer, and the pharmacokinetics of specific anticancer drugs in children.

2 Effect of Developmental Processes on Drug Disposition

2.1 Effect of Development on Drug Absorption

The majority of new anticancer agents, including tyrosine kinase inhibitors, are all administered orally. Therefore, understanding how developmental processes affect drug absorption is of utmost importance. Several of the older anticancer agents can also be administered orally, including methotrexate, 6-mercaptopurine, etoposide, topotecan, irinotecan, cyclophosphamide, and temozolomide. Several of these drugs, such as cyclophosphamide and topotecan, have antiangiogenic properties when given at low doses and are being evaluated in children for “metronomic” therapy, consisting of continuous or long-term daily oral administration [12–14].

Oral drug absorption is dependent on both physicochemical and physiological factors. Physicochemical factors, which can vary among individual agents and formulations, consist of molecular weight, size and shape of the dosage form, degree of ionization under physiological conditions, and solubility at the site of absorption. These characteristics are drug specific and are not subject to maturational changes. However, because many young children are unable to swallow whole tablets or capsules, the clinician must consider that crushing or dissolving tablets for extemporaneous administration to children may alter oral absorption. Whether absorption will be increased or decreased depends upon the physicochemical properties of the drug, and the clinician is advised to consult appropriate references prior to altering commercially manufactured dosage formulations.

Physiological factors can vary among individuals as well as with maturation. Knowledge of the age-related changes in factors associated with drug absorption will enhance the ability of the clinician to use oral anticancer drugs appropriately in children. These factors include gastric emptying time,

gastric pH, bile salt production, bacterial colonization of the GI tract, gastrointestinal transit time, and pancreatic function.

Gastric emptying time is prolonged in children relative to adults [15], and this can reduce or delay the peak concentration of drugs administered orally and, furthermore, delay the therapeutic effect. The rate of gastric emptying is directly related to gestational and postnatal age and the type of feeding [16–18]. Furthermore, meals with high caloric density can increase gastric emptying time further in premature infants [19]. Gastric emptying time approaches values comparable to adults within the first 6–8 months of life.

Gastrointestinal transit time, which can also affect the absorption of orally administered drugs, has been less extensively studied in children than gastric emptying time. Intestinal transit times of 3–13.1 h have been reported for full-term neonates aged 3–5 days [20, 21]. After 45 days, breast-fed infants had a longer transit time (>10 h) than infants who were fed formula (<10 h) [22]. In comparison to adults, who usually have an intestinal transit time of about 24 h, it is possible that infants could have reduced absorption of some agents. The frequency of defecation decreases with age, so that 85 % of children 1–4 years of age defecate once or twice daily. A proportionate decrease of high-amplitude propagating contractions (e.g., slow transit) occurs in toddlers compared to adults [23].

Gastric acid secretion and pH strongly influence gastrointestinal absorption. At birth, gastric pH is neutral due to the presence of amniotic fluid in the stomach. However, within hours, pH rapidly falls to 1.5–3.0. Acid secretion peaks during the first 10 days of life and decreases from 10 to 30 days after birth [24]. Gastric pH usually reaches adult values by 2 years of age. The volume of gastric acid secretions approaches adult values by 3 months of age. The lack of acidity in the gut could decrease the absorption (and hence bioavailability) of anticancer drugs that are weak acids and increase the absorption of weak bases. For example, methotrexate absorption is reduced by coadministration with milk, which effectively reduces the acidity of the gut [25].

Both the rate of bile acid synthesis and the bile acid pool size are decreased in neonates compared to adults [26, 27]. These changes may alter the disposition of drugs that undergo enterohepatic recirculation, such as irinotecan. Also, the absorption of lipid-soluble drugs may be decreased in neonates. However, within the first year of life, bile acid synthesis and pool size increase to adult values.

Pancreatic enzyme activity is low at birth and is even lower in premature infants [27]. Lipase activity is low in the neonate and in combination with low bile acid production could reduce the gastrointestinal absorption of lipid-soluble drugs. However, even with low pancreatic lipase, the neonate is able to absorb 90–95 % of dietary fat through gastric and intestinal lipases [28]. Lipase activity increases 20-fold during the first 9 months of life to reach adult values [16, 29]. The secretion of both amylase and trypsin remains low for the first year of life [17, 30, 31].

Colonization of the gastrointestinal tract by bacterial flora varies with respect to age, type of delivery, and type of feeding. Before birth, the gut lacks bacterial flora; thereafter, the gut acquires bacteria from the environment. For example, a vaginal birth would lead to colonization of the gut by the mother's vaginal and large intestinal flora [32]. Due to the presence of certain antibodies in the breast milk, an infant who is fed breast milk will acquire different types of bacteria compared to an infant who is fed formula [32]. These changes in bacterial colonization of the gastrointestinal tract have implications for drug absorption and metabolism as will be discussed later in Sect. 5.6.

Absorption of many oral drugs is limited by intestinal metabolism and efflux transporters, particularly of the ATP-binding cassette (ABC) family [33]. P-glycoprotein (P-gp, ABCB1) is a transporter that is capable of effluxing many anticancer drugs. There is limited information on the ontogeny of P-gp in the GI tract. Although no changes in apparent oral clearance of the P-gp substrate fexofenadine were seen in patients 6 months to 12 years of age [34], another study has shown that the bioavailability of the P-gp substrate cyclosporine changes with age only in a subset of patients with certain *ABCB1* alleles [35]. Similar genotype-dependent changes with age were seen in gefitinib apparent oral clearance (Clinton Stewart, unpublished studies). Gene expression levels of *ABCB1* in the duodenum varied widely in children [36]. Although the relation to intestinal P-gp activity is not clear,

lymphocyte P-gp activity was elevated in lymphocytes from children <6 months of age [37]. On top of age-related changes, genetic polymorphisms in drug transporters may contribute to the interindividual variability in the absorption of orally administered anticancer drugs [38].

2.2 *Effect of Development on Drug Distribution*

Drug distribution describes to what extent a drug partitions to various body compartments after its administration. Often, drug distribution is described by a single pharmacokinetic parameter, the volume of distribution (V_d), which relates the drug dosage to the plasma concentration. However, drug distribution may also be described by physiologically-based pharmacokinetic models which attempt to mechanistically describe drug distribution to various tissues [39]. Similar to gastrointestinal absorption, drug distribution in children is dependent on the interaction of physiochemical (e.g., molecular size, ionization, lipid solubility) and physiological factors. Physiological factors relevant to drug distribution that change with age include body and organ size, body composition, levels of plasma proteins, tissue binding characteristics, vascular perfusion, and expression of drug transporters.

Body composition, which can be expressed as the relative proportion of total body water, total extracellular water, and total body fat, varies widely from neonates to adolescents [15]. Total body water (as a percentage of total body weight) falls from 85 % to 77 % in the premature and full-term neonate, respectively, to 73 % at 3 months, 59 % at one year, and 55 % at 12 years of age [40]. The clinical relevance of this observation is that relatively water-soluble drugs (e.g., topotecan) will have larger volume of the central compartment in infants compared with adults, whereas lipid-soluble drugs (e.g., etoposide or SN-38, the active metabolite of irinotecan) will have smaller volumes of the central compartment. Similarly, extracellular water falls from 45 % of total body weight in the full-term neonate to 33 % at 3 months, 28 % at one year, and 20 % in the adult [40]. Also, total body fat increases from infancy to about ten years of age. In boys during puberty, the percentage of total body weight that is fat begins to decrease at about age 17 to a mean of 12 %. Conversely, in girls, the percentage of body weight that is fat rapidly increases during puberty to as much as 25 % [41]. These changes suggest that during adolescence, sex-related differences in volume of distribution play a more important role than they do in younger children or adults.

Drug distribution in children may also be affected by age-related changes in plasma proteins. Extent of protein binding depends on a variety of factors such as amount of plasma proteins and the presence of endogenous substances that may compete for binding (e.g., bilirubin). Protein binding is reduced in infants due to the presence of fetal albumin and decreased albumin, γ-globulin, and α_1-acid glycoprotein. The concentrations of these proteins do not approach adult values until about 1 year of life [42]; thus, an infant given highly protein-bound drugs (e.g., etoposide or SN-38) will likely have decreased protein binding and an increased fraction unbound. This could lead to an increase in the systemic exposure to the unbound and putatively active drug. For anticancer drugs, which have a very narrow therapeutic range, this could potentially be associated with toxicity. Theoretically, increases in the unbound fraction of a drug could also potentiate antitumor activity; therefore, the predicted changes in toxicity and efficacy resulting from changes in the unbound drug fraction should be balanced when selecting appropriate dosages of highly protein-bound drugs in children. Even though protein concentrations are relatively stable from two years to adulthood, other factors such as disease or malnutrition can decrease plasma protein concentrations and create similar circumstances, potentiating both toxicity and efficacy of protein-bound drugs.

Tissue binding also affects drug distribution and changes with maturation. The absolute mass of tissue available for binding in each organ will increase with age. Therefore, younger children who have smaller organs have less tissue available for drugs to bind. This could lead to a greater amount

of free drug in the plasma and greater exposure to the drug. Each tissue has a different affinity for drug binding based upon the physicochemical properties of the drug, and the maturational changes in the amount and composition of such tissues may significantly alter drug distribution.

Drug distribution to certain compartments, in particular the brain and cerebrospinal fluid (CSF), is limited by the blood–brain barrier (BBB) and blood–CSF barrier (BCB). This is of particular importance to pediatrics, since brain tumors account for 20–25 % of all malignancies in children under the age of 15 and are the leading cause of death from childhood cancer. Little is known about the development of the BBB and BCB in regard to the penetration of anticancer drugs [43]. Indeed, endothelial tight junctions are still maturing during the third trimester [44]. However, CNS drug penetration may also be altered by disease state. For example, brain tumors affect the BBB, resulting in higher than expected drug penetration to the tumor [45].

Drug transporters also control anticancer drug penetration to the brain and CSF. P-gp expression was seen in brain microvessel endothelial cells as early as 8 weeks of gestation [46] and expression levels increased with maturation [47]. The expression pattern of P-gp, breast cancer resistance protein (BCRP; ABCG2), and multidrug resistance protein 1 (MRP1) in full-term infants was similar to adults, but the immunostaining was less intense [47], suggesting postnatal ontogeny of drug transporters at the BBB and BCB may influence the penetration of anticancer drugs which are substrates for these transporters.

2.3 Effect of Development on Drug Metabolism

Metabolism is a major route of clearance for many anticancer drugs. On the other hand, several anticancer prodrugs (e.g., cyclophosphamide, ifosfamide) need to be activated by hepatic enzymes. The ratio of liver weight to body weight is greater in infants and young children and decreases with age [48, 49]. On the other hand, liver microsomal protein content is decreased in neonates (26 mg/g liver), and increases with age, not reaching a maximum until approximately 30 years of age (40 mg/g liver) [50, 51]. To some extent, the opposing effects of relative liver mass and liver microsomal protein content may cancel each other out. Furthermore, the importance of the ontogeny of specific drug-metabolizing enzyme isoforms involved in clearing a drug may outweigh general changes in liver mass or protein content. An overview of the ontogeny of phase I and phase II enzyme systems is given below.

2.3.1 Phase I Enzymes

The cytochrome P450 (CYP) enzyme system is a crucial pathway in the metabolism of xenobiotics, including many anticancer drugs. Furthermore, numerous medications used in the supportive care of children with cancer are substrates, inducers, or inhibitors of CYP450 enzymes, which alter the metabolism, efficacy, and toxicity of anticancer agents that are metabolized via this pathway.

Many CYP enzymes develop early in fetal life, reflecting their role in maintaining homeostatic levels of endogenous substrates [52]. After birth, total CYP enzyme expression increases until adult levels are reached at approximately 1 year of age [53]. However, each individual CYP isoform has a unique ontogeny. The understanding of CYP ontogeny has been complicated by studies using different methods to measure expression (mRNA and protein) and activity, measured either in vitro using human liver microsomes or in vivo with a selective probe drug. The expression of mRNA, protein, and activity levels does not necessarily correlate, which further complicates the large interindividual variability in expression and activity.

CYP1A2 is the major CYP1A isoform expressed in the liver [54]. Expression is low in the fetus, and compared to other CYP isoforms, expression does not increase until relatively late [55]. CYP1A2 becomes detectable by 1–3 months of age and reaches adult expression levels by 1 year [56].

CYP2B6 hepatic protein expression was twofold higher in livers from individuals older than 1 month of age compared to those less than 1 month of age, but expression did not change from 1 month through 18 years of age [57]. Another study showed a sevenfold difference between protein expression of infants aged less than one year compared to older children and adults, although the samples size was limited to 10 per group [56]. Renal CYP2B6 has also been detected in kidney microsomes from children [58].

CYP2C activity is low in the fetus and newborn [59, 60], with concomitant low enzyme expression levels [52]. CYP2C9 mRNA and protein levels rise rapidly after birth. As demonstrated clinically by diazepam metabolism, urinary metabolites increase rapidly after birth and then remain stable until 5 years of age [61]. Unlike CYP2C9, which reaches adult protein expression levels rapidly after birth, CYP2C19 expression levels increase slowly over the first 6 months of life [62].

CYP2D6 ontogeny has been well examined at the level of mRNA, protein, microsomal activity, and in vivo activity using the selective O-demethylation of dextromethorphan. CYP2D6 protein content and activity is associated with increasing gestational age in prenatal livers, but a large increase in protein expression and activity occurs within the first week of life that is independent of gestational age [63, 64]. In vivo CYP2D6 activity corresponded to genotype-predicted activity by 2 weeks of age [65]. After this point, there is no correlation of CYP2D6 expression or activity with postnatal age [63, 65, 66].

Relevant CYP3A family isoforms include CYP3A4, CYP3A5, and the fetal isoform CYP3A7. CYP3A7 shares 95 % identity in the coding region with CYP3A4 [67]. Substrate specificity of the two isoforms is similar, although differences have been shown [68–70]. CYP3A7 hepatic expression and activity peaks around 1 week after birth and subsequently declines over the first year of life to very low levels [68]. However, while CYP3A7 activity is decreasing, CYP3A4 activity is increasing. There is limited CYP3A4 activity in the fetal liver, but expression increases after birth and adult activity is reached by 1 year of age [56, 68]. Clinical studies of midazolam hydroxylation, catalyzed by CYP3A4, have also shown that activity is reduced in infants, particularly preterm infants, and increases over the first year of life [71, 72].

2.3.2 Phase II Enzymes

Phase II enzymes catalyze conjugation reactions. Examples include the uridine 5′-diphosphate glucuronosyltransferase (UGT) family and glutathione transferase (GST) family. Phase II reactions are important in the disposition of a number of anticancer drugs used in children. For example, the active irinotecan metabolite SN-38 is glucuronidated by UGT isoforms, and the active metabolite of cyclophosphamide, 4-hydroxycyclophosphamide, is conjugated to glutathione through GST isoforms.

Sixteen different isoforms of UGT have been identified. Moreover, some UGT isoforms are polymorphic, which may affect the interindividual variability in the disposition of UGT substrates. Irinotecan [73–75] and acetaminophen are metabolized by isoforms in the UGT1A family, which is subject to genetic polymorphism and undergoes maturational changes. UGT1A1 is virtually undetectable in the fetus, and its activity increases immediately after birth, and adult levels are attained by 3–6 months of life [52]. The activity of UGT1A3 is approximately 30 % of adult levels in neonates. UGT1A6 (for which acetaminophen is a substrate) activity slowly increases after birth to 50 % of adult levels at 6 months and does not reach adult levels prior to 10 years of age. The implications of these developmental changes in the expression and activity of the UGT1A for the metabolism of irinotecan in children will be discussed later in this chapter. UGT2B7, which catalyzes the metabolism of morphine to morphine-6-glucuronide (100-fold more potent analgesic than morphine), has approximately 10–20 % of adult activity by 15–27 weeks of fetal life. Adult levels of UGT2B7 activity are reached by 2–30 months of life [76]. However, the complete ontogeny of all of the UGT isoforms has not yet been determined, so the full impact of the development of UGT on drug metabolism, especially anticancer drugs, is unknown.

The cytosolic GST isoforms, thought to be the most important for drug metabolism [77], are divided into six subfamilies: GSTA, GSTM, GSTO, GSTP, GSTT, and GSTZ. GSTA and GSTM are expressed at low levels in the fetal liver, and expression increases rapidly after birth [78]. The GSTP isoform expression is highest in early gestation and progressively decreases to very low levels in adults [78]. In addition to the liver, cytosolic GST isoforms are also expressed in the kidney and lung [79].

2.4 Effect of Development on Renal Excretion

Many anticancer drugs and their metabolites are excreted renally; thus, it is important to understand the effect of maturation on renal function. Renal clearance is dependent upon glomerular filtration, tubular secretion, and tubular reabsorption processes, which do not mature at the same rate. As with adults, anticancer drugs and supportive therapy (e.g., cisplatin, radiocontrast dye, aminoglycosides, or amphotericin B) can alter renal function. Also, the presence of disease (e.g., from high burden of leukemic blasts, renal obstruction) can impair renal function. Thus, the clinician must accurately measure renal function in the pediatric patient; however, accurate measurement of renal function in this population presents challenges.

At birth, the glomerular filtration rate (GFR) in full-term infants is 20–40 mL/min/1.73 m^2 [80, 81], and it increases rapidly during the early postnatal period. The reason for this increase is not nephrogenesis, which is complete by 36 weeks gestation [82]. Rather, the rapid change in GFR is due to increased renal blood flow; at birth, renal blood flow is only 5–6 % of cardiac output and reaches adult values of 15–25 % of cardiac output by one year of age [52]. After a rapid increase in GFR over the first 2 weeks of life, GFR rises steadily and approaches adult values at approximately 12 months of age [83]. Because lean body mass increases more rapidly than GFR after one year of life, serum creatinine gradually increases during childhood to reach the normal adult values of 1.0–1.5 mg/dL during adolescence [84].

Preterm infants show much lower GFR than full-term infants. Furthermore, preterm infants are slower to develop normal GFR secondary to incomplete nephrogenesis [84, 85]. Although such young infants are rarely treated for cancer, the clinician must consider not only postnatal age but also postconceptional age to determine appropriate dosages of renally excreted anticancer drugs in light of the development of GFR.

While a detailed discussion of all of the available methods for evaluation of renal function in children is beyond the scope of this chapter, the major advantages and disadvantages of some of the most commonly used methods are outlined in Table 1. Despite its limitations (e.g., cost and radioactivity), Tc-99m DTPA clearance is the best method for assessing glomerular filtration rate in children with cancer [89]. Recently, the use of cystatin C (CysC) has been proposed as a replacement for estimating GFR based on serum creatinine (SCr), since serum CysC levels are not as dependent on age, gender, and muscle mass. In a group of 536 children aged 1–18, estimation of GFR with CysC showed less bias than estimation based on SCr [91]. Another study of 80 pediatric cancer patients indicated that CysC may be superior to SCr particularly for estimating GFR in young children less than 3 years of age [92].

In an uncomplicated pediatric patient, the clinician might depend upon a paradigm of renal developmental physiology to predict the capacity to eliminate a renally excreted drug. However, in a child with altered renal function due to multiple factors (e.g., maturational changes, malignancy, anticancer drugs, supportive care drugs, sepsis), the clinician cannot rely solely on this paradigm and must measure renal function. Although it is recommended that Tc-99m DTPA clearance be determined, in those institutions not able to perform these studies, the clinician must utilize the best available method to assess renal function when dosing renally excreted drugs in children with altered renal function.

Table 1 Methods to evaluate renal function in children with cancer

Method	Advantages	Disadvantages
Nomogram (e.g., Schwartz: plasma creatinine) [86, 87]	1. Avoids urine collection 2. Rapid 3. Robust for children of normal body habitus [88] 4. Relatively inexpensive	1. Maternal creatinine present in neonates 2. Reflects GFR only under steady-state conditions 3. Clinician must recall many proportionality constants for specific demographics 4. Invasive sampling required
24-hour urine collection for creatinine clearance	1. Accurate if entire urine volume in 24 h can be collected and quantified 2. Noninvasive 3. Relatively inexpensive	1. Difficult to collect and quantify 2. Impractical to measure urine volume accurately for young child (e.g.. diapers) 3. Time delay (at least a 24-h turnaround time from initiation of test)
Cystatin C	1. Avoids urine collection 2. Rapid 3. More accurate than serum creatinine	1. More expensive than serum creatinine 2. Invasive sampling required
Radionuclide clearance (^{99m}Tc-DTPA or ^{51}Cr-EDTA)	1. Sensitive marker of GFR even in the presence of impaired renal function [89] 2. Rapid 3. Avoids urine collection	1. Expensive 2. Requires administration of radiopharmaceutical 3. Invasive sampling required

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Renal tubules are important for active secretion and active and passive reabsorption. Development of tubular secretion, which is immature at birth, lags behind that of glomerular filtration [52]. In particular, tubular secretion of organic anions is only about 20–30 % that of adults at birth and reach adult levels at 7–8 months of age [93]. Little is known about the ontogeny of individual transporters expressed in human renal tubules.

3 Methods for Dosing Anticancer Drugs in Pediatrics

Several reviews have recently addressed the question of the selection of the appropriate method to dose anticancer drugs [94–97]. Many of the issues regarding dosing anticancer drugs in adults that were addressed in these reviews may be relevant in children, although many unique challenges exist to dosing drugs in children. It is common practice to normalize drug doses to a measure of body size (e.g., total body weight, BSA) with the intention of reducing interindividual variability in drug systemic exposure and thus response. A potential source of variability in calculation of drug doses in children arises from the different methods used to BSA. Although the initial approaches to measuring BSA utilized direct measurement techniques, current practices rely instead upon nomograms developed from early studies. In 1916, the DuBois formula for calculation of BSA was developed by measuring the BSA of nine subjects (including only one child) and conducting regression analysis of the known height and weight of subjects to yield $\mathrm{BSA} = W^{0.425} \times H^{0.725} \times 71$ [98]. Height (or supine length) in centimeters and weight in kilograms are the major components of the most frequently used formulas for calculating BSA. Therefore, one of the primary determinants of accurate assessment of BSA is accurate measurement of height and weight, which can be difficult to obtain in children. Calculation of BSA from regression is further limited by the potential for either over- or underestimation of true BSA in a proportion of the population.

BSA was selected for its perceived correlation with organ function associated with drug elimination, although these assertions are not supported by current evidence [99, 100]. In adults, BSA has correlated with a pharmacokinetic parameter for only a limited number of drugs [97, 99, 101, 102].

Although liver volume measured by MRI in children with cancer correlated with BSA [48], clearance of hepatically metabolized drugs does not necessarily correlate well [94, 101]. Furthermore, creatinine clearance has not been shown to correlate with BSA. Lean body weight correlates more strongly than BSA with physiological volumes. Infants have proportionately higher BSA to weight than older children, so drug dosages based on BSA rather than weight may be too large for infants. BSA-normalized doxorubicin clearance was significantly greater in children >2 years old than children <2 years old; however, no statistically significant difference was observed in weight-normalized doxorubicin clearance [103]. In order to minimize anthracycline-induced cardiotoxicity, it has been suggested either to use lower mg/m^2 doses of doxorubicin in young children or to dose doxorubicin in young children in terms of mg/kg, but no further studies have been published to validate this recommendation. Discrepancies in age-related changes in etoposide clearance have also been observed, depending on whether clearance is BSA normalized or weight normalized. These are reviewed below in Sect. 5.4. In addition, specific dosing recommendations based upon body size will be discussed for individual drugs later in this chapter.

We expect that dosing recommendations based on patient genotype will increase as our knowledge of pharmacogenetics expands, and prospective genotyping of patients is incorporated into clinical trials. However, clinicians should be aware of the source of data leading to these dosing recommendations, since genotype–phenotype relations confirmed in adults may not be relevant to pediatric populations. For example, the *UGT1A1*28* polymorphism has been used as a marker to adjust the dosage of irinotecan to reduce toxicity in patients with the 7/7 genotype. However, this polymorphism was not associated with toxicity in children, presumably because of the different regimen (i.e., lower dosage and protracted dosing schedule) of irinotecan that children commonly receive [104].

4 Practical Issues for Clinical Pharmacology Studies in Children with Cancer

4.1 Blood Collection

In addition to the effect of maturation leading to differences between children and adults in drug disposition, the clinician must also consider the practical aspects of pharmacokinetic studies conducted in children. Often children will have indwelling venous access for administration of anticancer drugs; however, this site may not be practical for obtaining pharmacokinetic samples because of the need for repeated use of the line and infection concerns, excessive blood loss due to flushing of the line, and potential for drug adsorption, which might lead to falsely high plasma concentrations. Therefore, children enrolled on pharmacokinetic studies often need two separate lines: a central line from which blood is drawn and a peripheral line where the anticancer agent is administered. Another potential option for the patient with a double-lumen Hickman or Broviac central line is to use the same line for sample administration and sample acquisition; however, this must be done with appropriate attention to catheter care (e.g., proper flushing and nursing care) and careful attention to the results of the pharmacokinetic study to determine if adsorption has occurred.

As noted above, children have a smaller intravascular volume than adults, which places a practical limit on the volume of blood that can be safely collected from each subject for pharmacokinetic studies. Currently, no formal recommendation is available for the amount of blood that can be obtained from children for pharmacokinetic studies. However, the FDA has published guidelines for industry which state that the volume of blood withdrawn should be “minimized” in pediatric studies and institutional review boards/independent ethics committees should review and may define the maximum amount of blood (usually on a milliliters (mL)/kg or percentage of total blood volume basis) that may be used for

pharmacokinetic studies. Some institutions use 5–7 % of total blood volume in 24 h or 3–5 mL blood/kg over 8 weeks as a guideline for the maximum amount of blood that can be withdrawn from a child for pharmacokinetic studies. Selection of the most informative sample times for pharmacokinetic studies in children is critical. However, even with appropriate selection of plasma sample times for pharmacokinetic studies, the clinician should consider that for the individual child, blood will be collected not only for pharmacokinetic studies but also for routine labs including serum chemistries, complete blood counts, bacterial cultures, and blood gases. In neonates and infants, even the discard volume from routine blood draws may be significant. Therefore, the clinician must coordinate pharmacokinetic studies with the clinical pharmacologist.

4.2 Population Pharmacokinetics and Limited Sampling

Population pharmacokinetic analysis, which employs nonlinear mixed-effects modeling, has been proposed as a method to determine optimal drug dosing in children [8, 105–107]. The decreased dependence on dense blood sampling and flexibility of collection times makes this approach ideal for pediatrics. Population pharmacokinetics can be used to identify relevant patient covariates, including metrics of body size, developmental processes, genetic polymorphisms, and concomitant medications. These correlations between patient-specific parameters and pharmacokinetic parameters can be useful to refine dosage selection as exemplified by carboplatin dosing based upon a patient's renal function and desired carboplatin systemic exposure. This approach can be extended to population pharmacokinetic–pharmacodynamic analysis, which is particularly useful for when the relationship between drug concentrations and drug response is not defined or may be different in children than in adults.

Limited sampling models can help maximize the information obtained from pharmacokinetic studies in children with cancer. By analysis of concentration–time profiles collected from serial plasma samples, a limited sampling model can be derived that will minimize the number of plasma samples necessary for a pharmacokinetic study yet maximize the information yielded from those samples [108]. A limited sampling strategy combined with Bayesian parameter estimation to adjust topotecan dosage to obtain a desired plasma topotecan lactone exposure has been used [109, 110]. Thus, with the application of these techniques, the clinician can derive much information from a child with cancer regarding the clinical pharmacology of a drug while remaining attentive to the volume of blood taken from the patient.

5 Pharmacokinetics of Specific Anticancer Drugs in Pediatrics

5.1 Methotrexate

Methotrexate competitively inhibits dihydrofolate reductase (DHFR), the enzyme responsible for converting folic acid to reduced folate cofactors, and interferes with several critical biosynthetic pathways, including DNA and RNA synthesis. Methotrexate is FDA approved for use in the treatment of acute lymphocytic leukemia (ALL) and osteosarcoma in adults and children. Furthermore, methotrexate is used extensively in both adults and children for nonmalignant diseases such as rheumatoid arthritis. New regimens containing methotrexate are currently being evaluated in pediatric clinical trials for the treatment of ALL, osteosarcoma, and brain tumors. Pharmacokinetic monitoring of patients receiving methotrexate has been shown to reduce morbidity and mortality associated with this drug [9, 111].

The volume of distribution of methotrexate is approximately 0.18 L/kg [112]; however, its steady-state volume of distribution ranges from 0.4 to 0.8 L/kg [113–117]. Methotrexate is approximately 50 % bound to albumin in plasma [118]. Methotrexate distributes into pleural fluid, ascites, and pseudocysts. Even though concentrations in these "third spaces" may be low compared to maximum serum concentrations, the movement of methotrexate from third spaces into the central compartment is slow, leading to a prolonged systemic clearance and potentially increased toxicity [119–121]. Physiologically based pharmacokinetic modeling (PBPK) has shown that the third space has the greatest effect on clearance when the fluid volume is large, the protein binding in the effusion fluid is greater than in plasma, and the rate of transfer from the effusion fluid to the central compartment is slow [122]. Thus, the presence of ascites, pleural effusions, and pseudocysts should be documented so that a sufficient duration of leucovorin rescue can be provided.

Methotrexate CSF concentrations range about 1.5–2.5 % of serum concentrations after a 24-h infusion [123–125]. However, if higher dosages are used ($\geq$5 g/m^2 over 24 h), it is possible to achieve cytotoxic concentrations ($\geq$0.01 μM) despite poor distribution into CSF [123–125]. To attain putatively cytotoxic CSF concentrations, methotrexate has been administered via the intrathecal route. Intrathecal dosage regimens based on BSA underdose children less than eight years old because they have larger CSF volumes relative to their BSA [126]. Due to these age-related changes in CSF volume, it has been suggested that intrathecal methotrexate dose should be based on age [127].

Metabolism is a minor route of elimination of methotrexate. The most abundant metabolite in plasma, 7-hydroxymethotrexate, is found in low concentrations (0.1–0.3 μM) and is 40–200 times less active than methotrexate against DHFR [128]. However, 7-hydroxymethotrexate is less soluble at physiological pH and may contribute to renal toxicity through precipitation in renal tubules [129]. The formation of 7-hydroxymethotrexate is mediated by aldehyde oxidase [130], an enzyme with unknown ontogeny. Also in both adults and children, folyl polyglutamate synthetase metabolizes intracellular methotrexate to methotrexate polyglutamates [131], which are more toxic to cells than methotrexate, because the polyglutamates are retained by cells for longer periods of time than methotrexate [132]. Methotrexate polyglutamates are at least as active as methotrexate and, therefore, have a major role in the cytotoxicity of methotrexate.

Renal excretion of methotrexate is the primary route of methotrexate elimination, with ~80 % excreted unchanged in the urine in 24 h through renal tubular secretion and glomerular filtration [128]. Less than 10 % of a dose is excreted as the 7-hydroxymethotrexate metabolite in the urine [133, 134]; however, this has been shown to increase when methotrexate is administered as a prolonged infusion [113]. Methotrexate is also excreted into the bile, however, to a much smaller extent (~10 %) than other routes of elimination. Although biliary excretion is not a major route of elimination, methotrexate can be reabsorbed from the gastrointestinal tract, which can prolong its terminal half-life [135]. Based on preclinical studies, several transporters appear to have important roles in eliminating methotrexate and 7-hydroxymethotrexate, including BCRP, MRP2, and MRP3 [136–138]. Furthermore, polymorphisms in the organic anion transporter polypeptide SLCO1B1 were associated with methotrexate pharmacokinetics [139].

Methotrexate systemic clearance appears to be dependent on age. Infants have a lower clearance than older children, which may reflect the maturation of glomerular filtration [140, 141]. On the other hand, methotrexate clearance is reported to be higher in children less than 10 compared to older children and adults [142–148] and decreases with age [149, 150]. However, this can be due to a variety of factors that can alter the disposition of methotrexate such as renal function, pleural effusion, and GI obstruction.

Hydration and urinary alkalinization can increase the renal clearance of methotrexate [151]. It is therefore recommended that patients are well hydrated with intravenous fluids and treated with sodium bicarbonate to alkalinize the urine prior to high-dose methotrexate therapy (i.e., IV fluid (with 40 mEq $NaHCO_3$/L) administered at the rate of 200 mL/m^2/h for at least 2 h before the start of high-dose methotrexate. Urinary pH should also be monitored during infusion and additional sodium bicarbonate therapy be administered if pH is less than or equal to 6.0. Acetazolamide may be used if systemic alkalosis limits the administration of bicarbonate for urinary alkalinization.

5.2 Oxazaphosphorines (Cyclophosphamide and Ifosfamide)

The oxazaphosphorine alkylating agents cyclophosphamide and ifosfamide are prodrugs that are activated by 4-hydroxylation via the CYP450 system to 4-hydroxycyclophosphamide and 4-hydroxyifosfamide, respectively. Cyclophosphamide is approved for acute nonlymphocytic leukemia (ANLL), acute lymphocytic leukemia, neuroblastoma, retinoblastoma, and Wilms' tumor. However, cyclophosphamide is also used in bone marrow transplantation, childhood germ cell tumors, Ewing's sarcoma, osteosarcoma, and rhabdomyosarcoma. Ifosfamide is used in Hodgkin's lymphoma, non-Hodgkin's lymphoma, Ewing's sarcoma, osteosarcoma, rhabdomyosarcoma, and Wilms' tumor.

Low-dose oral cyclophosphamide is being examined as maintenance therapy in several pediatric clinical trials [12, 14]. Bioavailability is 85 % in adults [152] but has not been examined in children. Ifosfamide and 4-hydroxyifosfamide CSF concentrations were almost as high as plasma concentrations in children [153], whereas cyclophosphamide concentrations were 20 % of plasma concentrations, with high interpatient variability for both drugs [154]. No effect of age was seen on CSF concentrations of either drug [154].

Metabolic deactivation of ifosfamide to 2- and 3-dechloroethylifosfamide by CYP3A4 and CYP2B6 also yields an equimolar amount of chloroacetaldehyde, which has been implicated in the neurotoxicity associated with ifosfamide [155, 156]. Coadministration of ketoconazole with ifosfamide reduced the formation of the active metabolite 4-hydroxyifosfamide, which may lead to reduced antitumor efficacy. Coadministration of rifampin with ifosfamide increased the metabolism of ifosfamide to both the active metabolite and the two inactive metabolites 2- and 3-dechloroethylifosfamide, as well as the neurotoxic by-product chloroacetaldehyde. Thus, administration of either the CYP inhibitor ketoconazole or the CYP inducer rifampin can reduce the therapeutic benefit of ifosfamide [156].

Wide interpatient variability has been reported in the disposition of cyclophosphamide in children [157]. Cyclophosphamide systemic clearance varied from 1.2 to 10.6 L/h/m^2. The half-life varied from 1.1 to 16.8 h and was significantly prolonged at high dosage levels. In a more recent study, Yule et al. have demonstrated that fluconazole inhibits the first step in the activation of cyclophosphamide in vitro [158]. Moreover, in a group of 22 children ranging in age from 2 months to 18 years, they have demonstrated a statistically significant reduction in clearance and increase in half-life of cyclophosphamide when fluconazole is coadministered.

Cyclophosphamide clearance is greater in children than in adults [157, 159, 160]. This may be due to relatively higher CYP3A activity [52]. However, the pharmacokinetics of cyclophosphamide have not been adequately studied in children less than 1 year of age, who do not have mature CYP2C and CYP3A function. 4-Hydroxycyclophosphamide is metabolized by aldehyde dehydrogenase [161] and glutathione S-transferase isoforms [162]. Thus, the ontogeny of these enzymes, which are not well studied, may also contribute to age-related changes in the disposition of 4-hydroxycyclophosphpamide. Therefore, not only should the clinician be aware of specific CYP450 drug interactions with cyclophosphamide and ifosfamide, but the clinician should also anticipate different patterns of metabolism of these drugs in children of various ages. The pharmacogenetics of cyclophosphamide have recently been reviewed [163], although few studies have been performed in children.

5.3 Vincristine

Vincristine is a naturally occurring alkaloid, which exerts its cytotoxic effects by binding to tubulin, thus stopping mitosis and leading to apoptosis. The FDA has approved vincristine as part of a combination regimen to treat a variety of adult and pediatric malignancies, including acute lymphocytic leukemia (ALL), Hodgkin's disease, and Wilms' tumor. Because it lacks myelosuppression as a

dose-limiting toxicity, vincristine is an attractive anticancer agent for use in combination with other myelosuppressive agents. In general, vincristine pharmacokinetics are associated with high intra- and interindividual variability in children; however, intraindividual variability is usually smaller than interindividual variability [164, 165]. The importance of vincristine disposition was demonstrated by Lönnerholm et al., who showed that standard risk ALL patients with lower vincristine exposures had a higher risk of relapse [166].

Vincristine is administered as an intravenous infusion or bolus dose due to poor oral absorption. Gidding et al. demonstrated that the vincristine volume of distribution at steady state is highly variable in children with ALL, non-Hodgkin's lymphoma, or Wilms' tumor (range, 56–1,165 L/m^2) [164]. Within 15–30 min after intravenous administration, vincristine is rapidly distributed, and greater than 90 % distributes from the blood to the tissue where it is tightly (but not irreversibly) bound [167]. Although CSF penetration of vincristine is poor [168], intrathecal administration is strictly contraindicated because of associated lethality.

Screening of a library of cDNA-expressed CYP450 enzymes showed that only CYP3A4 and CYP3A5 significantly metabolized vincristine. The major metabolite (M1) formed by the CYP3A family was due to the oxidative cleavage of the piperidine ring [169]. M1 was also the major metabolite formed by human liver microsomes and human hepatocytes [170, 171]. Metabolism by CYP3A4 reduced vincristine cytotoxicity, indicating that this metabolite has little activity [172]. CYP3A5 had 9–14-fold higher intrinsic clearance in forming the major metabolite than CYP3A4 [169]. The rate of vincristine metabolism was related to CYP3A5 expression in both human liver microsomes and hepatocytes [170, 171]. Because 70 % of African Americans are CYP3A5 expressers, compared to 20 % of Caucasians, Renbarger et al. compared vincristine-associated neurotoxicity in pediatric patients with ALL [173]. They found that African Americans experienced less neurotoxicity (4.8 % vs. 34.8 % of patients), had less severe neurotoxicity (average NIH Common Terminology Criteria for Adverse Events grade of 1 vs. 2.72), and had fewer dose interruptions and reductions.

Preclinical models show that biliary excretion of unchanged vincristine may be an important route of elimination [174–176]. Experiments with inhibitors and inducers of P-gp showed that this transporter may mediate the efflux of vincristine into the bile [174–176]. P-gp was also involved in the renal elimination of vincristine in the mouse and dog [174, 177]. The relation between vincristine disposition in humans and measures of biliary function have been investigated. Two reports [165, 178] showed that vincristine clearance was inversely related to alkaline phosphatase and gamma-glutamyltransferase, although these enzymes are not specific to biliary function. Other investigators have seen no correlation between vincristine clearance and total bilirubin, alkaline phosphatase, or albumin level [164].

There is evidence for age-related changes in vincristine clearance in infants. Two studies found that vincristine clearance is reduced in patients less than 1 year of age compared to older patients [164, 167]. Woods et al. demonstrated a high incidence of neurotoxicity in infants smaller than 0.5 m^2 and recommended that children with a BSA of less than 1 m^2 receive dosages based on body weight [179]. Delayed clearance of vincristine and the large ratio of body surface area to weight in infants could cause the observed increase in toxicity. In children older than 1 year, studies have shown either a very weak relationship or no relationship to age [164, 167, 179, 180]. Nonetheless, vincristine clearance appears to be several fold higher in children with ALL than adults with lung cancer, regardless of whether or not they received CYP450-inducing drugs [167, 181].

The influence of genetic polymorphisms on in vivo vincristine clearance and outcome is not yet clear. *CYP3A5* genotype was related to the in vitro rate of vincristine metabolism in human liver microsomes. Based on the *CYP3A5*3*, **6*, and **7* genotypes, predicted high CYP3A5 expressers had a fivefold higher median estimated hepatic clearance than low CYP3A5 expressers [170]. However, *CYP3A* polymorphisms were not associated to neuropathy-related impaired motor performance in a study of 34 children with ALL [182]. Similarly, polymorphisms in *ABCB1* have been shown to affect transport of vincristine in vitro [183], but little influence of *ABCB1* polymorphisms has been seen

clinically. In a study of 52 childhood ALL patients, there was an association between *ABCB1* 3435/2577 haplotype and the elimination half-life of vincristine, but there was no association with vincristine exposure, indicating limited relevance [184].

Drug–drug interactions contribute to the interindividual variability seen with vincristine pharmacokinetics in children and adults. Drug–drug interactions with vincristine may be mediated by induction or inhibition of CYP3A isoforms or P-gp [185, 186]. The clinically important drug interactions with vincristine have been recently reviewed and include azole antifungals, cyclosporine, corticosteroids, and CYP450-inducing anticonvulsants [187]. While it is unknown whether the induction of the clearance of the vinca alkaloids has a clinically significant consequence (i.e., reduction in efficacy or toxicity), inhibition of vincristine clearance has been shown to augment toxicity [185].

5.4 Etoposide

Etoposide, a topoisomerase II inhibitor that can be administered orally or intravenously, is used in the treatment of children with acute lymphocytic leukemia, acute myelogenous leukemia, acute nonlymphocytic leukemias, Ewing's sarcoma, non-Hodgkin's lymphoma, neuroblastoma, retinoblastoma, rhabdomyosarcoma, and Wilms' tumor. High-dose etoposide is also used in conditioning regimens for bone marrow transplantation.

Chen et al. determined that the mean (range) bioavailability of etoposide in 12 children with relapsed ALL is 60.6 % (17.6–91.2 %) [188]. By evaluating the disposition of oral etoposide in 16 patients ranging in age from 3 to 73 years in comparison to intravenous etoposide in 18 different children ranging in age from 0.8 to 17 years, Wurthwein et al. determined a similar mean apparent bioavailability of 59 % [189]. The results from preclinical models of P-gp- and Mrp2-deficient mice [190] and rats treated with verapamil [191] indicate that these transporters limit oral absorption of etoposide.

Etoposide is extensively plasma protein bound (90–94 %) [192]. However, high inter- and intraindividual variability in etoposide protein binding has been observed [193]. Although only 0.3 % of the plasma etoposide concentration is reached in the CSF in children with ALL, it is thought that even these low levels of etoposide can be cytotoxic to leukemic blasts, depending on the inherent sensitivity of each individual child's disease to etoposide [194]. The ratio of etoposide CSF and plasma concentrations in children with ALL is comparable to those observed in adults with brain tumors without tumor invasion of the ventricles or subarachnoid space (0.7 %) [195]. Therefore, it is unlikely that age-related changes exist in the penetration of etoposide into the CSF; changes in CSF penetration are more likely due to the effects of specific disease (e.g., disruption of the blood–brain barrier).

The metabolism of etoposide has been summarized by Yang et al. [196]. In vitro, CYP3A4 catalyzes 3′-demethylation of etoposide, with minor contributions from CYP1A2 and CYP2E1 [197]. CYP3A4 also catalyzes the in vitro O-demethylation of etoposide to the catechol metabolite, with a lesser contribution from CYP3A5 [145–147]. Etoposide administration could slightly induce CYP3A4 protein levels [198], and formation of the catechol metabolite increases over multiple days of etoposide dosing [199]. In human liver microsomes, 40-fold variability in the formation of the catechol metabolite was observed, which may account for some of the interindividual variation in the disposition of etoposide [200].

Wurthwein et al. have reported a population pharmacokinetic model of the disposition of high-dose etoposide in children ranging in age from 9.6 months to 23.7 years [193]. The concentration–time data were best fit by a three-compartment model with a clearance of 15.5 mL/min/m^2 and a terminal half-life of 44.2 h. This clearance value is similar to several other reports [201–203]. Patients with a very low or very high body mass index (BMI) had etoposide clearances similar to patients with a normal BMI [204], even in a morbidly obese patient [205]. Administration based on BSA has been

recommended due to decreased variability in etoposide exposure compared to dosing based on body weight [206].

In eight children ranging in age from 4.2 to 22 years with refractory solid tumors, 45 % of the parent drug was excreted unchanged in the urine [203]. This is in the range of 30–70 % reported for adults [207]. The etoposide elimination rate was significantly decreased in children with renal impairment [208]. An inverse relationship was observed between serum albumin levels and etoposide renal clearance in children, which suggests that changes in albumin concentration might influence the amount of free drug available for renal clearance [203]. Based on a study of two children with external biliary drainage, biliary excretion is a minor route of etoposide elimination, with less than 2 % of the dose excreted into the bile [209]. Preclinical models show that P-gp is important for etoposide elimination [190], but presumably P-gp contributes through renal elimination and not biliary elimination. The *ABCB1* 3435C>T genotype correlated with etoposide clearance in children with ALL [210]. Polymorphisms in CYP3A5, GSTP1, UGT1A1, and the vitamin D receptor also correlated with etoposide clearance, but only in African Americans [210].

Age-related changes in the disposition of etoposide have not been clearly established. A negative correlation between BSA-normalized clearance and age was reported by Sonnichsen et al., but this was based on oral dosing with systemic clearance calculated by fixing the bioavailability term [211]. A similar negative correlation was also seen in children with ALL with age ranging from 0.4 to 18.7 years [210]. Interestingly, no correlation was seen in the same population when patients were receiving prednisolone. Although a negative correlation was seen between age and etoposide clearance normalized to body weight in a study of children aged 9.6 months to 23.7 years, no correlation was seen when clearance was normalized to BSA [193]. Similarly, another study showed that AUC, dose normalized by BSA, was independent of age, but when normalized to body weight was directly correlated with age [206]. Another study showed no correlation of etoposide clearance with age in a population of 78 children, whether adjusted to weight or BSA [212]. Infants less than one year of age seem to have similar clearance values to older children [201–203, 212, 213], although these populations did not include very young infants.

Etoposide is subject to a number of drug–drug interactions in children. Clearance of etoposide was 47.4 mL/min/m^2 when pediatric ALL patients were receiving prednisolone, which induces CYP3A enzymes and P-gp, compared to 29.2 mL/min/m^2 without prednisolone [210]. Cyclosporine increased the exposure to etoposide approximately twofold [214, 215] and increased the rate of toxicities in patients who did not receive an etoposide dose reduction [214]. It is recommended that patients administered cyclosporine receive half the etoposide dose. In 17 children with neuroblastoma, administration of etoposide 2 days after cisplatin treatment resulted in a 31–36 % increase in etoposide exposure compared to administration of etoposide 21 days after cisplatin treatment, with a concomitant increase in toxicity [145–147]. Children with solid tumors on enzyme-inducing anticonvulsant therapy and receiving high-dose etoposide had a mean etoposide clearance of 23.7 mL/min/m^2 compared to 13.4 mL/min/m^2 for patients not on anticonvulsants [202].

5.5 Topotecan

Topotecan, a water-soluble camptothecin analog, is a topoisomerase I inhibitor. Topotecan undergoes a reversible, pH-dependent conversion from lactone form to carboxylate form at physiological pH [216–218]. Topotecan has been approved by the FDA as second-line treatment of small-cell lung cancer and ovarian cancer in adults; however, it has been shown to be effective in a variety of pediatric solid tumors and leukemias. Topotecan has been evaluated as a single agent or in combination in clinical trials in children to treat neuroblastoma [219], medulloblastoma [109], glioblastoma [220], ALL [221], rhabdomyosarcoma [222], Wilms' tumor [223], and retinoblastoma [224].

The oral bioavailability of the intravenous solution of topotecan in children is approximately 30 % [225], which is similar to that reported in adults [226]. After oral administration, topotecan peak lactone plasma concentrations occurred from 0.75 to 2 h. Large interpatient variability was noted in oral topotecan pharmacokinetics, whereas intrapatient variability was relatively small. Preclinical models show that oral absorption of topotecan is limited by BCRP [227], and patients heterozygous for the ABCG2 421C>A polymorphism, which reduces efflux activity, had 1.34 higher oral bioavailability than patients with the wild-type allele [228]. Inhibition of transporters with elacridar increased topotecan bioavailability to approximately 100 % and reduced interpatient variability in absorption [229].

The topotecan volume of distribution (Vd_{ss}) has shown high interindividual variability. In adults, it is as large as 160 L; however, in children, it has been reported to be 73 ± 27 L/m^2 [230] and 97 ± 116 L/m^2 [231]. Plasma protein binding of topotecan is 7–35 % in adults and children [232], which is reasonably low. Alterations in plasma proteins in children, whether age related or disease related, will likely not alter topotecan disposition or pharmacologic effect. A study in adults showed that topotecan also distributes into third spaces and pleural effusions without sequestration [233]. Although distribution into third spaces did not appear to influence topotecan disposition, inadequate data was presented to fully evaluate this conclusion. Cytotoxic concentrations of topotecan can be achieved in the CSF [234], and the CSF exposure is dependent on the length of topotecan infusion [235]. A model has been developed to estimate ventricular CSF topotecan lactone concentrations based on plasma concentrations [236, 237]. Distribution of topotecan into the brain parenchyma and CSF is controlled by P-gp, BCRP, and MRP4 [238–240]. Coadministration of gefitinib, which inhibits these transporters, increases brain penetration of topotecan in mice [241]. Intrathecal administration of topotecan is currently being explored [242, 243].

Topotecan is metabolized by the CYP450 enzyme system to *N*-desmethyl topotecan, which has peak concentrations in plasma that are only 0.7 % of parent topotecan [244]. Also, both topotecan and *N*-desmethyl topotecan have been found to exist as an *O*-glucuronide [245, 246]. However, topotecan primarily undergoes renal elimination with 49–70 % of a dose recovered in the urine as unchanged drug [247].

The renal excretion of topotecan includes both glomerular filtration and tubular secretion [248]. A study of adults receiving topotecan demonstrated that patients with impaired glomerular filtration, as measured by creatinine clearance, had decreased topotecan renal clearance. Thus, it is recommended that adult patients with creatinine clearance <39 mL/min receive topotecan at reduced dosages. Similarly, topotecan clearance was only 5.3 L/h/m^2 in a child with severe renal dysfunction [249] although topotecan clearance was 15.5 L/h/m^2 in an anephric child [250]. When compared with an age-, SCr-, and BSA-matched cohort of patients, the topotecan clearance was not statistically different, although the Wilms' tumor patients had significantly reduced GFR, as measured by Tc-99m DTPA clearance. It is likely that other mechanisms (i.e., increase in drug transporters, other unspecified metabolic pathways) may have compensated for the anticipated decrease in topotecan renal clearance. Therefore, based upon these data, it is not recommended that topotecan dosage be altered in children with decreased renal function as measured by Tc-99m DTPA clearance.

Studies of pharmacokinetically guided topotecan dosing to attain discrete target area under the concentration–time curves (AUC) have been conducted in multiple populations of children with cancer. Thus far, pharmacokinetically guided dosing has been successfully applied to reduce interindividual variability in topotecan lactone systemic exposures [109, 251]. A population pharmacokinetic analysis of topotecan in 162 children showed that BSA, GFR, phenytoin coadministration, and age were related to topotecan clearance [252]. Consistent with renal development, patients less than 6 months of age had decreased topotecan clearance compared to patients older than 6 months of age, although the number of infants was too small to fully define age-related changes in topotecan disposition. A study in adults showed serum cystatin C to be a better predictor of topotecan clearance than serum creatinine [253], although this has not been validated in children.

5.6 *Irinotecan*

Irinotecan also inhibits topoisomerase I and is a prodrug that must be activated by carboxylesterase to form SN-38, the active moiety of irinotecan. Similar to topotecan, irinotecan undergoes reversible pH-dependent hydrolysis from lactone to carboxylate at physiological pH. The FDA has approved irinotecan for use in adults with colorectal cancer. Although irinotecan has not yet been approved for pediatric use, phase I trials in children have shown that irinotecan is active in a variety of solid tumors [254, 255].

Although irinotecan is only 12 % bioavailable in children (2–19 years) with solid tumors, the ratio of SN-38 to irinotecan AUC is greater for oral administration (0.76 ± 1.43) than for intravenous administration (0.10 ± 0.06), suggesting that SN-38 is formed presystemically after oral administration of irinotecan [256]. Coadministration of the tyrosine kinase inhibitor gefitinib, which inhibits the activity of several ABC transporters, increased the bioavailability of oral irinotecan to 42 % and reduced the apparent clearance of irinotecan and SN-38 by ~37 %, leading to an increase in SN-38 systemic exposure [257].

SN-38 is highly protein bound. Children with serum albumin within normal limits (3.0–4.4 g/dL) exhibited approximately 3.4 % unbound SN-38 (range, 1.4–6.5 %) [255]. Thus, the clinician must be aware of possible interactions between irinotecan and other highly protein-bound drugs and pathophysiological changes that can alter a child's serum albumin.

Irinotecan is rapidly metabolized by carboxylesterase to form the active metabolite SN-38. Irinotecan is also metabolized by CYP3A to 7-ethyl-10-[4-(1-piperidino)-1-amino]-carbonyloxycamptothecin or NPC [258] and 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino] carbonyloxycamptothecin or APC [259]. NPC can be subsequently converted by carboxylesterase to SN-38, whereas APC cannot [260]. SN-38 can be metabolized by both hepatic UGT [261] and CYP450 isoforms [258, 259]. The importance of CYP3A activity was demonstrated in adults, where dosing patients according to their CYP3A phenotype, determined by midazolam clearance, reduced variability in irinotecan and SN-38 exposure and resulted in a fourfold reduction in the occurrence of severe neutropenia [262].

SN-38 is glucuronidated by the UGT1 family. Ciotti et al. [73] have demonstrated that COS-1 cells transfected with UGT1A7 were 12–40-fold more active in the conjugation of SN-38 to glucuronide than nine other isoforms of the UGT1A family, including UGT1A1, which had been previously demonstrated to be the predominant isoform responsible for this reaction [75]. In addition, Hanioka et al. have demonstrated that recombinant human UGT1A3, UGT1A6, and UGT1A9 expressed in microsomes of insect cells catalyze the glucuronidation of SN-38 [74]. Because the expression of the UGT1A family increases as a child matures [52], young children may be subject to an increased risk of SN-38 toxicity until the expression of UGT1A isoforms reaches adult levels.

Bacterial colonization of the gastrointestinal tract has implications for irinotecan disposition and toxicity, since bacterial-expressed β-glucuronidase can cleave SN-38 glucuronide to parent SN-38, which will increase large intestine SN-38 exposure and increase the risk of diarrhea [263]. In theory, very young children in whom GI flora is not fully established would be less susceptible than adults or older children to irinotecan-induced diarrhea due to a relative lack of bacterial β-glucuronidase. The use of cephalosporin antibiotics is now routinely used to reduce β-glucuronidase levels. Administration of cefixime beginning 5 days prior to irinotecan therapy in children with solid tumors allowed dose escalation of daily oral irinotecan from 40 to 60 mg/m^2 [256].

Although the FDA suggests that the *UGT1A1*28* genotype be used to adjust the irinotecan dosage to avoid toxicity in adults, no relation was seen between the *UGT1A1*28* genotype and either neutropenia or diarrhea in children, presumably because of the low-dose, protracted schedule that children receive [104]. Polymorphisms in the transporter genes *SLCO1B1* and *ABCC1* also correlated with neutropenia in adults [264]. These findings have not been confirmed in children.

The clearance of irinotecan lactone in children is approximately 55.5 L/h/m^2 [265], which is similar to that observed in adults [266, 267]. A population analysis of 82 children aged 1–21 years showed

that SN-38 clearance was dependent on age, as well as bilirubin levels. SN-38 clearance was greater in patients less than 10 years of age [268].

Patients who received enzyme-inducing anticonvulsants (EIAs) exhibited greater clearance of irinotecan lactone and lower SN-38 lactone AUC values than those who did not receive EIAs [265]. Because those who received EIAs did not have a significantly greater median AUC of APC than those who did not receive EIAs, induction of CYP3A4 may not explain the alteration in the pharmacokinetics of irinotecan in the presence of EIAs. It is possible that EIAs may increase the biliary excretion of irinotecan through induction of ABC transporters. When possible, EIAs should be avoided in children receiving irinotecan; if anticonvulsants are required, the newer nonenzyme-inducing anticonvulsants should be considered [265]. Intrapatient dosage escalation for pediatric patients receiving EIAs has also been explored [269]. A study of three patients indicated that SN-38 exposure is significantly decreased when irinotecan is coadministered with ifosfamide; however, no drug interaction with temozolomide was noted [270, 271].

5.7 Temozolomide

Temozolomide is a methylating agent that is approved for treatment of newly diagnosed glioblastoma multiforme and refractory anaplastic astrocytoma in adults and is undergoing evaluation for use in pediatrics to treat brain tumors such as high-grade gliomas [272, 273] and sarcomas [274]. Temozolomide undergoes spontaneous base-catalyzed hydrolysis to form the methyl triazene, MTIC, which is the active methylating species [275, 276].

Similar to adults, absorption of temozolomide is rapid in children, with maximal plasma concentrations occurring within 60 min [277]. The bioavailability of temozolomide is near 100 % in adults [278], and it is likely to be similar in children, although it has not been examined specifically in this population. Increasing gastric pH by administration of an H_2-receptor antagonist did not alter the bioavailability of temozolomide [279]. In the case of children who are unable or are too young to swallow temozolomide capsules, temozolomide capsules can be opened carefully and reconstituted in applesauce, apple juice, or orange juice. No clinically relevant difference has been observed in temozolomide pharmacokinetic parameters using this method of administration.

The volume of distribution of temozolomide in children was 12.6 L/m^2 [280]. Plasma protein binding is only 15 %. Temozolomide shows good CSF penetration, with CSF exposures about 20 % of plasma exposures in adults [281]. Using positron emission tomography (PET) in combination with plasma sampling, Rosso et al. predicted that the tumor to plasma exposure ratio was 1.3, compared to a normal brain to plasma exposure ratio of 0.9 in adult glioma patients [282]. The difference may be accounted for by changes in the tumor vasculature. Tumor penetration of temozolomide may be modulated by antiangiogenic agents through a process of vascular normalization [283]. The CC allele of the 1236C>T polymorphism in the *ABCB1* gene was predictive of survival in glioblastoma patients treated with radiation therapy and temozolomide [284], although it is not clear whether this was due to an effect on the temozolomide disposition or cellular drug penetration.

Because temozolomide is spontaneously converted to MTIC, activation is not dependent on hepatic metabolism [278]. The rate of degradation of temozolomide in a liver homogenate was identical to that in phosphate buffer, indicating little or no hepatic metabolism [285]. Circulating levels of MTIC are low compared to temozolomide concentrations [286]. MTIC degrades through acid catalysis, forming the methyldiazonium ion, which ultimately leads to O^6-methylguanine formation in DNA, plus the inactive metabolite, AIC [278]. The major route of elimination is via the kidney with only a small amount of biliary excretion [286].

The clearance of temozolomide in children has been recently examined in many pharmacokinetic studies and is similar to adults, with limited interpatient and intrapatient variability [277, 280, 287–293]. Although one study saw a slight increase with age in BSA-normalized oral clearance [280],

another study saw no effect of age on oral clearance [292]. However, no studies have examined infants less than 1 year of age, where temozolomide disposition may be different than older children due to reduced renal elimination. No other drugs have been shown to have a clinically significant interaction with temozolomide, and temozolomide does not appear to affect the disposition of irinotecan [294] or O^6-benzylguanine [289], with which it is commonly coadministered.

5.8 Imatinib

Imatinib is a tyrosine kinase inhibitor that targets the abnormal *bcr-abl* tyrosine kinase. It is FDA approved for treatment of Philadelphia chromosome-positive (Ph+) CML in adults and children, Ph+ ALL in adults. Imatinib has also been tested for treatment of pediatric solid tumors [295] and CNS tumors [296] expressing the c-Kit or PDGF receptors, which are also inhibited by imatinib.

Imatinib mesylate has a high oral bioavailability of 98 % in adults [297], despite being a substrate for P-gp [298]. The time to maximal plasma concentrations was approximately 3 h in children [299], which is similar to values reported in adults [297]. Scored 100 mg tablets, which are better for incremental pediatric dosing, are bioequivalent to imatinib capsules [300]. Absorption of imatinib was not significantly affected by agents raising the gastric pH [301, 302].

Protein binding of imatinib and its active metabolite CGP74588 was similar, ranging from 70 % in healthy volunteers to 92 % in AML patients, with high binding to α1-acid glycoprotein [303]. Imatinib does not have high CSF penetration; CSF levels were approximately 1 % of plasma levels in leukemia patients [304, 305]. One reason for limited brain and CSF concentration is likely the expression of P-gp and BCRP transporters [306].

Imatinib is metabolized by several CYP450 isoforms, including CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5 [307]. The main active metabolite, CGP74588, is formed primarily by CYP3A4 and CYP3A5, with a lesser contribution by CYP2D6 [308], although CYP3A5 [309] and CYP2D6 [310] genotypes did not correlate with imatinib disposition. Inhibition of CYP3A4 with ketoconazole resulted in a 40 % increase in imatinib exposure, but a 13 % decrease in CGP74588 [311]. In adults, imatinib half-life was decreased by enzyme-inducing anticonvulsant drugs [312], and imatinib trough levels were reduced up to 2.9-fold [313]. Similarly in children with gliomas, enzyme-inducing anticonvulsant drugs decreased imatinib exposure by 60 % and doubled the CGP74588 to imatinib ratio [314]. Imatinib itself can inhibit the metabolism of the CYP3A4 substrate simvastatin [315], indicating it may inhibit its own metabolism.

Imatinib is cleared through a mix of metabolism and biliary and renal excretion. Biliary excretion was studied in two patients with biliary stents [316]. Biliary excretion accounted for about 18 % of the dose in the first patient, but in the second patient, who had liver dysfunction, biliary excretion only accounted for 2 % of the dose. However, another study showed that imatinib exposure and renal clearance were similar in adult patients with and without liver dysfunction [317]. *ABCB1* genotype affected imatinib steady-state apparent clearance [309]. Polymorphisms in *ABCG2* and *CYP3A5* were associated with poor response to imatinib in patients with CML [318], although in another study of 82 patients with gastrointestinal stromal tumors, these genotypes did not correlate with the apparent clearance of imatinib [319].

Similar to adults, substantial interpatient variability has been reported for the disposition of imatinib in children [295, 299, 320, 321]. Accumulation of imatinib at steady state was 1.7-fold [299]. In 22 patients aged 3–22 years, weight-normalized dosing did not reduce interpatient variability in imatinib exposure [299]. However, in a population analysis that combined 33 children and 34 adults with solid tumors, body weight explained a significant amount of variability in the apparent clearance and apparent volume [321]. Similarly, in a population analysis of 41 children and young adults with leukemia or solid tumors aged 6–24 years, body weight explained variability in apparent clearance and apparent volume and correlated better with these parameters than BSA [320]. No age-related

changes in imatinib disposition independent of body size have been reported, and pharmacokinetic parameters for children are similar to adult values [320, 321]. However, the disposition of imatinib has not yet been examined in infants, where reduced CYP3A activity would be expected to augment imatinib exposure.

5.9 Gefitinib and Erlotinib

Gefitinib and erlotinib are tyrosine kinase inhibitors that target the epidermal growth factor receptor (EGFR). Gefitinib was approved for adults with non-small cell lung cancer, but this has been restricted to patients who have benefited from the drug or patients enrolled on an IRB-approved clinical trial. Erlotinib is approved for adults with non-small cell lung cancer or pancreatic cancer. Gefitinib and erlotinib have been examined in pediatric trials of solid tumors and CNS tumors [236, 237, 272, 273, 322, 323].

The bioavailability of gefitinib after a single dose was 57 % and 59 % in healthy adults and cancer patients, respectively [324], whereas bioavailability for erlotinib was reported as 59 % in health adults [325] and 76 % in cancer patients [326]. Peak plasma concentrations of gefitinib were reached at 2.3 h in children with solid tumors [323]. A similar time to peak concentrations (2–4 h) was reported for erlotinib tablets, but the time was reduced to less than 1 h when children were administered erlotinib as an oral solution [272, 273, 322]. Similar values are reported in adults for erlotinib tablets, but gefitinib absorption is slower, with peak plasma concentrations occurring 5–7 h after the dose [327]. In adults, food increased exposure to erlotinib by 100 %, compared to only 35 % for gefitinib [327].

The volume of distribution of gefitinib is about tenfold higher than erlotinib in adults [327]. The volumes of distribution of gefitinib and erlotinib have not yet been determined in children. Gefitinib plasma protein binding was reported to be 90 % in normal human plasma [328] but 99.7 % in plasma from pediatric cancer patients [323]. Erlotinib is 92–95 % bound to plasma proteins [329].

Both gefitinib and erlotinib are extensively metabolized and have little urinary excretion. Gefitinib is metabolized mainly by CYP3A4, CYP3A5, CYP2D6, and CYP1A1 [330, 331]. CYP2D6 catalyzes rapid metabolism in vitro, but human liver microsomes from CYP2D6 poor metabolizers do not have a reduced rate of metabolism [331]. Clinically, CYP2D6 poor metabolizers have a twofold higher mean gefitinib exposure than CYP2D6-extensive metabolizer, but the high degree of variability makes this genotype of little predictive use [332]. Erlotinib is also metabolized by CYP3A4, CYP3A5, and CYP1A1, which can produce its active OSI-420 metabolite [330]. The exposure of this metabolite is approximately tenfold lower than erlotinib exposure in children [322]. Unlike gefitinib, erlotinib is also metabolized by CYP1A2 [330].

Both erlotinib and gefitinib are substrates and inhibitors of P-gp and BCRP [333–336]. Because of its ability to inhibit ABC transporters in the gut, gefitinib has been combined with oral irinotecan in children with solid tumors, which increased the bioavailability of irinotecan by fourfold [257].

Apparent oral clearance of gefitinib in children with solid tumors was 14.8 L/h/m^2, with almost eightfold variability in clearance values, and the half-life was 11.7 h [323]. The apparent clearance of erlotinib was 3.1 L/h/m^2 in a similar population of children, with a half-life of 8.7 h [272, 273]. No age-related changes in clearance were seen in either study, although infants were not included in either study.

6 Summary and Conclusion

Recent advances in cellular and molecular biology techniques have led to an increased understanding of pediatric clinical pharmacology and in many instances a more rational approach to dosing drugs in children. As described in this chapter, a stronger scientific basis for dosing drugs in children will be

derived from an increased understanding of the effects of maturation on the physiological processes affecting drug disposition, concomitant drug therapy, and disease states. Moreover, a further evaluation of the most appropriate dosing approach (e.g., dose per weight, height, BSA, age) in the pediatric population is warranted. Because the importance of pharmacokinetic and pharmacodynamic studies in this age group has been highlighted, an improved understanding of the clinical pharmacology of many anticancer drugs in children has been gained. In addition, technology advances have become readily available to enhance the conduct of pharmacokinetic studies. This improvement in computer hardware and software has encouraged investigators to pursue additional aspects of clinical pharmacokinetic studies, including population pharmacokinetic studies, on a more routine basis in this age group. This approach is viewed as one method to identify covariates that can be used to develop novel dosing approaches. Finally, pharmacogenomic analysis in children with cancer will further explain the observed interindividual variability in pharmacokinetics, toxicity, and efficacy. Establishing phenotype–genotype correlations will improve the care of children with cancer.

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Clinical Pharmacology in the Older Adult

Patricia W. Slattum and Jürgen Venitz

Abstract Drug pharmacokinetics are influenced by a number of patient-specific factors, including age. Interindividual variability in physiology increases with age, making it necessary to consider the patient's overall condition, "physiologic age," disease states, and concurrent medications when applying general knowledge of pharmacokinetic differences in older adults to the care of individual patients. Geriatric patients may demonstrate altered bioavailability, absorption, distribution, metabolism, and renal excretion of drugs. Most clinically significant pharmacokinetic changes in advanced age can be attributed to altered renal and hepatic metabolic function. The prevalence of cancer among older adults is high and increasing, resulting in increasing exposure to chemotherapeutic agents in geriatric patients. Overall, age-related renal impairment is the major cause of dose modifications for chemotherapeutic agents in older adults, and the (estimated) creatinine clearance (CLcr by the Cockcroft–Gault method) serves as a good predictor for a patient-individualized dosing regimen. Apparent age-related effects on hepatic drug metabolism/biliary excretion have been observed, but usually do not lead to dose adjustments; however, metabolic drug–drug interactions (i.e., inhibition or induction of drug metabolizing enzymes or drug transporters in the liver and/or GI tract) can be very important in older adults, as they are more likely to receive comedications for diseases unrelated to their cancer with the potential for drug–drug interactions. Recommended dosage adjustments for select chemotherapeutic agents are summarized in this chapter.

Keywords Geriatric • Clinical pharmacology • Pharmacokinetics • Aging physiology

1 Introduction

Clinical response (i.e., therapeutic efficacy/benefit or toxicity/harm) to medication in an individual patient is the net result of the interaction of a number of complex processes. These processes can be categorized into two broad areas (1) those affecting pharmacokinetics or the relationship between the dosing regimen (administered dose, route and rate of administration) and the concentrations of the

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drug in the systemic circulation and (2) those affecting pharmacodynamics or the relationship between concentrations of the drug in the systemic circulation and the observed pharmacologic response(s). Drug absorption into, distribution throughout, metabolism in, and excretion from the body comprise the processes determining drug pharmacokinetics. Drug–receptor interactions, concentrations of the drug at the receptor, and secondary, homeostatic compensatory mechanisms determine a drug's pharmacodynamics. Pharmacokinetics and pharmacodynamics are affected by a number of patient-specific factors including age, sex, ethnicity, genetics, disease processes, and prior and present drug exposure. This chapter will focus on the effects of advanced age on pharmacokinetics.

In clinical decision making for the older adult patient, it is important to recognize that older adults may also experience an unexpected clinical response to a medication due to the impact of factors other than their age such as concurrent diseases and co-administered medications. Despite the fact that much less is known about pharmacodynamic changes in older adults than changes in pharmacokinetics, the potential for altered pharmacodynamics must also be considered.

1.1 Definition of "Older Adult"

"Older adult" has generally been defined as age 65 years or older, although many other chronological definitions have been applied. Some researchers have enrolled patients as young as 50 years old as "older adult," while others have studied only those patients in their 1980s or older as "older adult." Although a chronological age is most often used to define older adult, it is important to recognize that older adults are a heterogeneous group, with individuals aging at varying physiological rates. Interindividual variation is much larger in the older adult population compared with the young [1]. The aging process has been described as a progressive decline in function that makes the individual more vulnerable to environmental insults and increases the risk of disease and mortality [2]. When decline occurs more obviously in one organ system than another, a disease is diagnosed. It is therefore difficult to distinguish between normal age-related changes and pathological states. Biological or physiological definitions of older adult have proved difficult to formulate, so chronological definitions of older adult remain the standard. The Food and Drug Administration's "Guideline for Industry Studies in Support of Special Populations: Geriatrics" arbitrarily defines the geriatric population as comprising patients aged 65 years or older, and the inclusion of older patients in clinical trials is encouraged to the extent possible [3].

1.2 The Older Adult Patient

Although many older adults successfully age and lead healthy, productive lives well into their later years, older adults as a group are more likely to suffer from chronic diseases and take more medications than their younger counterparts. The aging process itself is associated with changes in physiology that may alter drug pharmacokinetics and pharmacodynamics. When applying general knowledge of pharmacokinetic alterations in the older adult to the care of an individual patient in the clinical setting, it is necessary to consider the patient's overall condition, "physiologic age," disease states, and concurrent medications.

Older adults are especially vulnerable to adverse reactions to medications. The incidence of adverse drug reactions is two to three times that found in younger adults but may be underestimated because of lack of detection and underreporting [4]. Many adverse reactions are preventable. Examples of preventable adverse effects include consequences of known drug–drug interactions and prescribing an inappropriate dosage for the older adult. The increased incidence of adverse reactions in the older

adult results from altered pharmacokinetics, altered pharmacodynamics, increased opportunity for drug–drug interactions, or inappropriate prescribing. Medication monitoring, where the therapeutic benefits and harms are reassessed over time, is important for improving safety and reducing the burden of adverse drug events [5]. In the USA, approximately one-half of hospitalizations related to adverse drug events are caused by medications that require regular monitoring, and errors in monitoring are more common than errors in initial prescribing [5]. Knowledge of basic pharmacokinetic differences in the older adult associated with age-related changes in physiology can be used to choose appropriate dosing regimens for the older adult and avoid preventable adverse drug reactions. Appropriate monitoring and recognition of possible adverse events can reduce drug-related morbidity in older patients when addressed promptly.

Since older patients tend not to tolerate anticancer agents either due to pharmacokinetic or pharmacodynamic heterogeneity, new metrics including frailty indices, biomarkers of aging or diseases associated with morbidity or mortality, or components of geriatric assessment are beginning to be incorporated into clinical trials to assess if older patients can be stratified for risk of adverse outcomes related to anticancer treatment [6].

Geriatric assessment incorporates demographic characteristics, physical function, disability, depression, and cognitive status which may be an aggregate of the generic performance status utilized by oncologists. Whether this approach will be successfully applied in oncology is yet to be determined.

1.3 Pharmacokinetic Studies in the Older Adult

Almost all of the information known about age-related changes in humans, including pharmacokinetics, has been learned from cross-sectional studies: In these studies, the variable under investigation is measured in groups of subjects of different ages at a single point in time. Age differences are then inferred from a comparison of the mean values for each group or from a regression of the variable on age. The cross-sectional approach assumes that average differences between age groups reflect the change that occurs in an individual with the passage of time, which may or may not be valid.

When studying chronological changes in a particular variable, there are three primary time-related factors that must be considered: the effects of age, the effects of an environmental change or historical event at a specified period in time (period effects), and the effects of being part of the group or cohort of individuals born at a particular time (birth cohort). Cross-sectional studies often confound age effects with birth cohort effects. Findings in a group of individuals aged 65 today may differ from those in a group of 65-year olds studied 25 years from now. These groups would be the same age but from different birth cohorts with different group experiences, such as improved access to health care. Cross-sectional studies can also suffer from selective mortality effects, because the oldest study cohorts include only those individuals who survived to reach old age, and these individuals may be unique for a variety of reasons.

Another approach to studying age-related changes is longitudinal studies: in these studies, repeated measurements of a variable are made on the same individual at various points in time. This approach measures individual rates of aging for the specified variable, rather than differences between age groups as in cross-sectional studies. Although the results of longitudinal studies may be a more reliable approach to studying age-related changes, longitudinal studies tend to confound age effects with the effects of an environmental change or historical event at a specified period in time (period effects). These studies are also very difficult to conduct, taking many years to complete. For this reason, pharmacokinetic studies are virtually always cross sectional in design [7].

Two general cross-sectional approaches are used to study pharmacokinetics in the older adult. The first is a formal pharmacokinetic study conducted either in healthy geriatric subjects or in older adult patient volunteers with the disease the drug is intended to treat. A relatively small group of

subjects is studied using intensive blood sampling in each individual. In this approach, very healthy older people are generally selected for participation in an attempt to ensure that advanced age, and not disease, is the primary factor under investigation. Often these studies include only relatively young geriatric subjects that can meet the stringent inclusion criteria, limiting the generalizability of the results to the very old or frail patient. Results of these studies must be considered along with pharmacokinetic studies in other populations, such as patients with renal impairment, when making therapy decisions for individual patients.

The second cross-sectional approach is the pharmacokinetic screening or population-pharmacokinetic study. These studies are typically conducted in conjunction with the main Phase 3 (or Phase 2) clinical trials program. Usually under steady-state conditions, a small number of blood samples for drug level determinations are collected and analyzed. When appropriately designed, the influence of demographic and disease factors on pharmacokinetics can be examined in this type of study. Although the data analysis is more difficult, the advantage to this approach is that age and other factors, as well as their interactions, can be evaluated [8]. However, age may be confounded with other factors such as concurrent medications, diet, and impaired renal functioning (see below).

2 General Pharmacokinetic Changes Associated with Aging

Normal aging is associated with changes in human physiology, and many of these changes contribute to altered pharmacokinetics in the older adult. These changes may be even more evident in frail or very old patients [1]. Drug absorption and bioavailability, distribution, metabolism, and renal excretion may be altered in geriatric patients. If these changes are not considered when dosing older adult patients, preventable medication-related problems may result.

2.1 Absorption and Bioavailability

Bioavailability of a drug is defined as the fraction of the administered dose reaching the systemic circulation after drug administration. Age-related changes in bioavailability depend on the route of drug administration, age-associated changes in the gastrointestinal tract and other organs of drug absorption, and age-associated changes in metabolism during the first pass through the liver or intestine. Despite changes in physiology with age, oral absorption and bioavailability of most drugs appear to remain unchanged in the older adult due in part to the large functional reserve capacity of the gastrointestinal tract [9].

Gastric pH, gastrointestinal blood flow, active drug transport processes, and gastrointestinal motility have been reported to be altered in the older adult to a variable extent [9]. Most drug absorption in the gastrointestinal tract occurs by passive diffusion, and the majority of studies indicate that there are no clinically significant changes in the rate or extent of drug absorption from the gastrointestinal tract. Absorption of vitamin B_{12}, iron, calcium, and magnesium, which are absorbed by active transport processes, has been shown to be impaired in older adults, but to date, effects of age have not been reported for gastrointestinal or hepatic transporter expression or function [9].

Intragastric metabolism and hepatic first-pass metabolism may be reduced in older adults resulting in increased drug bioavailability. Studies with levodopa, for example, have shown that older adults experience a threefold increase in availability of levodopa related to a reduction in gastric wall content of DOPA decarboxylase [10]. Some drugs, which undergo a high rate of first-pass metabolism, such as propranolol and labetalol, demonstrate increased oral bioavailability due to decreased first-pass extraction, while other drugs undergoing extensive first-pass metabolism, such as verapamil and propafenone, show no change in bioavailability in older adults [9].

Absorption and bioavailability for non-oral routes of administration (intramuscular, rectal, buccal, transdermal) and sustained release dosage forms have not been as well studied in older adults. There are insufficient data to draw conclusions regarding the potential for age-related changes in drug absorption and bioavailability by these routes [9].

2.2 Distribution

Age-related changes in body composition and plasma protein binding may affect drug distribution in older adults. Older adults tend to have decreased lean body mass, increased body fat, and decreased total body water [9]. Interestingly, older individuals with high levels of physical activity are not different from those with low activity levels with respect to fat-free mass and fat mass [11]. Lipid-soluble drugs may show an increased volume of distribution, and water-soluble drugs may show a decreased volume of distribution in geriatric patients related to these changes in body composition. For example, older adults have an approximately 20 % lower volume of distribution than young individuals for ethanol, which distributes in body water [12, 13]. Changes in body composition, resulting in changes in volume of distribution, may necessitate changes in loading doses of some drugs for older adults.

Age-related changes in plasma protein binding do not generally result in clinically significant changes in drug therapy for elderly patients. Generally, plasma protein binding of drugs remains unchanged or is decreased in older adults. Serum albumin concentrations may be decreased in older adults by 15–20 %, but this is often related to renal dysfunction, hepatic disease, or frailty [14].

2.3 Hepatic Metabolism

Hepatic metabolism is one of the major routes of clearance for certain drugs in humans. The rate and extent of hepatic drug biotransformation depends on hepatic blood flow and hepatic enzyme content, affinity, and activity. Hepatic metabolism, usually inactivation, but sometimes activation, of drugs and environmental toxins occurs through Phase I oxidative pathways (oxidation, deamination, or hydroxylation) or Phase II conjugative pathways (acetylation, glucuronidation, or sulfation). Not all pathways of hepatic drug metabolism are equally efficient. Hepatic biotransformation results in a metabolite, which may be pharmacologically active or inactive, and may be eliminated from the body or further metabolized before elimination.

Interest in potential age-associated changes in drug metabolism is significant because of the need to reduce the risk of adverse drug reactions and drug interactions in older adults. A number of age-related changes in physiology that may impact hepatic drug metabolism in elderly patients have been reported, but the effect of age on hepatic metabolism remains controversial. Much of the literature in this area has been conflicting. Early studies attributed observed changes in drug clearance in older adults to changes in hepatic enzyme activity and more recently to decreased liver size and hepatic blood flow. In vitro tests of enzymes have been inconsistent with results of in vivo studies. Despite these controversies, several generally accepted principles of the effect of aging on hepatic drug metabolism have emerged.

Hepatic blood flow has been shown to decline by about 40 % with age, in parallel with a decline in cardiac output [9, 15]. For drugs with a high hepatic extraction ratio, where hepatic clearance depends primarily on the rate of drug presentation to the liver through hepatic blood flow, aging is associated with decreased hepatic drug clearance [15]. Phase I oxidative metabolism of some drugs appears to decline with aging [1, 9, 16], despite the fact that in vitro hepatic enzyme activity does not appear to be altered by age. Reduction in hepatic oxygen diffusion resulting from age-related changes in

hepatocyte volume and surface membrane permeability and conformation is one proposed explanation for reduced oxidative drug metabolism observed with aging [15]. The interplay between drug transporters and drug metabolizing enzymes may also play a role [9]. Hepatic enzymes can be inhibited and induced by concurrent drugs and other compounds. Changes in hepatic enzyme induction with aging remain controversial. Phase II conjugative metabolic pathways appear to be unchanged with aging.

When prescribing for the older adult patient, age-related changes in drug metabolism should be considered. From a pharmacokinetic point of view, drugs which are metabolized exclusively by Phase II conjugative mechanisms are preferred in older adults. For oxidatively metabolized drugs with a high extraction ratio (high-clearance drugs), dosages should generally be reduced due to decreased hepatic blood flow [17]. Dosages for drugs with a low extraction ratio (low-clearance drugs) should be reduced as well [15]. After initial dosing, doses can be adjusted based on patient response and tolerability. The potential for significant drug–drug interactions, particularly resulting from hepatic enzyme inhibition in elderly patients on multiple medications, must be carefully considered.

2.4 Renal Excretion

Altered renal elimination of drugs is probably the most clinically important pharmacokinetic difference between older adult and young patients. Renal clearance depends, in part, on renal blood flow, which delivers drugs and metabolites to the kidneys for elimination. Elimination from the kidneys then occurs through glomerular filtration, tubular secretion, and tubular reabsorption. With aging, renal blood flow rate declines as cardiac output declines, resulting in decreased glomerular filtration rate as measured by creatinine clearance (CL_{cr}) in older adults. Although there is considerable interindividual variability, declining creatinine clearance with age (about 10 % per decade after age 20) is consistently reported in the literature, but not all older individuals have a decline in renal function [9]. Changes in kidney function with age may be more associated with hypertension and heart disease than with aging itself, and therefore age alone may not have as great impact on renal excretion of drugs than previously thought [9]. The most important aspect of renal function to monitor clinically is the glomerular filtration rate (GFR). Most decisions about drug dosing for renally excreted drugs can be made based on the estimated GFR. Clinically, creatinine clearance (CL_{cr}) is used to estimate GFR. Serum creatinine alone is not a good indicator of renal function in the older adult population because muscle mass, and therefore creatinine production, declines with age [9]. Thus, an apparently "normal" serum creatinine value can result when both creatinine formation and elimination are reduced. Several empiric algorithms have been proposed to estimate creatinine clearance.

One frequently used method was developed by Cockcroft and Gault [17], where CL_{cr} is calculated based on the patient's age, body weight, and measured serum creatinine concentration:

$$\mathrm{CL_{cr}}\left(\mathrm{ml/min}\right)=\frac{\left(140-\mathrm{age}\left(\mathrm{years}\right)\cdot\mathrm{body\ weight}\left(\mathrm{kg}\right)\right)}{72\cdot\mathrm{serum\ creatinine\ concentration\ (mg/dl)}}.$$

For females, the result is multiplied by 0.85. This formula is less accurate for estimates in the very high or low range and when renal function is changing rapidly.

Another commonly used method is the "Modification of Diet in Renal Disease" (MDRD) formula [18]:

$$\mathrm{CL_{cr}\ (ml/min/1.73\ m^2)} = 186\ (\text{serum creatinine } (\mu\mathrm{mol/L})/88.4)^{-1.154}\cdot \text{age (years)}^{-0.203}.$$

For females the result is multiplied by 0.742. Note the difference in units between the Cockcroft–Gault method (ml/min) and MDRD (ml/min/1.73 m^2), as the latter is normalized for body surface area (BSA) while the former is not.

Another approach to estimate GFR based on serum creatinine has been proposed: the Chronic Kidney Disease Epidemiology Collaboration equation [19]. These equations were derived using different patient populations with different ranges of kidney function and age. The validity of each of them for use in estimating GFR in older adults has been advocated and challenged [18–23]. Current consensus is to continue using the Cockcroft and Gault equation for estimating GFR for the purpose of individualizing drug dosing regimens in older adults, although this may change as more studies are conducted using the newer algorithms [20, 22]. More recently, serum cystatin C has been proposed as a better endogenous marker of GFR than serum creatinine for older adults because it does not depend on muscle mass [21].

3 The Effects of Age on the Pharmacokinetics of Chemotherapeutic Agents

3.1 Introduction

Old age is playing an increasing role in the treatment of cancer since the prevalence of cancer in geriatric patients is high and increasing [24]: 60 % of all cancers occur in patients aged 65 years and above, and older adults constitute a growing portion of the overall population with 20 % of the population expected to be above 65 years by the year 2030.

This will lead to an increased use of anticancer agents by geriatric patients. In addition to the physiological effects that aging may have on the pharmacokinetic characteristics of these agents, it has to be noted that the likelihood of polypharmacy due to noncancer, age-related chronic illnesses may lead to an increased incidence of drug–drug interactions [25]. Quite a few of these interactions are pharmacokinetically based by inhibition of gastrointestinal and/or hepatic metabolism (cytochrome P450 dependent, CYP) or drug transporters by concurrent medications.

3.2 Examples

Previous published review papers [24–31] have reviewed the primary literature, describing the effects of aging on the pharmacokinetic properties of chemotherapeutic agents. In the primary references, most of the clinical studies assessing age effects were small, cross-sectional trials and evaluated plasma concentrations of the drug of interest, and in some cases, their active metabolites. A large portion of these studies report changes in systemic exposure (e.g., peak plasma concentration, area under the curve, and terminal half-life) rather than more meaningful pharmacokinetic parameters such as volume of distribution, specific organ clearances, and/or oral bioavailability, if appropriate, which would require IV administration to differentiate effects on systemic drug disposition from effects on (oral) drug absorption. Therefore, as pointed out above, it is sometimes difficult to assess whether physiological aging, concurrent medications (drug–drug interactions), or other confounding covariates are responsible for the observed age differences in systemic exposure. In addition, it is usually very difficult to interpret the results mechanistically (i.e., what pharmacokinetic process(es) may be affected by age-related changes—except for the reduction in renal drug excretion).

Table 1 (adapted from [28]) lists the major routes of elimination and dose-limiting toxicities for commonly used classes of anticancer drugs. As shown in Table 1, most of the newer, targeted anticancer agents, such as TKI, as well as chemopreventive agents, such as AI, SERM, and SRI, are highly plasma protein bound and primarily eliminated by multiple metabolic Phase I (CYP dependent) and

Table 1 General properties of anticancer drugs (adapted from [28]; supplemented with [31–36])

Drug class	Route of elimination	Dose-limiting toxicity
Antimetabolites	Renal excretion	Myelosuppression mucositis
Alkylating agents	Hepatic metabolism and renal excretion of metabolites	Myelosuppression, cardiac, renal, CNS
Platinum analogs	Renal excretion	Renal, neurotoxicity, myelosuppression
Anthracyclines	Hepatic metabolism and biliary excretion	Cardiac toxicity
Vinca alkaloids	Hepatic metabolism and biliary excretion	Peripheral neurotoxicity
Taxanes	Hepatic metabolism and biliary excretion	Neutropenia and neurotoxicity
Camptothecins	Chemical conversion and renal or biliary excretion	Diarrhea
Tyrosine kinase inhibitors (TKI)	Hepatic metabolism and biliary excretion	Hypertension
Aromatase inhibitors (AI)	Hepatic metabolism and biliary excretion	n/a
Selective estrogen receptor Modulators (SERM)	Hepatic metabolism and biliary excretion	n/a
Steroid 5α-reductase inhibitors (SRI)	Hepatic metabolism (CYP3A, glucuronidation) and biliary excretion	n/a

Phase II (glucuronidation) pathways as well as biliary excretion of parent drug and metabolites, while renal excretion of parent drug or metabolites is negligible [32–35]. For the drugs used for cancer chemoprevention, this finding is presumably the consequence of their chemical similarity to endogenous steroid hormones. As a result of these basic pharmacokinetic properties, old age is not expected to play a major role in the disposition of these drugs, while drug–drug interactions can be a very important source of variability in drug response. Two notable exceptions exist: For tamoxifen, a SERM, older women (>80 years) showed a two- to threefold elevation in circulating concentrations compared to younger women (<40 years); the reason behind this observation is unknown [32]. For dutasteride, a SRI, a considerable increase in plasma half-life with increasing age has been demonstrated [19], again, for reasons not understood. Nevertheless, age-related effects on hepatic metabolism do not lead to recommended starting dose adjustments for any of these agents.

On the other hand, most of the antimetabolites, especially the antifolates and platinum analogs, are subject to a high degree of renal excretion and, at the same time, have serious dose-limiting toxicities. Therefore, the physiological age-related decline in renal function (see Sect. 2.4) has indeed a major impact on their risk–benefit assessment and does require dose adjustments with old age, based on renal function (see below).

Resulting from the general drug properties listed in Table 1, Table 2 lists individual drugs whose pharmacokinetics are known to be affected by age, the likely mechanism of that age effect, and the need for dose modification in older adults [26–31, 36–41]. Note that f_e indicates the fraction of the total dose renally eliminated unchanged after IV administration, i.e., unaffected by oral bioavailability (see Sect. 2.4).

As can be seen from Table 2, by far the major reason for dose modification in older adults is the age-related impairment in renal excretory function. Therefore, Table 3 (adapted from [27]) illustrates the recommended dose modifications based on renal function for selected anticancer agents. The values listed in that table indicate the dose-multiple relative to a standard dose for a patient with normal renal function, e.g., 0.75 means 75 % of the standard dose.

Overall, it is apparent that age-related renal impairment is the major cause of dose modifications in older adults, and the (estimated) creatinine clearance (CL_{cr} by the Cockcroft–Gault method; see Sect. 2.4) serves as a good predictor for a patient-individualized dosing regimen. Apparent age-related effects on hepatic drug metabolism/biliary excretion have been observed, but usually do not lead to dose adjustments; however, metabolic drug–drug interaction (i.e., inhibition or induction of drug-metabolizing enzymes or drug transporters in the liver and/or GI tract) can be very important in older adults, as they are more likely to receive comedications for diseases unrelated to their cancer with the

Table 2 Age effects on the pharmacokinetics of specific anticancer drugs

Drug	Mechanism of age effect	Dose modification
Antimetabolites		
Methotrexate	Renal elimination (f_e: 44–100 %)	Yes
Pemetrexed	Renal elimination (f_e: 70–90 %)	Yes [38]
Pralatrexate	Renal elimination (f_e: 30–40 %)	Unknown [39]
5-FU in presence of a DPD inhibitor only	Renal elimination (f_e: 77 %)	Yes
Fludarabine	Renal elimination (f_e: 60 %)	Yes
Gemcitabine	Hepatic metabolism	No
Alkylating agents		
Ifosfamide	Distribution into body fat reduced	No
Melphalan	Renal elimination	Yes
Platinum compounds		
Cisplatin	Renal elimination (f_e: 90 %)	Yes
Carboplatin	Renal elimination (f_e: 100 %)	Yes
Oxaliplatin	Renal elimination	No, severe renal failure only
Anthracyclines		
Idarubicin	Accumulation of renally eliminated metabolites	Yes [31]
Mitoxantrone only	Hepatic metabolism (f_e: 10 %)	No, hepatic failure
Vinca alkaloids		
Vinblastine	Hepatic metabolism	Yes [30]
Vinorelbine	Biliary excretion	No, severe hepatic failure only
Taxanes		
Paclitaxel	Hepatic metabolism	No, potential for DDI; severe hepatic failure
Docetaxel		
Topoisomerase inhibitors		
Topotecan	Renal elimination (f_e: 30 %)	No, moderate/severe renal failure only
Irinotecan	Metabolism/biliary excretion of active metabolite (SN38)	No
Etoposide	Renal elimination	Yes
Others		
Lenalidomide	Renal excretion (f_e: 85 %)	Yes [41]

Table 3 Dose modification algorithms for selected chemotherapeutic drugs based on renal function (adapted from [27])

	Renal function (creatinine clearance)		
Drug	<60 ml/min	<45 ml/min	<30 ml/min
Bleomycin	0.70	0.60	Unknown
Carboplatin	Use Calvert formula based on glomerular filtration rate		
Carmustine	0.80	0.75	Unknown
Cisplatin	0.75	0.50	Unknown
Cytarabine	0.80	0.50	Unknown
Dacarbazine	0.80	0.75	0.65
Fludarabine	0.80	0.75	0.65
Ifosfamide	0.80	0.75	0.70
Melphalan	0.65	0.50	Unknown
Methotrexate	0.85	0.75	0.70

potential for drug–drug interactions. Furthermore, age-related effects on drug absorption are rare since most agents are given intravenously, while for oral drugs, it is difficult to separate pre-systemic and systemic effects from oral exposure information only (see above). Finally, age effects on drug distribution are difficult to observe and are unlikely to result in dose modifications.

These overall conclusions may change in the future, however, when cancer treatment will involve further long-term chemoprevention and biologically targeted disease modification, where most agents will be given orally and are less likely to be renally eliminated. Furthermore, due to the polypharmacy in the older adults, the likelihood of clinically significant drug–drug interaction at the level of drug absorption, first-pass and systemic metabolism will continue to increase.

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Organ Dysfunction Trials: Background, Historical Barriers, Progress in Overcoming Barriers, and Suggestions for Future Trials

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Abstract In the past, patients with cancer, who also have hepatic or renal dysfunction, have carried a triple burden. Not only have they suffered from cancer and organ dysfunction, but until recently, their oncologists have had inadequate information to tailor doses of drugs to their degree of organ dysfunction. In the past, phase 1 clinical trials in patients with organ dysfunction were either performed long after drug approval or not at all. This chapter provides background, a review of barriers to the conduct of clinical trials in this population, information about how these barriers have been largely overcome, and suggestions for conducting organ dysfunction trials in the future.

Keywords NCI organ dysfunction working group • Hepatic dysfunction • Renal dysfunction • Special population studies • Dose determination • Drug development

1 Introduction

In oncology, most clinical trials exclude cancer patients who have significantly impaired hepatic or renal function, resulting in a lack of data on safety and dosing in these populations [1]. Such patients are treated with empirically determined doses, often lower than standard doses, due to concerns regarding increased toxicities and poor tolerance. As most anticancer drugs are cleared by the liver or kidney, this concern is justified and highlights the need to conduct well-designed, carefully monitored studies in such patient populations to generate dosing guidelines. However, given the high-risk population such trials are difficult to conduct since patients with organ dysfunction often have generally

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poor medical status and confounding comorbid conditions, making enrollment of eligible patients a challenge. Therefore, such trials are multi-institutional and have to be conducted in centers with significant drug development expertise. The National Cancer Institute's (NCI's) Organ Dysfunction Working Group (discussed below), the Southwest Oncology Group (SWOG) Early Therapeutics Committee, and the Cancer and Leukemia Group B (CALGB) Pharmacology and Experimental Therapeutics Committee have focused their efforts in conducting such trials safely and efficiently. Each group has multiple leading drug development centers that participate, reducing the accrual time and study completion to a little over a year [2]. Clinical safety, toxicity, and pharmacokinetic data obtained from these trials are then used to develop dosing guidelines for the treatment of patients with varying degrees of organ dysfunction. These trials encompass both the pharmacologic and toxicity-driven approaches in establishing the recommended doses. The following chapter highlights the approach and issues surrounding the conduct of organ dysfunction trials in oncology.

1.1 Overview

To understand hepatic and renal dysfunction clinical trials, it is necessary to have a working knowledge of how organ dysfunction can affect the pharmacokinetic (PK) and pharmacodynamic (PD) profiles of drugs, the underlying cause of the organ dysfunction, and the laboratory and clinical evaluation to assess the dose and associated risks of a given treatment. PK reflects drug metabolism, distribution to tissues, excretion, and, for non-parenterally administered drugs, drug absorption rates. Pharmacodynamics reflects the effect of the drug on its purported target and its off-target effects that lead to both the efficacy and toxicity of an agent. Most patients with only mild organ dysfunction do not have significant perturbations in pharmacokinetics or pharmacodynamics and therefore can usually be administered at the doses established for patients with normal organ function. However, most anticancer drugs have relatively low therapeutic indices thus organ dysfunction that can alter the pharmacokinetics such that exposures vary can have significant impact on the safety of an agent. Even if parameters such as clearance may be unaltered by the organ dysfunction, other pharmacokinetic indices such as the free fraction may be altered accounting for increased toxicity and/or potentially increased efficacy. For example, low serum albumin levels in patients with hepatic dysfunction may cause a higher than normal free fraction of the drug, increasing the agent's toxicity.

1.2 Liver Impairment

Hepatic dysfunction may dramatically alter drug pharmacokinetics and pharmacodynamics because the liver is the major organ involved in drug metabolism and excretion. Liver dysfunction, regardless of the underlying etiology, raises considerable concern about administration of full therapeutic doses of drugs to patients with liver impairment. Liver dysfunction can also lead to alteration in other organ functions such as the kidney, further impacting drug metabolism and clearance. This becomes a greater consideration for anticancer agents as an appreciable number of these agents have a narrow therapeutic index.

1.3 Renal Impairment

Renal dysfunction can significantly alter parent drug and drug metabolite excretion and potentially alter drug absorption. Diminished renal clearance also affects the nonrenal disposition of drugs. Kidney function impairment causes a cascade of pathological and physiological alterations in every

organ system of the body, including the liver. Many studies have shown that loss of renal function can result in decreased hepatic clearance of drugs [1, 3–5]. The mechanism by which this occurs remains unclear, but studies have shown that as the kidneys fail, key enzymatic systems in the liver, intestine, and kidney become inhibited, thus affecting metabolism of some drugs.

In chronic renal failure, downregulation of selected isoforms of phase 1 liver metabolism (oxidation, reduction, or hydrolysis), and specifically the hepatic cytochrome P450 (CYP450), occurs, probably due to decreased gene expression and accumulation of circulating factors that modulate CYP450 activity (uremic toxins) [6]. Phase 2 metabolic reactions in the liver (glutathione S-transferases, UDP-glucuronosyltransferases, *N*-acetyltransferases, amino acid *N*-acyltransferases, sulfotransferases) are also reduced in chronic renal failure [7]. Although most evidence comes from preclinical in vitro and in vivo studies, chronic renal failure is associated with a decrease in the expression of specific liver CYP450 isoforms secondary to reduced mRNA levels in humans [6, 7]. Phase 2 reactions in chronic renal failure have not been examined as extensively, but studies suggest that phase 2 enzyme activities, such as glucuronidation and acetylation, may also be suppressed in renal failure, probably due to the presence of uremic toxins [8, 9].

Reduced nonrenal clearance in patients with renal failure has also been observed for drugs that are not extensively metabolized, and reduced CYP450 activity does not explain this phenomena [10–12]. Thus, in patients who have renal dysfunction, other mechanisms may contribute to altered drug clearance. Chronic renal failure increases drug exposure by decreasing intestinal first-pass metabolism and by decreasing drug excretion; the latter is the result, in part, of decreased P-glycoprotein levels in renal failure. Chronic renal failure has been shown to downregulate intestinal CYP450 gene expression [3, 4, 7]. Furthermore, renal impairment may alter hepatic metabolism, plasma protein binding, and drug distribution. These pharmacokinetic changes cause, in turn, pharmacodynamic changes.

2 Etiology and Laboratory Evaluation

2.1 Hepatobiliary Dysfunction

In addition to the myriad causes of hepatic dysfunction (too numerous to review in this chapter), oncology patients may also have liver dysfunction secondary to their tumors and/or previous treatments. Patients with cancer may have hepatocellular damage from liver primaries or from liver metastases, cholestatic liver lesions from tumor-related obstruction, or liver dysfunction from previously administered antineoplastics and/or radiation.

Hepatobiliary impairment results from three types of damage: hepatocellular damage, cholestatic damage, and combined cholestatic and hepatocellular damage. The former is, by far, the more important for determination of drug pharmacokinetics and pharmacodynamics. This damage is detected by increases in aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGT), alkaline phosphatase, and total and direct bilirubin. The ability to synthesize clotting factors, vitamin K, and albumin is impaired in the face of hepatocellular damage, and increases in prothrombin time (PT) and partial thromboplastin time (PTT) and decreases in albumin are frequently detected. Cholestatic damage most often results from gallstones, malignant biliary obstruction, and primary biliary cirrhosis, although other conditions may also contribute. Cholestatic damage, in contrast to hepatocellular damage, is detected by an abnormal ratio of direct bilirubin to total bilirubin, because of inability to conjugate bilirubin, as well as by increases in alkaline phosphatase.

The tests described above are not very useful as a measure of drug-metabolizing capabilities of the liver. Alternative approaches have been employed to assess hepatic metabolism and clearance of drugs. One approach involves analysis of the metabolism of a surrogate drug. Low-dose midazolam

Table 1 Methods used to estimate the degree of renal function

- Direct determination of glomerular filtration rate (GFR)
- Measurement of serum creatinine (SCr). The accuracy of this method depends on the laboratory involved
- Determination of creatinine clearance (CrCl) using serum creatinine and measurements obtained from 24-h urine collection. This method takes into account muscle mass variations, which cause differences in the generation of creatinine, but may overestimate GFR because the kidneys secrete creatinine in addition to filtering it. This method is inaccurate if suboptimal urine collections are done and/or if urine collection specimens are not promptly and correctly processed. It has the disadvantage of being relatively more complex than simply measuring serum creatinine

Table 2 Formulas used to estimate creatinine clearance [11, 15–17]

Originator(s)	Formula
The Cockcroft–Gault method	CrCl (male) = ([140 − age] × weight in kg)/(SCr × 72) CrCl (female) = CrCl (male) × 0.85
The Jelliffe formula	CrCl (mL/min) = {98 − [0.8 × (age − 20)]} × [1 − (sex × 0.1)/[(SCr × 0.814)/72 × (BSA/1.73)], where actual body weight (ABW) is measured in kilograms and SCr is measured in micromoles per liter
The Martin formula	GFR (mL/min) = {163 × ABW × [1 − (0.00496 × age)] × [1 − (0.252 × sex)]}/SCr
The Wright formula	GFR (mL/min) = {[6,550 − (38.8 × age)] × [1 − (0.168 × sex)] × BSA}/SCr
The Modification of Diet in Renal Disease (MDRD) formula	$186 \times (\mathrm{SCr})^{-1.154} \times (\mathrm{age})^{-0.203} \times 0.742$ (if the subject is female) or ×1.212 (if the subject is Black)

BSA is measured in square meters, height in centimeters, age in years (nearest 10 years for Jelliffe formula), and sex = 0 (male) or 1 (female)

is used as a surrogate to measure hepatic drug clearance for CYP3A metabolic studies. Intravenous ^{14}C-erythromycin was used as a surrogate for CYP3A4 activity, but it is no longer available for commercial use. ^{14}C-erythromycin surrogate testing has been correlated with docetaxel clearance and the associated myelosuppression [13]. This use of ^{4}C-erythromycin surrogate testing, however, is limited by exposure to radioactive materials, patient noncompliance, and the need for frequent and expensive sampling. Furthermore, patients may not breathe into the balloon adequately and multiple samples may be required for optimal accuracy, thereby increasing time and expense. These factors may limit its advantages over midazolam sampling for phenotyping hepatic drug-metabolizing activity [14].

2.2 Renal Dysfunction

There are many etiologies of renal impairment including intrinsic renal disease, direct injury, and drug- and toxin-induced injury. A detailed review of these is beyond the scope of this chapter. Patients with cancer often have additional reasons for renal dysfunction, as they do for hepatobiliary dysfunction. Renally cleared drugs can be affected by (1) the kidney's altered metabolic capacity, (2) altered renal excretion pursuant to altered renal blood flow or cancerous involvement of the organ, or (3) production of toxic compounds that damage the kidneys. There are multiple methods to assess renal dysfunction, as shown in Table 1.

For purposes of drug development, GFR is the most adequate method for measuring renal function. However, direct determination of GFR using insulin clearance or exogenous filtration markers, such as cold iothalamate, iohexol, and hot radionuclides, is too cumbersome for routine application. On the other hand, indirect determination of GFR can be achieved by estimating creatinine clearance (CrCl). More than 25 different formulas to estimate CrCl have been developed although only a few are in common clinical use, as shown in Table 2 [15–17]. The National Kidney Foundation of the United

States has recommended using either the formula proposed by Cockcroft and Gault or the Modification of Diet in Renal Disease (MDRD) [15, 18, 19]. Both formulas use SCr, age, and gender to estimate renal clearance, but the Cockcroft and Gault formula is shorter, easier to calculate, and has been in use for approximately 20 years longer. The Cockcroft–Gault formula is preferred by some researchers, including those from the Cancer and Leukemia Group B (CALGB), while the Jelliffe formula is preferred by other researchers including those from the Gynecologic Oncology Group (GOG). The MDRD, although not widely accepted nor widely employed for oncology drugs, is preferred by the National Institutes of Health (NIH) and industry for studies that are done either to evaluate drugs for patients with chronic kidney disease or to identify candidates for living kidney donation [19–22]. BSA indexing of GFR is still controversial although it avoids overdiagnosis of renal impairment in patients who have low body surface areas and underdiagnosis of renal impairment in patients with high body surface areas; thus, it could eliminate a source of variability between groups in randomized studies of interventions in patients with chronic kidney disease [18, 19, 23–28].

3 Barriers to Clinical Trials in Patients with Organ Dysfunction

3.1 Overview

One of the major problems in oncologic drug development has been the delay and difficulty of determining the appropriate dose and schedule for patients with organ dysfunction. The challenge of finding an acceptable therapeutic index for antineoplastic drugs for this patient population would be difficult under any circumstances, considering the unpredictability of drug metabolism and clearance. This difficulty has been compounded manyfold by significant barriers to conducting timely phase 1 clinical trials in these populations. Commercial interests, FDA standards, and practical barriers for investigators have all conspired against performing these trials.

3.2 Commercial and Regulatory Barriers

The pharmaceutical industry's bottom line has been to speed new oncology agents to market by excluding patients with organ dysfunction from early and pivotal oncology trials [29]. This attitude was tacitly accepted by the Food and Drug Administration (FDA) by approving antineoplastics with fairly limited pharmacokinetic and pharmacodynamic information from patients with organ dysfunction treated with the study drug. Although the FDA has traditionally encouraged pharmacologically guided dosing for drugs used in infectious diseases, cardiology, neurology, and other specialties, it has not done so routinely for oncology products.

3.3 Practical Barriers

Even when investigators have chosen to perform organ dysfunction trials, there were practical barriers to doing so. Patient accrual has been arduous because many potential patients have comorbid illnesses and poor overall medical status which made them ineligible. Single institutions or a small group of collaborating institutions often took years to accrue the requisite numbers of patients with varying degrees of organ dysfunction [2].

It was often difficult to complete trials, even if well enrolled, because patients' organ dysfunction too often follows a trajectory of rapid decline, independent of drug administration; this was particularly true for patients with severe dysfunction. While progressive organ dysfunction was not a major issue in renal dysfunction trials, it was formidable in some hepatic dysfunction trials. In the face of rapidly progressive hepatic dysfunction, some patients could only be followed for a single cycle or less [14].

4 Progress in Overcoming Barriers

4.1 How Has Progress Been Achieved?

Barriers to conducting organ dysfunction trials have been largely overcome in the last 15 years.

The cooperative groups in oncology, the US National Cancer Institute (NCI), and the US FDA have been largely responsible for this progress.

4.2 Cooperative Groups and the National Cancer Institute

Two cooperative groups have developed committees whose explicit goals are to efficiently accrue to and conduct phase I clinical trials in patients with renal and hepatic dysfunction. Similarly, the NCI has developed and sponsored its own highly structured program and group. The Cancer and Leukemia (CALGB) Pharmaceutical and Therapeutics Committee, the Southwest Oncology Group (SWOG) Early Therapeutics Committee, and the NCI-Sponsored Organ Dysfunction Group (NCI-ODG) each perform phase 1 dose-escalation trials. Each committee is composed of multiple institutions from leading cancer centers which have extensive drug development expertise. Their trials provide safety/toxicity and pharmacokinetic data, which provides the foundation to build guidelines and recommendations for drug administration to other organ-impaired patients.

4.3 Hepatic Dysfunction Trial Design and Conduct and *Cohort Stratification by Severity*

4.3.1 Trial Design/Conduct

Approximately 60–100 patients are evaluated in a hepatic dysfunction trial. Patients on hepatic dysfunction trials are stratified, according to their degree of organ function/dysfunction. Typically, the first cohort stratification group is composed of patients who have normal organ function, and subsequent stratification groups are composed of patients with increasingly severe organ dysfunction. Thus, the patients in the first group serve as controls. Each severity cohort receives sequentially increasing dose levels of the study agent using a modified Fibonacci escalation design. Dose levels are expanded according to the standard 3 + 3 dose design; if one patient, at a dose level, experiences a dose-limiting toxicity, an additional three patients are enrolled in that cohort. The maximally tolerated dose is defined as the highest dose at which ≤ 1 out of at least six patients experiences a significant drug-related toxicity. After the maximally tolerated dose is established for each stratification, up to another

12 patients are dosed at that drug level; this is done to confirm that the pharmacokinetic and safety data from initial maximally tolerated dose cohort is consistent with the data from the larger sample of patients. Dose escalation occurs simultaneously in each cohort with normal, mild, moderate, and severe organ impairment although patients with greater impairment never receive a drug dose that has not already been proven to be safe in subjects with less impairment.

A unique feature of phase 1 studies in subjects with organ dysfunction, as opposed to other phase 1 oncology trials, is that toxic events in one cohort of a particular stratification group will lead to changes in the dose-escalation schema for the next, more organ-impaired stratification group. For example, if unacceptable toxicity is observed in ≥2/6 in cohort "X" of the less severely organ-impaired stratification group, patients in the next more severely organ-impaired stratification group will only be assigned to receive doses below those given previously to cohort "X." Additionally, although accrual into the different organ dysfunction stratification groups may occur concurrently or sequentially, there are unusual cases in which the study agent is believed to actually cause hepatotoxicity; when this scenario occurs, accrual to the next more severe hepatic stratification group cannot begin until sufficient safety data has been gathered from patients in the less severe hepatic dysfunction stratification group. The rate-limiting step for these trials is delay in enrolling patients who are healthy enough to meet all eligibility criteria but have the requisite degree of organ dysfunction; enrollment of patients with severe organ dysfunction is particularly challenging [30–32].

A different trial design paradigm was recently used by the CALGB in a hepatic dysfunction trial of sorafenib (and in a renal dysfunction trial); CALGB focused on defining sorafenib pharmacokinetics in a patient population rather than individual subgroups [33]. All patients received a single standard dose of sorafenib, followed by intensive pharmacokinetic sampling. Using this design, most patients were evaluable for the primary pharmacokinetic endpoint, but the design provided far less toxicity information. A shortcoming of this design is that oncologists base dose modifications on toxicity rather than pharmacokinetics [33].

4.3.2 Stratification by Severity

A variety of hepatic function scoring systems, which make use of commonly available information, have been developed. A few of these are the Child–Pugh Classification for alcoholic cirrhosis and portal hypertension, shown in Table 3 below; the Mayo risk scores for primary biliary cirrhosis and primary sclerosing cholangitis; and the Mayo End-Stage Liver Disease (MELD) score [34, 35].

None of these, however, are very helpful in the determination of optimal oncologic drug dosing. Although the Child–Pugh Classification of Liver Dysfunction is still being used by trialists in oncology, it has several flaws. The indices used to determine the CPC score of albumin, prothrombin time (PT)/international normalized ratio (INR), and encephalopathy can be affected by conditions other than liver dysfunction such as poor nutrition, sepsis, and metabolic disorders. Additionally, stratifying patients to Child–Pugh Classification category C and sometimes to category B may be futile as these patients may be encephalopathic and, therefore, unable to understand and give informed consent. In response to these issues, CALGB developed and the NCI Organ Dysfunction Group (NCI-ODG) uses their own classification system, shown in Table 4. In the latter system, patients are classified by their degree of liver dysfunction (i.e., mild, moderate, and severe) based on serum levels of transaminases and bilirubin, laboratory tests routinely obtained during cancer therapy. This classification is also not perfect although it has an advantage over the Child–Pugh Classification system because of its simplicity. NCI-ODG and CALGB hepatic dysfunction trials are stratified according to their classification system, making it possible for community oncologists to adopt the dosing guidelines, based on these trials with minimal effort.

Table 3 Child–Pugh classification of liver dysfunction

	1	2	3
Encephalopathy grade	None	1 or 2	3 or 4
Ascites[1]	Absent	Slight	Moderate
Serum bilirubin (mg/dL)	<2	2 to 3	>3
Serum albumin (g/dL)	>3.5	2.8 to 3.5	<2.8
Prothrombin time (sec prolonged)	<4	4 to 6	>6

[1]Ascites: slight – asymptomatic, Moderate – requires intervention

	Encephalopathy Grading (EEG required for Gr. 2, 3, 4)
0	Normal consciousness, personality, neurological exam, EEG
1	Restless, sleep disturbed, irritable/agitated, tremor, impaired handwriting, 5 cps waves
2	Lethargic, time-disoriented, inappropriate, asterixis, ataxia, slow triphasic waves
3	Somnolent, stuporous, place-disoriented, hyperactive reflexes, rigidity, slower waves
4	Unrousable coma, no personality/behavior, decerebrate, slow 2-3 cps delta activity

Childs A (mild dysfunction) 5-6 points

Childs B (moderate dysfunction) 7-9 points

Childs C (severe dysfunction) 10-15 points

Table 4 CALGB/NCI classification of liver dysfunction

Group	Group A	Group B	Group C	Group D	Group E
Liver function	Normal	Mild	Moderate	Severe	Liver transplant
Total bilirubin	≤ULN	B1: ≤ULN B2: >1.0×–1.5× ULN	>1.5×–3× ULN	>3× ULN	Any
SGOT/AST	≤ULN	B1: >ULN B2: any	Any	Any	Any

4.4 Renal Dysfunction Trial Design and Conduct and Stratification by Severity

Renal dysfunction trial design is conceptually the same as that for the hepatic dysfunction trials. Renal dysfunction trials depend on very accurate GFR measurements because cancer drugs often have a narrow therapeutic index in patients with normal renal function and an even narrower one in patients with impaired kidneys [31, 32]. The NCI-ODG used GFR-based dosing of carboplatin in order to achieve a targeted area under the curve [34–36]. In an Action Letter, dated October 1, 2010, CTEP/NCI announced that by December 31, 2010, all clinical chemistry laboratories in the United States

Table 5 Stratifications for renal function by the CALGB/NCI-ODG and the FDA [18]

Stratification	CALGB/NCI-ODG	FDA
Acceptable function	CrCl > 60 mL/min	CrCl > 80 mL/min
Mild dysfunction	CrCl = 40–59 mL/min	CrCl = 50–79 mL/min
Moderate dysfunction	CrCl = 20–39 mL/min	CrCl = 30–49 mL/min
Severe dysfunction	CrCl < 20 mL/min	CrCl < 30 mL/min

would measure serum creatinine using standardized isotope dilution mass spectrometry and informed investigators conducting CTEP-sponsored trials that "measurement of serum creatinine" by the IDMS method could result in an overestimation of GFR in some patients with normal renal function. The letter provided new guidance for AUC-based dosing of carboplatin.

There are several ways to score renal dysfunction. The CALGB, NCI-ODG, and the FDA's renal function scoring systems are somewhat different (see Table 5).

4.5 How Successful Have the NCI and Cooperative Groups Been?

The efficiency with which SWOG, CALGB, and the NCI-ODG committees have operated is evident by their very impressive track records. Some trials have been completed in less than 1.5 years, a dramatic improvement from the duration of similar trials performed before the committees took action [2]. A partial list of successful dose-escalation trials conducted since 2003 is shown in Table 6.

4.6 FDA Initiatives

If the FDA's previous stance on organ dysfunction trials was limited in the past, the guidelines which it subsequently promulgated went far to rectify its position. In 1998, they issued "Guidance for Industry: Pharmacokinetics in Patients with Impaired Renal Function—Study Design, Data Analysis, and Impact on Dosing and Labeling" (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM204959.pdf). The FDA also provided a renal function classification system, shown in Table 5. The system adopted was originally produced by the National Kidney Foundation. In 2003, the FDA also issued a set of guidelines to industry about pharmacokinetics in patients with hepatic dysfunction (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM072123.pdf) (Table 7).

5 Next Steps: Learning from the Past and Adjusting to the Future

5.1 Perspective

It is patently clear that recent efforts have resulted in an efficient system by which phase 1 clinical trials, in patients with organ impairment, can be and are conducted. Patients with cancer and hepatic or renal dysfunction can now receive certain oncology drugs—with the knowledge that the therapeutic index has been determined for their level of organ dysfunction. It is to be hoped that future trials will be conducted even earlier in the development of new anticancer agents, before these are licensed for widespread use [30–32]. Nonetheless, if these efforts are to be successful in the future, researchers and the FDA must learn from the past and be aware of new considerations when they arise.

Table 6 A partial list of hepatic and renal dysfunction trials completed between 2003 and 2008

Agent	Study	Cooperative group	Number of patients	Classification	Results	References
Bortezomib	Activity and safety of bortezomib in multiple myeloma patients with advanced renal failure: a multicenter retrospective study	NCI-ODG	24 (retrospective)	Advanced renal failure	The standard 1.3 mg/m^2 dose appeared well tolerated in patients with mild to moderate renal dysfunction	[41]
Bortezomib	Bortezomib in recurrent and/or refractory multiple myeloma. Initial clinical experience in patients with impaired renal function	NCI-ODG	256	Varying renal function (10 with CrCl ≤30 mL/min)	Patients with CrCl ≤ 50 mL/min (n = 52 patients) had similar rates of discontinuation and similar adverse event profiles	[42]
Bortezomib		NCI-ODG	51	Cohort I, AST > ULN, bilirubin within normal limits or cohort IB, bilirubin >1–1.5 × ULN; cohort II, bilirubin >1.5–3× ULN (AST any); cohort III, 3× ULN (AST any)	Patients with moderate to severe hepatic dysfunction (cohorts II and III) should have dose reduced to 0.7 mg/m^2 per injection	[43]
Carboplatin	Pharmacokinetics and dosage reduction of *cis*-Diammine (1,1-cyclobutanedicarboxy-lato) platinum in patients with impaired renal function	CALGB	22	Cohort I, CrCl ≥ 40 mL/min; cohort II, CrCl 20–39 mL/min; cohort III, CrCl 0–19 mL/min	Patients with reduced CrCl had increased thrombocytopenia	[44]
Erlotinib	Phase I and pharmacokinetic study of erlotinib for solid tumors in patients with hepatic or renal dysfunction: CALGB 60101	CALGB	55	Cohort 1, AST ≥ 3× ULN; cohort 1a, albumin <2.5 g/dL; cohort 2, direct bilirubin of 1–7 mg/dL; cohort 3, creatinine of 1.6–5.0 mg/dL	Patients with renal dysfunction tolerate 150 mg of erlotinib daily and seem to have an erlotinib clearance similar to patients without organ dysfunction. Patients with hepatic dysfunction should be treated at a reduced dose (i.e., 75 mg daily) consistent with their reduced clearance	[45]

Gemcitabine	Phase I and pharmacokinetic trial of gemcitabine in patients with hepatic or renal dysfunction: Cancer and Leukemia Group B 9565	CALGB	40	Cohort I, AST ≤2× ULN and serum bilirubin <1.6 mg/dL; cohort II, serum bilirubin 1.6–7.0 mg/dL; cohort III, serum creatinine 1.6–5.0 mg/dL and normal liver function	Patients with elevated AST tolerated gemcitabine without increased toxicity, no dose reduction needed. Patients with elevated bilirubin have increased toxicity so dose reduction is recommended. Patients with increased creatinine had increased sensitivity to gemcitabine, no firm dosing recommendations	[46]
Ibandronate	Renal safety and pharmacokinetics of ibandronate in multiple myeloma patients with or without impaired renal function	Multi-institutional trial	40	Renal function grading based on creatinine clearance (0: >80; 1: 50–79; 2: 30–49, and 3: <30 mL/min)	In patients with compromised renal function, there was no evidence of acute nephrotoxicity with ibandronate	[47]
Imatinib	Phase I and pharmacokinetic study of imatinib mesylate in patients with advanced malignancies and varying degrees of renal dysfunction: a study by the National Cancer Institute Organ Dysfunction Group	NCI-ODG	60 adults	Varying degrees of renal function	Daily imatinib doses up to 600 or 800 mg were well tolerated by patients with mild and moderate renal dysfunction, respectively, despite their having increased imatinib exposure	[36]
Imatinib	Phase I and pharmacokinetic study of imatinib mesylate in patients with advanced malignancies and varying degrees of liver dysfunction: a study by the National Cancer Institute Organ Dysfunction Group	NCI-ODG	89 adults with varying solid tumors	Stratified into four groups according to serum total bilirubin and AST	The maximal recommended dose of imatinib for patients with mild liver dysfunction is 500 mg/day. Dosing guidelines for patients with moderate and severe liver dysfunction remain undetermined	[48]
Irinotecan	Phase I and pharmacokinetic trial of irinotecan in patients with hepatic or renal dysfunction or with prior pelvic radiation: Cancer and Leukemia Group B 9863	CALGB	35	Cohort I, AST ≤3× ULN and serum bilirubin <1.0 mg/dL; cohort II, serum bilirubin 1.0–7.0 mg/dL; cohort III, serum creatinine 1.6–5.0 mg/dL and normal liver function; cohort IV, prior pelvic XRT and normal liver and renal function	Patients with elevated bilirubin had increased toxicity; elevated AST, creatinine, or prior pelvic radiation did not increase toxicity	[49]

(continued)

Table 6 (continued)

Agent	Study	Cooperative group	Number of patients	Classification	Results	References
Oxaliplatin	Oxaliplatin pharmacokinetics and pharmacodynamics in adult cancer patients with impaired renal function. National Cancer Institute Organ Dysfunction Group Study	NCI-ODG	34	Stratified by 24-h urinary CrCl into four renal dysfunction groups: Group A (control) CrCl, > or =60 mL/min; Group B (mild) CrCl, 40–59 mL/min; Group C (moderate) CrCl, 20–39 mL/min; and Group D (severe) CrCl, <20 mL/min	Oxaliplatin pharmacokinetics is altered in patients with renal impairment, but a corresponding increase in oxaliplatin-related toxicities is not observed	[50, 51]
Oxaliplatin	Dose-escalating and pharmacologic study of oxaliplatin in adult cancer patients with impaired hepatic function: a National Cancer Institute Organ Dysfunction Group study	NCI-ODG	60	Variable hepatic function or liver transplantation	Oxaliplatin at 130 mg/m^2 every 3 weeks was well tolerated in all patients with impaired liver function. Dose reductions of single-agent oxaliplatin are not indicated in patients with hepatic dysfunction	[52]
Paclitaxel	Phase I and pharmacokinetic trial of paclitaxel in patients with hepatic dysfunction: Cancer and Leukemia Group B 9264	CALGB	81	Cohort I, AST ≤2× ULN and serum bilirubin <1.5 mg/dL; cohort II, serum bilirubin 1.6–3.0 mg/dL; cohort III, serum bilirubin >3.0 mg/dL	Patients with serum bilirubin >1.5 mg/dL had increased toxicity; dose reduction is necessary	[53]
Sorafenib	Phase I and pharmacokinetic study of sorafenib in patients with hepatic or renal dysfunction: Cancer and Leukemia Group B 60301	CALGB	138	Cohort I, serum bilirubin ≤ULN, AST ≤ ULN and CrCl ≥60 mL/min; cohort II, bilirubin more than ULN but ≤1.5× ULN and/or AST > ULN; cohort III, CrCl 40–59 mL/min; cohort IV, bilirubin 1.5–3× ULN (any AST); cohort V, CrCl 20–39 mL/min; cohort VI, bilirubin 3–10× ULN (any AST); cohort VII, CrCl <20 mL/min; cohort VIII, albumin <2.5 mg/dL (any bilirubin or AST); cohort IX, hemodialysis	Patients with severe hepatic or renal dysfunction (cohorts IV–IX) did not tolerate sorafenib well. No significant associations between the pharmacokinetics parameters and cohorts were observed	[33]

Table 7 Guidance for industry: pharmacokinetics in patients with impaired hepatic function: study design, data analysis, and impact on dosing and labeling

This guidance recommends that drug manufacturers consider conducting pharmacokinetics studies in patients with impaired hepatic function if:
• The drug or active metabolite is subject to substantial hepatic metabolism or excretion
• The drug/metabolite's hepatic metabolism and/or excretion is less than 20 %, but evidence indicates it has a narrow therapeutic range
• One or more of the hepatic pathways of elimination might become important in the event of renal failure
• The drug's metabolism is unknown and no data suggest that hepatic elimination routes are minor, in which case the drug should be considered extensively metabolized

5.2 A Lesson from the Past: Inclusion Criteria

McHayleh and colleagues recently analyzed renal function in 12,575 patients who were enrolled in CTEP-sponsored phase 1 clinical trials from 1979 to 2005 to evaluate the percentage of patients with acceptable renal function according to three different formulas (Cockcroft–Gault, Jelliffe, and Levey) and GFR according to MDRD [37]. Distributions of CrCl and GFR were defined, and patients were classified as having normal renal function or mild, moderate, or severe renal dysfunction. Approximately 40 % of these patients had mild renal dysfunction, as defined by FDA criteria (i.e., CrCl between 59 and 79 mL/min). There was no increase in hematologic or non-hematologic adverse events between those with mild dysfunction and those with normal renal function, as defined by FDA criteria. Ivy and collaborators recently completed a review of data from all patients who have participated in the National Cancer Institute's Cancer Therapy Evaluation Program (CTEP) trials [38]. They applied NCI-ODG criteria for renal dysfunction and found that most of these trials mandated that participants have "acceptable" renal function (i.e., CrCl $\geq$ 60 mL/min); a small proportion of these trials was conducted in subjects with varying degrees of renal dysfunction (Dr. Percy Ivy, personal communication). They also found that patients who were specifically enrolled in renal dysfunction trials because of mild renal impairment (i.e., CrCl between 40 and 59 mL/min) generally tolerated drugs at the same doses as did patients with normal renal function. The maximally tolerated dose for patients with only mild dysfunction was, generally speaking, the same as the maximally tolerated dose for patients with "acceptable" renal function. If subjects with mild renal impairment were excluded from future renal dysfunction oncology phase 1 trials, as these researchers suggest, time to trial completion would be shortened without compromising the ability to obtain *critical* information. Possibly, as past experience continues to be evaluated, other lessons will be gleaned and applied to future trials.

5.3 Adjusting to the Future

5.3.1 Adjusting to the Future: Changes in Incidence of Mild, Moderate, or Severe Organ Dysfunction

It is possible that, over time, there will be changes in the epidemiology of organ dysfunction. A decrease in renal dysfunction overall has already been documented [37]. In the future, severe organ dysfunction, for example, may become much more common than mild dysfunction. Should this occur, it may become necessary for phase 1 trial groups to open more, or different, investigative sites such as those with very active transplant and dialysis programs. Although there are only hints that some incidence patterns may be changing, it is likely that the "graying of America" will contribute to this in the future.

Furthermore, advances in the treatment of organ dysfunction, particularly early organ dysfunction, *as well as* financial constraints on treatment of advanced organ dysfunction will likely also impact the incidence.

5.3.2 Adjusting to the Future: Changes in Drug Formulation

Changes in drug formulation are occurring and are expected to occur more commonly in this century. New drug formulations will have implications for phase 1 trials. For example, it is likely that nanoparticle technology will be applied more frequently in the development of new oncology drugs, and new study designs may be needed to accommodate this [39].

5.3.3 Adjusting to the Future: The Challenge of Success

Another challenge for future organ dysfunction trials may be a direct result of the success of phase 1 organ dysfunction trials. Having successfully completed numerous single-agent trials, the next frontier may be conducting phase 1 combination trials for the evaluation of widely accepted oncologic drug regimens. Such trials, if possible, will pose unique pharmacokinetic and pharmacodynamic challenges. Yet another, somewhat less challenging frontier may be conducting single-agent organ dysfunction trials in which the study drug is known to be toxic to the already impaired organ. The purpose of such trials would be to gather information to answer the question "are patients with impaired organs more likely to be predisposed to experience organ-specific known adverse events?" This type of trial would probably need to be done in the late post-market period because it may require large numbers of patients. For example, the NCI-ODG phase I trial of the hepatotoxic pazopanib, a tyrosine kinase inhibitor of VEGFR, was conducted in patients with hepatic impairment [40]. This trial does not have anywhere near the power to detect a possible correlation between baseline hepatic dysfunction and drug-related hepatotoxicity.

6 Conclusions

Patients with impaired renal and hepatic function and cancer present a substantial challenge to the practicing oncologist. Such patients have historically been underrepresented in oncology drug development clinical trials, leading to a paucity of data regarding how best to treat these patients. In the absence of data, oncologists may arbitrarily reduce doses of active chemotherapeutic agents in a desire to prevent excessive toxicities. Recently, there have been impressive efforts to systematically study anticancer agents in organ-impaired patients and evaluate dosing, toxicities, and pharmacokinetics to make recommendations regarding their use. However, given that patients with hepatic and renal dysfunction constitute high-risk populations, trials in them must be conducted with intensive patient monitoring at centers by physicians with expertise in drug development. Due to the overall medical status of such patients, most may not meet eligibility criteria set forth in the trials, making patient accrual an important consideration for the timely completion of these high-priority studies. However, the clinical relevance and importance of the data generated from such trials justifies the ongoing emphasis and resources being employed to conduct such studies.

Tremendous progress has been made in overcoming barriers to clinical investigation in these special populations, but the future will hold new challenges. In keeping with the spirit of the Americans with Disabilities Act and Health Care Reform, it is hoped that people with organ "disabilities" will be afforded cancer treatments which are safe and effective for them just as other patients without organ "disabilities" are. The future, of course, is in our own hands.

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Drug Formulations: How these Affects Anticancer Drug

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Abstract Delivery of anticancer drugs to tumors is often critically dependent on the pharmaceutical drug formulation. In this chapter, we will illustrate how the formulations of anticancer drugs can affect their pharmacokinetics and pharmacodynamics. This will be exemplified by discussing a number of nanomedicines that have been developed to improve drug delivery and efficacy of anticancer agents.

Keywords Liposomes • Polymer-drug conjugates • Polymeric micelles • Drug formulation • Pharmacokinetics and pharmacodynamics • Antibody-drug conjugates • Drug delivery • Drug efficacy • Anticancer drugs • Nanomedicines • Tumor-targeted nanomedicines

1 Introduction

The idea that anticancer agents ideally should act like "magic bullets," which target and destroy tumor cells while causing no damage to healthy tissues, was initially introduced by Paul Ehrlich about a century ago [1]. Today, this idea is still relevant and all kinds of strategies have been employed to approach this "magic bullet" concept, some being more successful than others. Relatively successful examples are targeted small molecule therapeutics (e.g., protein kinase inhibitors) that are rationally designed to target specific mutated molecules that drive the progression of individual cancers [1, 2]. Another example of targeted therapy are monoclonal antibodies that are engineered to recognize and

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Table 1 Examples of tumor-targeted nanomedicines

Nanomedicines	Tumor targeting
Liposomes	Active and passive
Polymer–drug conjugates	Active and passive
Polymer–protein conjugates	Passive
Polymeric micelles	Passive
Protein–drug conjugates	Passive
Lipoplex/polyplex	Passive
Antibody–drug conjugates[a]	Active

[a]For further information, please see the chapter on "Antibody–Drug Conjugates"

bind specifically to proteins on the surface of cancer cells, eventually leading to cancer cell death [2], or that target specific ligands (e.g., VEGF (vascular endothelial growth factor)), resulting in the inhibition of angiogenesis [3].

However, not only the mechanism of action of an anticancer agent determines its efficacy and adverse effects, but also the way an agent is delivered to a tumor can play an important role herein. Despite the fact that targeted agents are designed to specifically exert their effect on tumor cells or on tumor vasculature, delivery issues often result in off-target effects and unwanted toxicity. Over the past decades, much effort has been put and progress has been made in the development of novel delivery strategies, including nanomedicines, with the aim to improve tumor targeting and penetration of both old and new anticancer drugs while minimizing the adverse effects of these agents. Nanomedicines are drug formulations that can be defined as "nanometer size scale complex systems, consisting of at least two components, one of which being the active ingredient" (see Table 1) [4].

The aim of this chapter is to illustrate how the formulations of anticancer drugs can affect their pharmacokinetics and pharmacodynamics. This will be exemplified by discussing a number of nanomedicines that have been developed to improve drug delivery and efficacy of anticancer agents. Despite the large number of preclinical studies that have been published on this topic, the focus will be on anticancer nanomedicines that have entered clinical studies and on marketed products.

2 Drug Delivery and Drug Penetration

Delivery and penetration issues have been an important motivation for the development of nanoscale drug formulations (Table 1). The principal objectives of such tumor-targeting nanomedicines are to alter anticancer drug pharmacokinetics and pharmacodynamics, specifically (1) to improve drug targeting, (2) to restrict access of drug to healthy tissues, thereby reducing nonspecific toxicity, and (3) to make sure that a sufficient amount of the drug can reach its pharmacological target [5]. To better understand the rationale behind the design of nanomedicines, and how these nanoscale drug formulations can affect the pharmacokinetics and pharmacodynamics of the incorporated anticancer drugs, some general aspects of drug delivery and characteristics of solid tumors are discussed.

2.1 Drug Delivery to Tumors

Most classical anticancer drugs (e.g., taxanes, anthracyclines, and platinum drugs) and targeted small molecule therapeutics (e.g., protein kinase inhibitors, proteasome inhibitors, and histone deacetylase inhibitors) show an unfavorable distribution upon intravenous and/or oral administration [6]. This is

partly the result of the fact that these agents are rapidly cleared from the systemic blood circulation and penetrate healthy tissues where they cause nonspecific toxicity. For orally administered drugs, metabolism and elimination upon first pass through intestinal epithelial cells and the liver can significantly lower the systemic drug exposure resulting in only a small fraction eventually reaching the tumor. Moreover, due to tumor characteristics, penetration of drug into solid tumors is often limited [7]. Therefore, relatively high doses of anticancer agents have to be administered to gain sufficient efficacy. As a result, healthy tissues are also exposed to relatively high drug concentrations, resulting in nonspecific side effects and toxicity. In other words, drug nonspecificity limits efficacy, which is best exemplified by cytotoxic anticancer drugs [8].

2.2 Drug Penetration into Solid Tumors

Most cells in our body are only a few cell diameters away from the nearest blood vessel enabling efficient delivery of nutrients and oxygen to the cells. In addition, a dense network of intact and highly organized blood vessels is a prerequisite for efficient delivery of most anticancer drugs. Poorly organized vascular architecture in solid tumors is mainly due to the high proliferation rate of tumor cells compared to blood vessel cells, resulting in a reduction in vascular density, imperfectly constructed leaky blood vessels, and compression of blood and lymphatic vessels by cancer cells. As a consequence, solid tumors often display hypoxia and an increased interstitial fluid pressure. In addition, lack of functional lymphatic vessels results in accumulation of metabolic waste product (i.e., lactic acid and carbonic acid) that lowers the extracellular pH in solid tumors. Together these factors determine the microenvironment of solid tumors and can seriously restrict the delivery and penetration of antitumor drugs into tumor cells [7]. The combinations of leaky blood vessels and the lack of functional lymphatic vessels can result in the accumulation of plasma protein in the extracellular space of tumors that leads to osmosis and high interstitial fluid pressure, which in turn restrict the drug penetration into tumor tissue. Cells in hypoxic areas of the tumor display a slower proliferation rate and are therefore less sensitive to classical cytotoxic agents, as these specifically kill rapidly dividing cells. Due to the low extracellular pH, the penetration of basic anticancer drugs into tumor cells is decreased since these compounds are cationic and charged in an acidic environment and therefore not taken up into the cells [9]. Tumor cells can also display multiple molecular mechanisms of drug resistance. One example is the ATP-binding cassette (ABC) multidrug efflux transporters that are localized in the cellular membrane of tumor cells and can actively extrude anticancer drugs, thereby rendering tumor cells resistant to chemotherapy [10]. The latter molecular mechanisms of drug resistance are beyond the scope of this chapter and are discussed in the "ABC Transporters" chapter.

3 Nanoscale Drug Delivery Systems

A selected overview of nanoscale drug delivery systems is given in Table 1. Because most nanomedicines have a relatively large molecular weight and size, they remain in the bloodstream for a prolonged time and mainly diffuse and accumulate at sites with excessively leaky microvasculature, like tumor tissues, while normal endothelium is much less permeable for these complexes [8]. This principle is called "enhanced permeability and retention" (EPR) and results in high local drug concentrations (Fig. 1) [5, 6]. EPR was firmly established in preclinical tumor models, and although it has thus far been proven difficult to convincingly demonstrate EPR in the clinic, it is likely that this principle also applies to tumors in humans. As a consequence of EPR, drug penetration into healthy tissues with normal vasculature is usually limited, resulting in lower toxicity and adverse effects. This often leads to a higher

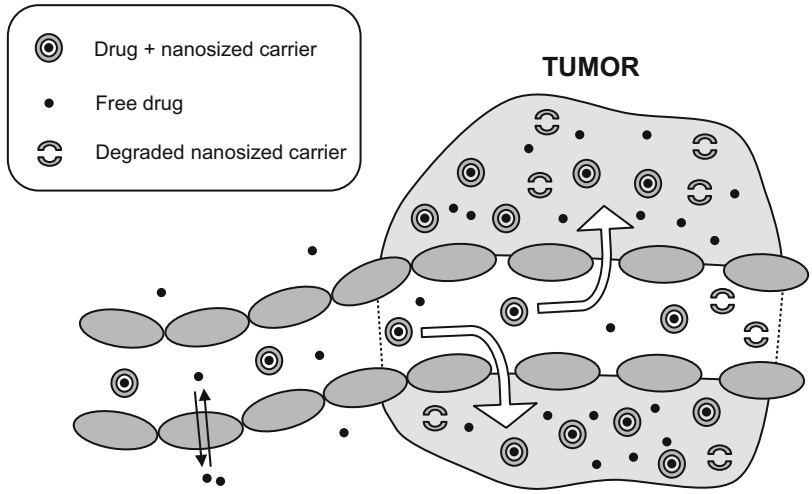

Fig. 1 Principle of enhanced permeability and retention (EPR). Because of their size, long-circulating nanosized carriers generally do not pass through normal vasculature, whereas free drug does. However, nanosized carriers can penetrate the leaky vasculature of solid tumors and degrade in the tumor interstitium, where they release free drug and create a high local drug concentration

maximum tolerated dose for anticancer drugs that are incorporated in nanomedicines, which may enable more intense treatment regimes compared to administration of free drug. However, by altering the pharmacokinetics, the EPR effect does not only reduce drug toxicity of anticancer agents that are incorporated in nanomedicines but can also alter the toxicity profile. For example, the dose-limiting toxicities for liposomal doxorubicin (Caelyx/Doxil) are hand–foot syndrome and stomatitis. This toxicity profile differs prominently from that of free doxorubicin administered by bolus or rapid infusion, whereas it resembles that of prolonged continuous infusion of free drug [11]. Nonetheless, when nanoscale drug delivery systems are often, in general less systemic drug needs to be administered to achieve sufficient drug concentrations in the tumor [8].

Important characteristics of the carrier material that is used in the nanosized drug formulations are biocompatibility and biodegradability. This means that the unloaded carriers must be nontoxic themselves and/or must be metabolized or degraded into nontoxic components that are cleared through the blood circulation. Small particles (<30 nm) are generally excreted via the urine, whereas (fragments of) carrier systems >30 nm are taken up and degraded by macrophages in the liver and the spleen [8]. The latter clearance mechanism is called the mononuclear phagocytic system (MPS) [12]. Importantly, the surface properties of nanosized carriers can importantly affect their clearance rate. This is explained by the fact that phagocytosis can be enhanced by opsonins, which are molecules that bind to foreign material (e.g., IgG and IgA antibodies), the complement cascade system, and mannose-binding lectin [13]. In other words, the easier the opsonins bind to carrier material, the more rapid this material will be cleared from the blood circulation.

3.1 Liposomes

Liposomes are the archetypal nanoscale drug delivery systems for anticancer drugs. Liposomes are spherical vesicles with a diameter of approximately 25 nm to 2.5 μm that are composed of a lipid bilayer surrounding an aqueous compartment in which hydrophilic drugs can be stored. Lipophilic drugs can be incorporated into or associated with the bilayer (Fig. 2), which consists of phospholipids

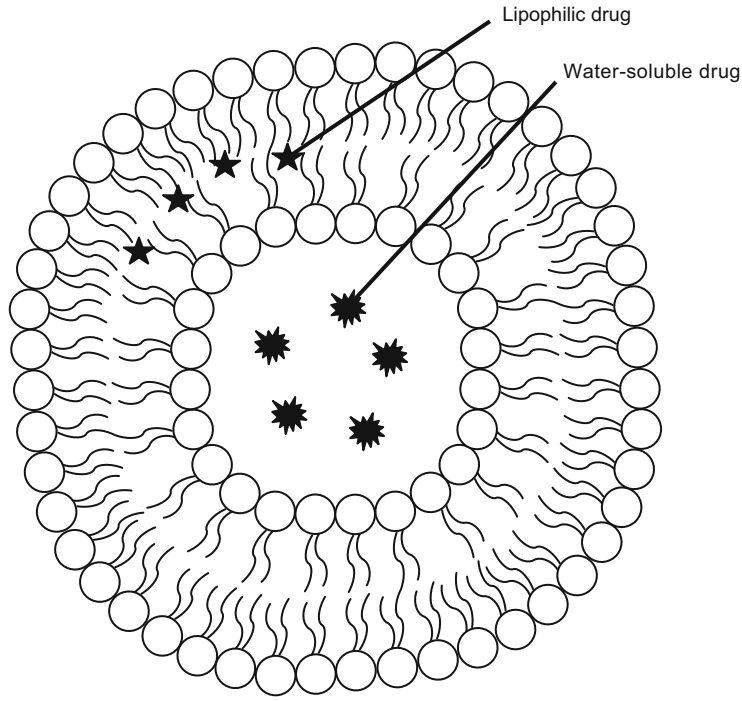

Fig. 2 Schematic representation of a liposome and its loading with various drugs

of a natural or synthetic origin and cholesterol molecules that have an amphiphilic nature and therefore spontaneously form a bilayer in an aqueous environment. Stability of liposomes depends on lipid composition and cholesterol content of the liposomal membrane, and by lyophilization, the stability and shelf life of liposomal drug formulations can be increased from several months to years [14]. There are multiple ways to load drugs into liposomes, including (1) addition of phospholipids to an aqueous saturated drug solution, so that drug will be encapsulated when the liposomes are formed, (2) dissolving the drug in an organic solvent and making use of solvent exchange mechanisms, (3) using lipophilic drugs, and (4) using a transmembrane pH gradient for drug loading [8]. The loading method can affect the release of drug from liposomes (e.g., precipitation of the drug within the liposomes can importantly slow down the rate of drug release) [15].

Conventional liposomal carriers for anticancer drugs passively target tumors by penetrating the leaky tumor vasculature (EPR principle) and localizing in the interstitial space of the tumor where the contents are released. However, after systemic administration, conventional liposomes are rapidly cleared by the MPS in the liver and spleen [16]. To prolong the half-life of liposomes in the systemic blood circulation, different methods have been employed including the use of synthetic phospholipids conjugated to polyethylene glycol (PEG) [17, 18]. PEG is an inert and biocompatible polymer that forms a hydrophilic layer around the liposomes, which are then classified PEGylated or "stealth" liposomes [19]. The PEG shield prevents recognition of liposomes by opsonins (antibodies, the complement cascade system, and mannose-binding lectin) and thereby markedly reduces liposomal clearance by the MPS [20, 21]. Figure 3 illustrates how liposomal drug formulations affect plasma pharmacokinetics and can improve the delivery of anticancer drugs. Upon intravenous administration, anticancer agents incorporated in liposomes are retained longer in the blood circulation compared to free drug (Fig. 3, left panel). This results in enhanced tumor penetration of liposomes by the EPR principle and, subsequently, in a higher drug exposure of the tumor than after intravenous administration of free drug (Fig. 3, right panel).

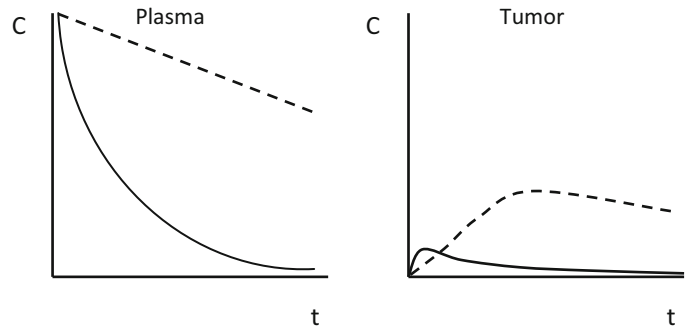

Fig. 3 Illustration showing how liposomal drug formulations can improve the delivery of anticancer drugs to a solid tumor. *Left panel*: plasma concentration–time curves after intravenous administration of free drug (*solid line*) or drug encapsulated in liposomes (*dashed line*). *Right panel*: concentration of free drug over time in the tumor after intravenous administration of free drug (*solid line*) or drug encapsulated in liposomes (*dashed line*)

Table 2 Examples of liposomal anticancer drugs that are currently approved or in clinical trials

Compound	Name	Indication	Status
Liposomal doxorubicin	Myocet, Caelyx (Doxil)	Breast, ovarian, Kaposi sarcoma	Approved
Liposomal daunorubicin	Daunoxome	Kaposi sarcoma	Approved
Liposomal vincristine	Onco-TCS	Non-Hodgkin lymphoma	Approved
Liposomal cytarabine	DepoCyte	Lymphomatous meningitis (intrathecal infusion)	Approved
Liposomal lurtotecan	OSI-221	Ovarian, head and neck, lung	Phase II
Liposomal cisplatin	SPI-77, Lipoplatin	Lung, pancreas, ovarian	Phase II/III
Thermosensitive liposomal doxorubicin	ThermoDox	Breast, liver	Phase III

Table 3 Pharmacokinetic properties in human of three commercial preparations of doxorubicin

	Free doxorubicin	Myocet	Caelyx/Doxil (PEGylated)
Dose (mg/kg)	1.2	1.8	1.5
AUC (mg h/L)	3.5	19.4	4,082
Clearance (mL/h)	25,300	9,520	23
Vss (L)	365	139	3.0
Half-life (h)	0.06/10.4[a]	<1/52.6[a]	84

Free doxorubicin is compared to doxorubicin encapsulated in conventional liposomes (Myocet) and in PEGylated liposomes (Caelyx/Doxil). Liposome diameter: 85–150 nm. Data are normalized using an average body surface area of 1.7 m^2 and an average body weight of 70 kg. Examples of representative studies [55–57]. Reprinted from [22] with permission from Dove Medical Press Ltd.
[a]Biphasic elimination

The clinical development of liposomal formulations of classical anticancer drugs focused on the anthracyclines, which are cationic amphiphiles that are conducive to efficient and stable liposomal entrapment [22]. Moreover, development of liposomal formulations seemed an attractive strategy to alter the pharmacokinetic and pharmacodynamic profile of anthracyclines with the aim to improve efficacy while reducing the risk of acute and cumulative cardiotoxicity. Accordingly, the conventional liposomal doxorubicin formulation Myocet and the PEGylated liposomal doxorubicin formulation Caelyx/Doxil were among the first liposomal anticancer drug formulations to be approved by the regulatory authorities (Table 2). The pharmacokinetic properties in human of the commercially available free doxorubicin, Myocet, and Caelyx/Doxil are summarized in Table 3 [22]. Liposomal doxorubicin shows a markedly decreased clearance and volume of distribution compared to administration

of free drug, resulting in a higher AUC compared to free doxorubicin. From Table 3, it is evident that PEGylation even further improves the pharmacokinetic profile of liposomal doxorubicin (i.e., the retention of the drug in the systemic circulation is further prolonged) enabling enhanced tumor penetration by the EPR principle (Figs. 1 and 3). Indeed, phase III clinical trials comparing either Myocet or Caelyx/Doxil to free doxorubicin showed at least comparable response rates and progression-free survival, whereas cardiac events and congestive heart failure were significantly reduced using the liposomal formulations [21, 23–25]. In patients with AIDS-related Kaposi sarcomas that display dense and highly permeable vasculature, Caelyx/Doxil showed increased efficacy and reduced cardiac toxicity over standard therapy [26]. The dose-limiting toxicities of Caelyx/Doxil are hand–foot syndrome and stomatitis, which is markedly different compared to free doxorubicin administered by bolus or rapid infusion and resembles that of prolonged continuous infusion of free drug [11]. The PEGylated liposomal doxorubicin formulation thus importantly affects the pharmacokinetics and pharmacodynamics of doxorubicin: it enhances tumor penetration and changes the toxicity profile of doxorubicin.

Given the success of Caelyx/Doxil, new liposomal formulations have been approved or are being evaluated in clinical trials (Table 2). A liposomal formulation of cytarabine (DepoCyte) has been designed for direct administration into the cerebrospinal fluid (CSF) and was recently approved for the treatment of patients with lymphomatous meningitis, a life-threatening complication of lymphoma [27]. DepoCyte is a sustained-release formulation that is a suspension of cytarabine encapsulated in multivesicular lipid-based particles, which are structurally distinct from lamellar liposomes. Multivesicular lipid-based particles consist of numerous non-concentric water-filled compartments, which have a diameter of approximately 3–30 μm [28]. After intrathecal injection, the biodegradation of the lipid membranes at body temperature leads to a gradually release of cytarabine into the cerebral spinal fluid resulting in a significantly prolonged drug exposure in the brain. DepoCyte has a half-life of 130–277 h compared with 3–4 h for conventional cytarabine [29]. Therefore, DepoCyte can be dosed once every 2 weeks, whereas for conventional cytarabine a spinal injection is required twice per week.

Another novel liposomal formulation that is currently being evaluated in a phase III trial is ThermoDox, a heat-activated liposomal encapsulation of doxorubicin [30]. The entrapped doxorubicin in ThermoDox is only released from the liposomes when focused heat (>40°C) is applied, aiming to achieve high doxorubicin concentrations in tumor tissue, while side effects in healthy tissues are reduced. The phase III trial is conducted in patients with primary liver cancer and evaluates the efficacy of ThermoDox in combination with radiofrequency ablation (RFA) compared to RFA alone, with progression-free survival being the primary endpoint [31]. Furthermore, numerous preclinical studies are conducted to develop novel liposomal anticancer drug formulations or to improve exiting formulation (e.g., improving the lipid bilayer or the protective coating).

In addition to passive tumor targeting, liposomes can be modified to enable active tumor targeting. For example, fragments of antibodies can be attached to the surface of PEGylated liposomes for selective tumor targeting of anticancer drugs. These so-called immunoliposomes can offer substantial benefits over the use of free drug and nontargeted liposomes (e.g., anti-ERBB2 immunoliposomes loaded with doxorubicin showed a significant greater antitumor activity than free drug or nontargeted liposomes in several tumor xenograft models) [32].

3.2 Polymer–Anticancer Drug Conjugates

Around 35 years ago, the concept of anticancer drugs conjugated to polymers was first introduced [33]. This idea has led to the development and approval of several biodegradable polymeric implants that function as subcutaneous depots for sustained release of luteinizing hormone-releasing hormone (LHRH) analogues (e.g., Zoladex and Lupron Depot) that are used for the treatment of prostate and other hormonal-dependent cancers (Table 4) [34]. In addition, biodegradable polymeric depots of

Table 4 Examples of polymer–anticancer conjugates that are currently approved or in clinical trials

Polymer–drug conjugate	Name	Indication	Status
Goserelin acetate implant	Zoladex	Prostate cancer	Approved
Leuprolide acetate implant	Lupron Depot	Prostate cancer	Approved
Carmustine implant	Gliadel	Brain tumors	Approved
HPMA copolymer–doxorubicin	FCE28068 (PK1)	Breast cancer, NSCLC	Phase I/II
HPMA copolymer–doxorubicin–galactose	FCE28069 (PK2)	Hepatoma	Phase I/II
HPMA copolymer–paclitaxel	PNU166945	–	Phase I – stopped
HPMA copolymer–camptothecin	MAG-CPT, PCNU166148	–	Phase I – stopped
HPMA copolymer–carboplatinum	AP5280	Solid tumors	Phase I
HPMA copolymer–DACH platinum	AP5346 (ProLindac)	Head and neck cancer	Phase II

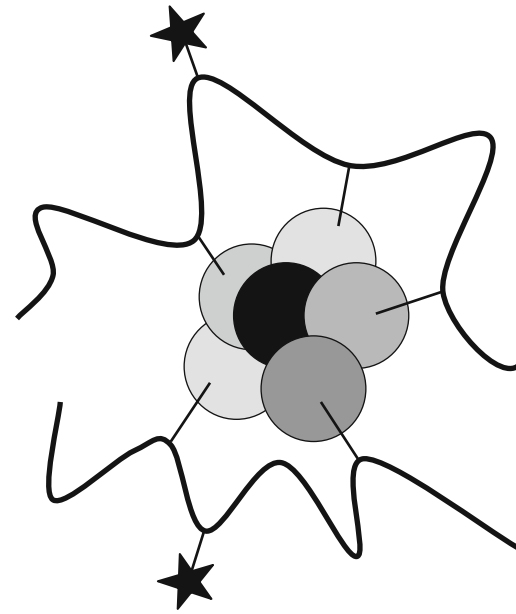

Fig. 4 Schematic representation of a polymer–drug conjugate, composed of three main components: polymer backbones (*curved lines*), biodegradable linkers (*straight lines*), and drug(s) (*spheres*). In addition to the drug(s), other moieties (*stars*) can be attached to the polymer backbones, including targeting residues and imaging agents. Note that different drugs can simultaneously be incorporated in one polymer–drug conjugate

anticancer agents are routinely implanted after resection of a brain tumor for local drug delivery in order to prevent tumor regrowth (e.g., Gliadel [35]). Gliadel is composed of controlled delivery polymers (poly[bis(p-carboxyphenoxy)propane-sebacic acid]) that protect carmustine from degradation. Gliadel is placed in the resection cavity, and on exposure to the aqueous environment, the anhydride bonds in the copolymers are hydrolyzed resulting in the release of carmustine, which diffuses into the surrounding brain tissue and produces an antineoplastic effect by alkylating DNA and RNA. If free carmustine is given systemically, its half-life is about 12 min, whereas for Gliadel, the brain concentration of carmustine is log orders higher than achievable by systemic carmustine administration [36]. Since the polymers in these implants slowly degrade, drug is continuously released and therapeutic drug levels can be maintained for several months with minimal systemic toxicity [5].

Furthermore, multiple polymer–anticancer drug conjugates for intravenous injection have been developed and are currently being evaluated in clinical trials (Table 4). These polymer–drug conjugates are typically constructed of three main components: (1) natural or synthetic (co)polymer backbone(s), (2) biodegradable polymer–drug linker, and (3) one, or sometimes more, anticancer agent (Fig. 4) [4]. In addition, also other components can be attached to the polymer backbone, such as residues for active tumor targeting and imaging moieties to aid preclinical pharmacokinetic

studies and to facilitate clinical imaging [5]. Similar to liposomes, the principle objectives of these polymer–anticancer drug conjugates are to alter the pharmacokinetics and pharmacodynamics by improving tumor targeting and reducing off-target toxicity. The delivery of polymer–anticancer drug conjugates relies on the EPR effect (Fig. 1).

Hydrophilic N-(2-hydroxypropyl) methacrylamide (HPMA) copolymers are among the most frequently used polymers for the delivery of anticancer drugs. In preclinical studies, HPMA was found to be nontoxic at doses up to 30 g/kg bodyweight, did not bind to blood proteins, and were not immunogenic [5]. HPMA copolymers have been conjugated to several classical anticancer drugs, including doxorubicin, paclitaxel, camptothecin, and carboplatinum (Table 4) [5]. Because the HPMA copolymer backbone is not biodegradable, the polymer–drug conjugates that were developed for clinical use had to have a molecular weight <40,000 g/mol to ensure eventual renal elimination [5]. Preclinical studies also indicated that conjugation of anticancer drugs to the hydrophilic HPMA copolymers can importantly improve the pharmacokinetics and pharmacodynamics [5]. First of all, the aqueous solubility of the HPMA copolymer–drug conjugates can be markedly increased compared to free drug, which is especially favorable for poorly soluble drugs like doxorubicin and paclitaxel. Furthermore, the hydrophilic HPMA copolymers prevented rapid uptake of the conjugate by the MPS in liver and spleen. This resulted in a markedly prolonged circulation time in the blood, which enables enhanced tumor penetration via the EPR principle (Fig. 1) [5]. Additionally, initial experiments revealed that early high plasma concentration is an important driving force for tumor penetration. Moreover, longer plasma circulation resulted in higher tumor accumulation [37, 38]. After accumulation in the tumor interstitium, the polar conjugates enter tumor cells by endocytosis [39–41]. Endocytosis is the process by which cells absorb polar molecules from outside the cell by engulfing them with their cell membrane, enabling these molecules to pass through the cell membrane and hydrophobic cellular plasma. Subsequently, the polymer–drug conjugates are taken up by intracellular lysosomes, and the linker, which attaches the drug to the polymer backbone, is degraded by protease enzymes and/or hydrolysis (depending on the linker composition) [5]. Therefore, drug release kinetics of polymer–drug conjugates is also important for therapeutic success. For instance, the linker should not degrade too early releasing the drug already in the blood circulation, whereas releasing the drug too slowly can completely eliminate activity and even lead to off-target toxicity (e.g., bladder toxicity caused by HPMA copolymer–camptothecin) [42–44].

In addition to passive tumor targeting, residues for active tumor targeting can be attached to the HPMA backbone. An example is HPMA copolymer–doxorubicin–galactose, which was designed to target liver cells via binding of the galactose residue to the asialoglycoprotein receptors on hepatocytes (Table 4). Preclinical studies showed that indeed ~80 % of the administered dose was taken up by the liver [45]. However, it was also found that saturation of the asialoglycoprotein receptors occurred at relatively low HPMA copolymer–doxorubicin–galactose dose [46]. Administration of this targeting conjugate by continuous intravenous infusion rather than by bolus injection was therefore considered a good strategy to avoid initially high blood concentrations and thereby receptor saturation [5, 47].

HPMA copolymer–doxorubicin conjugates were the first synthetic polymer-based anticancer conjugates to enter the clinic [48]. Phase I and II clinical trials with HPMA copolymer–doxorubicin (FCE28068 [49, 50]) and HPMA copolymer–doxorubicin–galactose (FCE28069 [47]) showed that these conjugates were 2- to 5-fold less toxic than conventional doxorubicin at equimolar dosages, whereas bone marrow-related dose-limiting toxicity was comparable to free drug administration. Importantly, no polymer-related toxicity or immunogenicity was seen, and despite an individual cumulative doxorubicin dose of up to 1,680 mg/m^2, no doxorubicin-related cardiotoxicity was observed [47, 49, 50]. The human pharmacokinetic profiles of FCE28068 and FCE28069 were largely consistent with preclinical results. FCE28068 remained in the systemic circulation for a long time (the terminal elimination half-life was 93 h), did not accumulate in the liver, and was largely eliminated via the kidneys (up to 75 % within 24 h) [49, 50]. FCE28069, which contains the liver targeting

residue galactose, indeed showed substantial liver accumulation (15–20 % of the dose after 24 h), and doxorubicin concentrations in the liver tumors were estimated to be 12- to 50-fold higher than would have been seen after the administration of free doxorubicin [47]. FCE28068 and FCE28069 showed antitumor activity in some of the chemotherapy refractory patients with breast cancer and non-small cell lung cancer [49, 50] and hepatocellular carcinoma [47], respectively. Overall, these promising results warrant further clinical development.

Additionally, two HPMA copolymer–platinates, the carboplatinum conjugate AP5280 and the oxaliplatin conjugate AP5364, were evaluated in phase I clinical trials [51, 52]. AP5280 showed prolonged plasma exposure with minimal renal toxicity and myelosuppression, toxicities that are typically observed with cisplatin and carboplatinum. However, the formation of platinum-DNA adducts after AP5280 administration was substantially lower than observed for therapeutic doses of cisplatin. Therefore, it remains to be determined whether AP5280 can actually increase platinum delivery to the DNA of tumor cells in man as has been shown in experimental models [51]. AP5364 (ProLindac) showed prolonged plasma exposure, was well tolerated, and showed evidence of antitumor activity [52]. Additional clinical studies with AP5280 and AP5364 are planned [48, 53].

However, not all polymer–anticancer drug conjugates that showed promising pharmacokinetics and pharmacodynamics and toxicity profiles in preclinical studies were also successful in the clinic (e.g., HPMA copolymer–paclitaxel [54] and HPMA copolymer–camptothecin [42–44]) (Table 4). A phase I clinical study with HPMA copolymer–paclitaxel had to be stopped because moderate neurotoxicity was observed [54]. Moreover, severe neurotoxicity was observed in additional animal studies (unpublished data). This unexpected side effect of HPMA copolymer–paclitaxel that was attributed to the altered pharmacokinetic behavior of paclitaxel administered as a polymer-bound drug emphasizes the necessity of thorough and protracted preclinical evaluation of novel drug–conjugate systems. Furthermore, the pharmacokinetic benefits of conjugation of paclitaxel to HPMA copolymers were limited because the drug was released too rapidly after administration [48]. Nonetheless, remission of skin metastases was seen in one patient with paclitaxel-refractory breast cancer at a relatively low paclitaxel dose of 100 mg/m^2 [54]. The HPMA copolymer–camptothecin (MAG-CPT) was evaluated in several phase I trials [42–44]. However, due to its labile linker that was designed to release the drug by hydrolysis in the slightly acidic pH in the tumor, camptothecin was also released during urinary excretion, with serious cumulative bladder toxicity as a consequence [42–44]. In addition, MAG-CPT did not show any clinical evidence of antitumor activity and its clinical development was discontinued.

Nonetheless, the promising clinical results for HPMA copolymer–doxorubicin conjugates and the HPMA copolymer–platinates have demonstrated benefits of these innovating nanoscale drug formulations, but further research is warranted. Given the importantly improved pharmacokinetics and pharmacodynamics and reduced toxicity compared to the administration of free drug, it is expected that copolymer–anticancer drug conjugates will be approved for clinical use in the near future.

4 Conclusion

The nanomedicines that are exemplified in this chapter show that the pharmaceutical formulation can importantly alter the pharmacokinetics and pharmacodynamics of the incorporated cytotoxic anticancer drugs. Most of the liposomes and polymer conjugates that are detailed in this chapter markedly prolong the systemic circulation of the encapsulated anticancer drugs, resulting in an increased penetration of the tumor by the EPR principle. At the same time, the volume of distribution of these nanomedicines is markedly reduced, which generally leads to fewer side effects because the penetration of drug into healthy tissues is reduced. However, the altered pharmacokinetic profile can also lead to an altered toxicity profile (e.g., hand–foot syndrome and stomatitis that was observed for liposomal

doxorubicin). Hence, although nanoscale drug formulations do improve the pharmacokinetics and pharmacodynamics of cytotoxic anticancer drugs, they do not completely transfer these drugs into Paul Ehrlich's idolized magic bullets, which only target and destroy tumor cells while causing no damage to healthy tissues. Nonetheless, the development of drug formulations that alter the pharmacokinetics and pharmacodynamics of anticancer drugs in a way that drug delivery and efficacy are improved while toxicity is reduced will remain an important and challenging area in cancer research.

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Nanotechnology in Cancer

Margit M. Janát-Amsbury and You Han Bae

Abstract Cancer nanotechnology, defined as nanotechnology applicable for diagnosis, prognosis, and therapy of cancer, offers exciting possibilities but also challenges. Although nanotechnology became fruitful in the field of diagnostics, to date the desire of improving current cancer treatments through nanotechnology has surpassed the research or development stage only in a handful of cases. However, efforts to clinically translate more of these treatments remain highly focused. Drug-bearing nanoparticles, which are usually injected directly into the bloodstream, are sent on a journey full of obstacles to overcome such as bypassing RES organs and crossing endothelial cells, as well as various membranes of cellular and extracellular compartments, before finally encountering tumor tissue. Therefore, a cornucopia of different nanoparticle designs varying in size, architecture, and surface properties has been designed to be deployed to tumor sites within the human body either passively or promoting the association with a particular cell or tissue type through targeting. Early clinical results with nanomedicines were indeed able to show reduced toxicity, improving patients' quality of life, but are still in need of stronger coupling with superior clinical efficacy to help justify the often high costs associated with this new class of therapeutics and their complexity. Nanotechnology aiming for the development of novel cancer therapeutics must focus in on potential, unforeseen toxicities and also consider the emerging role of epigenetics. The following chapter introduces most of the basic nanoconstructs developed to date, which inevitably will continue to evolve and eventually find an important place in health care, though to become the cancer medicines of tomorrow, nanotechnology's future success and translation must early on be connected to diagnostic and therapeutic potential for clinical applications. To be equally aware of potential benefits, risks and costs will be necessary to stimulate future research with emphases on how to improve therapeutic outcomes such as the extension of life in patients with cancer in times burdened with unsustainable healthcare costs.

Keywords Cancer • Nanotechnology • Diagnosis • Treatment • Clinical • Polymers

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1 Background

1.1 Nanoscale in Cancer Nanotechnology

The prefix "nano" stems from the Greek word nanos, which translates to "dwarf." When used in the metric system, it refers to a factor of one billionth (10^{-9}). The nanoscale usually refers to a length from 1 nm up to 100 nm. Most nanostructures or nanomaterials fall within this range; however, some commonly used biomedical nanoparticles range up to 300 nm in diameter without relinquishing the "nano" prefix.

As illustrated in Fig. 1, 1 nm will hold 7 oxygen atoms or 3–4 water molecules in a row. The hemoglobin molecule is approximately 5 nm and resides within a red blood cell that measures approximately 7,000 nm in diameter. A human hair ranges from 50,000 to 100,000 nm in thickness. Using recognizable objects as examples, a sphere with a diameter of 1 nm compared to a soccer ball has the same proportional relationship as the soccer ball compared to Earth.

1.2 Definition and Scope of Cancer Nanotechnology

Nanotechnology comprises the design and development of novel medical and nonmedical devices with sizes measurable on a nanoscale. Nanotechnology is being applied to cancer in two broad areas: the development of various nanoparticles, which can be uploaded with drugs or imaging agents and then delivered to tumors and high-throughput nanosensor devices for detecting the early biological signatures of cancer. When combined, such technologies are anticipated to lead to earlier diagnosis, better treatment and monitoring, and reduced adverse effects for patients with cancer [1].

In addition to the attributes noted, nanoparticles also possess multifunctionality. Many of the nanoparticles discussed in this chapter are designed to be multifunctional, meaning that one particle can possess capabilities for detection, diagnosis, imaging, and/or treatment in combination. This chapter summarizes the diagnostic and therapeutic potential of nanomedical applications in the present and future.

1.3 Enhanced Permeability and Retention Effect

Vasculature, extracellular matrices, and interstitial fluid pressure are all compromised in a tumor environment. These alterations create both barriers and advantages for the delivery of nanocarriers.

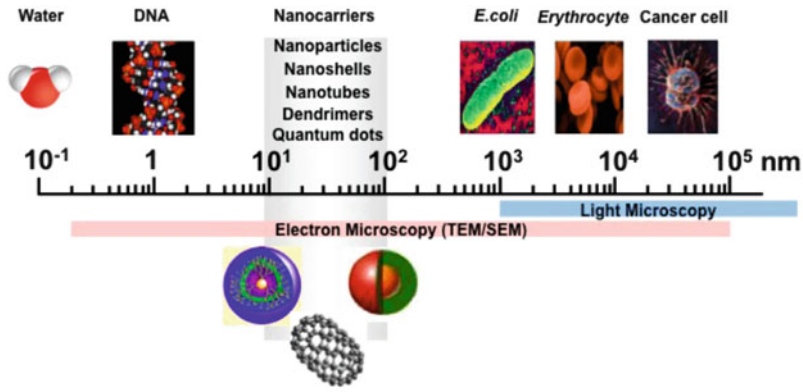

Fig. 1 Scales of nanocarriers and biomolecules

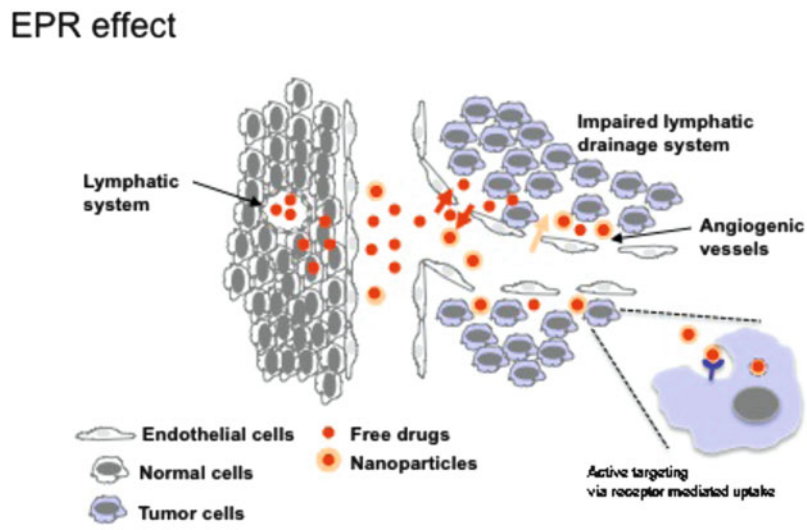

Fig. 2 Enhanced permeability and retention (EPR) effect, increased accumulation of particular vehicles in tumor tissues than in normal tissues because of the loose structure of angiogenic vessels, and lack of lymphatic system in tumor tissues

The enhanced permeability and retention effect (EPR) is a phenomenon responsible for passive localization and accumulation of nanoparticles in tumors. The passive entry of nanoparticles with a size of smaller than 400 nm into tumors [2] is attributed to leaky tumor vasculature. This leakiness is caused by defective endothelial cells allowing gaps, widened inter-endothelial junctions, defective base membranes and pericytes, and an increased number of transendothelial channels [3] as illustrated in Fig. 2. The enhanced permeation is coupled with a severely compromised lymphatic drainage system, resulting in retention effect [4].

1.4 Cancer Cell-Specific Interactions

Nanoparticles actively target drugs to cancerous cells based on the molecules expressed on their cell surface. Active targeting is combined with passive accumulation to improve drug efficacy, achieving greater tumor reduction with a lower drug dose. However, tumor tissue heterogeneity must be taken into account when developing active targeting systems based on cell-specific interactions.

A good example of a clinically relevant targeted therapy is the use of human epidermal growth factor receptor 2 (HER2) protein shown in Fig. 3. There are now a number of nanoparticles conjugated with the anti-HER2 monoclonal antibody trastuzumab, which are being extensively investigated with promising results in preclinical applications for breast cancer [5]. Trastuzumab recognizes a specific protein, the HER2/neu on certain cancer cells, and signals the body's immune system to destroy the cell. Further, it will interrupt signaling, which stops the cancer cells' further division and growth.

Folate-receptor-targeted therapy for the treatment of cancer is another example of successful active targeting technology. Vitamin folate is required for cell division. Rapidly dividing cancers overexpress folate receptors and access folate circulating in the bloodstream. In the case of ovarian cancer, 80–90 % of cases demonstrate an overexpression of folate receptors. A phase II clinical trial (PRECEDENT) is currently recruiting patients with platinum-resistant ovarian cancer to evaluate PEGylated liposomal doxorubicin in combination with EC145, a folate-receptor-targeted desacetylvinblastine hydrazide, with encouraging results [6].

Nanotechnology products, which currently are already available on the market, are listed in Table 1.

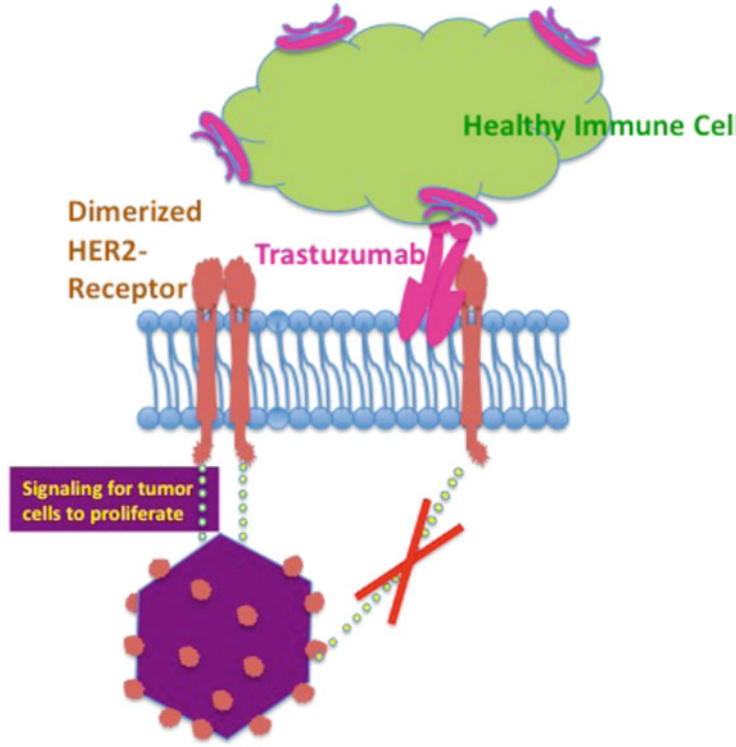

Fig. 3 cerbB2/Trastuzumab. Trastuzumab binds to HER2+ tumor cells, identifying them for destruction by healthy immune cells. Dimerized HER2 receptors signal to tumor cells to proliferate. Trastuzumab interrupts downstream signaling preventing tumor cells from proliferation

Table 1 Nanotechnology products on the market

Nanomedicine products on the market			
Compound	Nanotechnology component	Indication	Company
Drug delivery			
DaunoXome	Liposomal dunorubicin	Kaposi Sarcoma	Gilead
Doxil/caelyx	Liposomal doxorubicin	Cancer Kaposi sarcoma	Ortho Biotch, Schering Plough
Depocyt	Liposomal cytarabine	Cancer	Skye Pharma, Enzon
Myocet	Liposomal doxorubicin	Breast cancer	Zeneus Pharma
Abraxane	Paclitaxel protein-bound nanoparticles	Cancer	Abraxis BioScience, AstraZeneca
In vivo imaging			
Resovist	Iron nanoparticles	Liver tumors	Schering
Feridex/Endorem	Iron nanoparticles	Liver tumors	AdvancedMagnetics, Guerbet
Protein delivery			
SMANCS			
Oncaspar	PEG-asparaginase	Leukemia	Enzon
Neulasta	PEG-GCSF	Neutropenia	Amgen
PEG-asys	PEG-inteiferonα2a	Hepatitis C	Genentech
PEG-intron	PEG-inteiferonα2b	Hepatitis C	Schering
Combination			
Opaxio (Xyotax)	Polyglutamate paclitaxel	NSCLC, ovarian Cancer	Cell therapeutics, Inc.

2 Detection, Screening, and Diagnosis

Detection of a broad array of molecular signals and biomarkers in real time characterizes some of the unique capabilities of nanotechnology. These capabilities provide new avenues for the early detection of disease, novel prognostic systems, and the means to administer personalized, custom-tailored, therapeutic strategies. Detection nanotechnologies providing such capabilities include arrays of nanocantilevers, nanowires, and nanotubes. These nanosystems yield fast, reliable, specific, and cost-effective detection platforms for single or multiple molecules in complex biological samples.

Micro- and nanobiotechnology in medical diagnostics can be categorized in two main areas: in vitro (biosensors and integrated devices) and in vivo (implantable devices, medical imaging) applications. In vitro diagnosis of medical conditions, including cancer, has traditionally been a laborious task. The disadvantages of this traditional approach involve sample deterioration, high cost, lengthy waiting times, less accurate results for extremely small sample quantities, difficulties in integrating parameters obtained from a wide variety of methods, and poor standardization of sample collection.

Miniaturization, parallelization, and integration of different functions on a single device have led to the development of a new generation of devices that are smaller, faster, cheaper and more accurate and do not require special skills. These analytical devices require much smaller samples and are designed to deliver more accurate and comprehensive biological data from a single measurement. The requirement for smaller samples also means less invasive and less traumatic methods of collection. Nanotechnology enables another refinement of diagnostic techniques, the high-throughput screening of samples.

2.1 Screening and Detection

Nanotechnology is revolutionizing biomarker screening for disease diagnosis and monitoring of therapy. Because each cancer type demonstrates a distinguished expression pattern, the simultaneous screening for multiple biomarkers may help identify cancer subtype(s).

2.1.1 Lab-on-a-Chip

Biosensors contain biological elements, such as an enzyme, that are capable of recognizing and "signaling" the presence, activity, or concentration of a specific biological molecule in solution [7]. Key attributes of biosensors are their specificity and sensitivity. Nanoanalytical tools like scanning probe microscopy or imaging mass spectrometry offer new opportunities for in vitro diagnostics, such as molecular pathology or highly integrated ultrasensitive biochip monitoring. Techniques derived from the electronics industry have enabled the miniaturization of biosensors, allowing for smaller samples and highly integrated sensor arrays, which take different measurements in parallel from a single sample. New higher specificity biosensors reduce the invasiveness of diagnostic tools and significantly increase the quality and quantity of the biological information provided. Such information collection can characterize the phenotype, genotype, metabolome, and/or proteome [8].

Several complex preparation and analytical steps can be incorporated into "lab-on-a-chip" devices, which can mix, process, separate fluids, analyze, and identify samples in real time. Prerequisites for "lab-on-a-chip" technologies are summarized in Table 2. Devices, which integrate all the above, will be able to measure thousands of signals derived in a single sample. Some nanobiodevices for diagnostics have been developed to measure parts of the genome or proteome using DNA fragments or antibodies as sensing elements and are thus called gene or protein chips [9]. 'Cells-on-chips' use cells as their sensing elements and are employed in pathogen and/or toxicology screening [10]. Integrated devices can be used in the early diagnosis of disease and for monitoring the response to therapy. New advancements in microfluidic technologies show great promise in the realization of a fully

Table 2 Prerequisites for "lab-on-a-chip" technologies

A.	User-friendly sample preparation techniques • Detection of smallest amounts of disease marker in minimal tissue/blood samples • Detect smallest quantities even when diluted in large sample volume (e.g. 5–0 cancer cells in 100 ml urine)
B.	Ultra-sensitive and label-free detection techniques • Faster, direct detection
C.	Synthetic recognition elements (i.e. sensors) • Increase sensitivity, specificity and ruggedness of recognition • Advancement in deposition techniques and surface chemistry Self assembly of biomolecules Hybrid conjugates of biomaterials with nanoparticles Molecularly Imprinted Polymers
D.	Biomimetic sensors using molecules

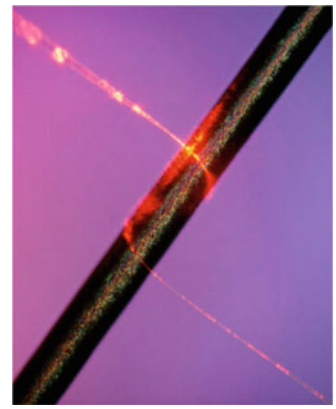

Fig. 4 This silica nanowire (glowing) is wrapped around a single strand of human hair. With kind permission from Mazur group, Harvard University

integrated device that directly delivers full data in a single sample. The aim of many is to develop in vitro diagnostic and surveillance/monitoring tools that are to be used in a standard clinical environment as "point-of-care" devices [11].

2.1.2 Nanowires

Nanowires capitalize on the properties of selectivity and specificity. They are able to detect the presence of altered genes associated with cancer and may help researchers pinpoint the exact location of the changes [12]. Nanowire-based biosensors, which are cost effective and easy to assemble, are being developed as single-nanowire chemoresistive sensor devices. One of these sensor devices performs CA-125 biomarker detection and quantification. The immunosensor shows excellent sensitivity with a lower detection limit of 1 U/ml CA-125 without any loss of performance upon exposure to CA-125 in spiked human blood plasma [13]. To be able to imagine the size and dimension, a nanowire is illustrated in Fig. 4, being wrapped around a single strand of human hair.

Nanowires are about five times smaller than a virus but are several times stronger than spider silk. Researchers have developed coated nanowires that bind to specific proteins that indicate the presence of prostate cancer before conventional tests can [14]. Other potential applications for nanowires include the early sensing of breast and ovarian malignancies. Nanowires are so small that doctors may 1 day implant them into the body as permanent health detectives that continuously monitor molecular signals.

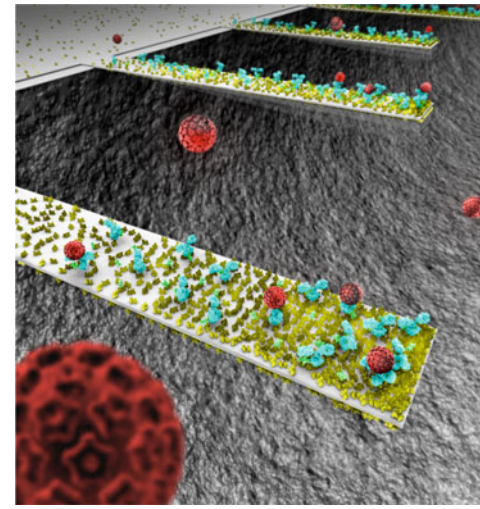

Fig. 5 http://news.uns.purdue.edu/UNS/html4ever/2006/060828.Bashir.nanocant.html

2.1.3 Cantilevers

Nanoscale cantilevers are nanoscopic and flexible beams anchored at only one end and built using semiconductor lithographic techniques. They function as sensors that are ideal for detecting the extremely small amount of molecules in biological fluids. Nanocantilevers can be coated with molecules capable of binding specific substrates, DNA complementary to a specific gene sequence, for example. Such devices, comprised of many nanometer-sized cantilevers, can detect single molecules of DNA or a specific protein. As a cancer cell secretes its molecular products, the antibodies coated on the cantilever fingers selectively bind to these secreted proteins. The physical properties of the cantilevers change as a result of the binding event. This change can be detected in real time and provides information about the presence, absence, and concentrations of various molecular expressions.

Nanomechanical cantilevers have been designed to electrically measure label-free prostate-specific antigen (PSA) and achieved a detection sensitivity of 10 pg/ml. PSA proteins are detected by simple electrical measurements of the resonant frequency change generated by the molecular interaction of the antigen and the antibody [15]. The nanocantilevers coated with antibodies, for example, will bend from the changes in surface tension when substrates that signal a malignancy bind to it [16]. Simply by monitoring nanocantilever deflections, specialists may someday identify the presence of cancer molecules that are now still undetectable. The nanocantilevers, resembling diving-board-like structures as seen in Fig. 5, are capturing viruses, which are represented as red spheres. It represents a new class of ultrasmall sensors for detecting viruses, bacteria, and other pathogens.

2.2 Imaging

Though imaging techniques and devices have tremendously improved during the last decade, current imaging methods still lack sensitivity, only detecting cancers once they have made a visible change in the tissue. By that time thousands of cells will have proliferated undetected and most likely have metastasized. Once visible, the nature of the tumor—malignant or benign—and the characteristics that might make it responsive to a particular treatment must be assessed through biopsies and histopathological evaluations. In the future, cancerous or even precancerous cells may be detected through nanodevices.

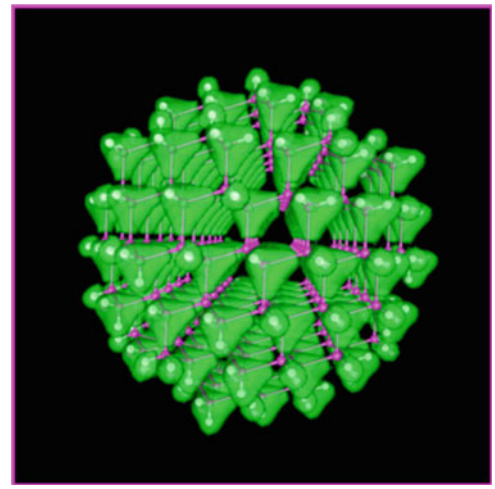

Fig. 6 The total electron charge density (shown in *green*) of a quantum dot of gallium arsenide, containing just 465 atoms (Image: Lin-Wang Wang, Lawrence Berkeley National Laboratory)

Fig. 7 Quantum dots sorted by size emitting light of different colors. Light-emitting core-shell Cd/Se/ZnS nanocrystals. D.V. talapin, A.L. Rogach, A. Kornowski, M. Haase, H. Weller. Highly Luminescent Monodisperse CdSe and CdSe/ZnS Nanocrystals synthesized in a Hexadecylamine-Trioctylphosphine Oxide-Trioctylphosphine Mixture. Nano Lett. 2001,1,207–211. With kind permission of Horst Weller Group, University of Hamburg

2.2.1 Optical Imaging

Quantum dots are colloidal fluorescent semiconductor nanocrystals (2–10 nm). The central core of quantum dots consists of combinations of elements from groups II to VI of the periodic table (CdSe, CdTe, CdS, PbSe, ZnS, and ZnSe) or III to V (GaAs, GaN, InP, and InAs), which are "overcoated" with a layer of ZnS. Figure 6 represents a quantum dot of gallium arsenide, containing just 465 atoms. Quantum dots emit different wavelengths of radiation depending on the type of a metal element used in their cores: for example, cadmium sulfide for ultraviolet to blue, cadmium selenide (seen in Fig. 7 sorted by size emitting light of different colors) for most of the visible spectrum, and cadmium telluride for the far red and near infrared. A dot's size determines its precise color within each range and the multiple colors of quantum dots provide a powerful tool for labeling and monitoring multiple cells and molecules simultaneously. Quantum dots are photostable, are resistant to photobleaching, and show exceptional resistance to photo and chemical degradation. They show size- and composition-tunable emission spectra and high quantum yield. Combined, these characteristics make quantum dots excellent contrast agents for imaging and labels for bioassays, because they can visualize certain types of cells or molecules inside the body [17]. A polymer coating enables researchers to take quantum dots a step further and attach molecules such as antibodies to their surfaces, which target organs, cells, and

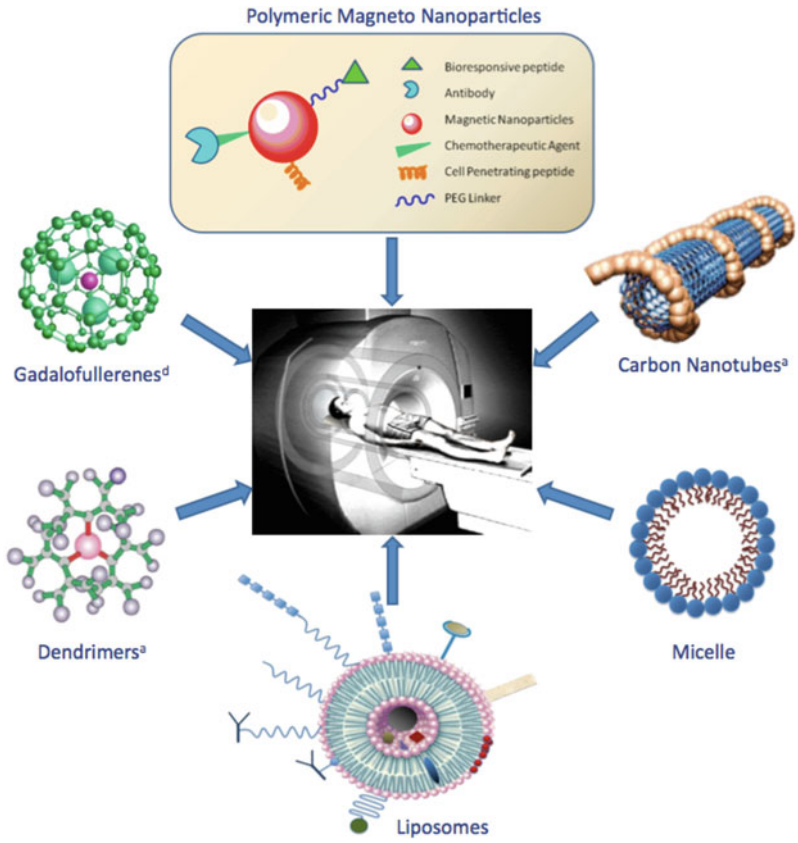

Fig. 8 Schematic diagram showing various types of contrast agents used for MRI (copyright image from Luna Innovations)

intracellular compartments. The coating also shields nearby cells from cadmium's toxicity [18]. When used in conjunction with MRI (magnetic resonance imaging), quantum dots can produce exceptional images of tumor sites. These nanoparticles are much brighter when compared to organic dyes, which are currently used in clinics. Additionally they only need one light source for excitation. This translates into higher contrast images at lower cost than currently used contrast agents [19]. While possessing great potential, the downside, however, is that quantum dots are usually made of toxic elements.

2.2.2 Magnetic Resonance Imaging

Nanoscale-targeted Magnetic resonance imaging (MRI) contrast agents illustrate the application of nanotechnology to diagnostics. Several types of nanoparticles for the enhancement of MRI contrast have been used clinically and in research protocols. A schematic diagram (Fig. 8) shows various types of contrast agents used for MRI. These include gadolinium- and iron oxide-based nanoparticles [20, 21]. For certain applications, nanotechnology might be the only way to secure in vivo diagnostic information [22–25]. Multiple-mode, nanosized imaging contrast agents that combine magnetic resonance with biological targeting and optical detection have been designed and demonstrate exceptional features [26–28]. Examples of liposomal imaging agents are ^{111}In, Mn, and Gd liposome with either soluble chelators (e.g., diethyleneamine pentaacetate [DTPA]) encapsulated inside a liposome or membrane-anchoring chelators (e.g., DTPA-stearylamine [DTPA-SA] or DTPA-phosphatidyl ethanolamine [DTPA-PE]) [29]. Encapsulation or membrane binding of imaging agents to liposomes

Table 3 Advantages of using SPIO

Minimum delays of about 10 min between injection (or infusion) and MR imaging extend the examination time
Cross-sectional flow void in narrow blood vessels may impede the differentiation from small liver lesions
Aortic pulsation artifacts become more pronounced

has been shown to reduce the risk of leakage of potentially toxic metals into the bloodstream or other body compartments [30]. Liposomes loaded with paramagnetic ions (e.g., Gd, Mn, Fe) have proven successful in the visualization of the lymphatic system [31], which may have application in detecting lymph node metastases [31, 32]. ^{111}In-labeled PEGylated liposomes (^{111}In-DTPA-PEGylated liposomes) have demonstrated distinctive characteristics in the visualization of lung [33, 34], head, and neck cancers [33, 34], glioblastomas [35], as well as other malignant tumors [33].

Magnetic nanoparticles are spherical nanocrystals of 10–20 nm of size with a Fe^{2+} and Fe^{3+} core surrounded by dextran or PEG molecules. Their magnetic properties make them excellent agents to label biomolecules in bioassays, as well as MRI contrast agents. They are also amenable to surface functionalization for active targeting in vivo or for in vitro diagnostics [36].

Superparamagnetic iron oxide-based colloids (SPIOs) are relatively new types of MRI contrast agents with a median diameter greater than 50 nm. These compounds consist of nonstoichiometric microcrystalline magnetite cores, which are coated with dextrans (in Ferumoxide®) or siloxanes (in Ferumoxsil®). After injection they accumulate in the reticuloendothelial system (RES) of the liver (Kupffer cells) and the spleen. At low doses circulating iron decreases the T1 time of blood; at higher doses the T2 effect predominates. SPIO agents are much more effective in MR relaxation than paramagnetic agents. Their major advantages are summarized in Table 3. Since hepatic tumors either do not contain RES cells or their activity is reduced, the contrast between liver and lesion therefore is improved. Superparamagnetic iron oxide particles cause noticeable shorter T2 relaxation times with signal loss in the targeted tissue (e.g., liver and spleen) with all standard pulse sequences. Magnetite, a mixture of FeO and Fe_2O_3, is considered one of the iron oxides. FeO can be replaced by Fe_3O_4. The use of these colloids as tissue-specific contrast agents is now a well-established area of pharmaceutical development. Feridex®, Endorem™, GastroMARK®, Lumirem®, Sinerem®, and Resovist® are examples with more patents pending, indicating more generations to come.

2.2.3 CT, X-Ray, PET, and Ultrasound

The combination of different imaging modalities is a promising new diagnostic approach. Examples of such combinations include positron emission tomography (PET) with MRI, magnetic resonance with ultrasound imaging or with electroencephalogram-based brain mapping, and ultrasound with optical technologies. These combinatorial methods provide advantages over a single modality. The fusion of magnetic resonance imaging and optical imaging modalities remains a challenge. In principle, this will require use of fluorescent nanoparticles as signal emitters, which function in both paramagnetic and infrared modes. Once this is achieved, nanotechnology may lead to the miniaturization of detection devices or the remote transduction of signals. In high-resolution measurements of electromagnetic fields, the development of new interfaces with nanostructured and/or biologically functionalized surfaces will improve the continuous monitoring of biological parameters dramatically. The ability to measure small local variations in temperature using radio frequency detectors has applications in identifying the onset and locus of many diseases, including cancer. To be fully successful, improved methods of image analysis and visualization, such as 3D optical reconstruction, real-time intracellular tomography, stereo imaging, virtual and augmented reality, holography, and in vivo imaging from optical catheters, and better endoscopic tools will be required.

3 Treatment

Current cancer therapies are predominantly confined to surgery, radiation, and chemotherapy. All three methods can damage normal, healthy tissues and often only partially remove the cancer. Nanotechnology offers the means to target selectively to cancer cells.

3.1 Chemotherapy/Photodynamic Therapy

The field of drug delivery has been utilizing nanotechnologies for several decades. Drug delivery is based upon three main requirements: (a) efficient encapsulation of drugs; (b) successful delivery of drugs to targeted, diseased regions of the body; and (c) successful release of that drug at the above-mentioned sites.

When investigating nanomedical approaches for drug delivery, most of them focus on the design of a system that improves a drug's bioavailability. Bioavailability refers to the presence of drug molecules in the right place, in the right concentration, and for the right amount of time. This can be achieved by molecular targeting of nanoengineered devices [37, 38]. Drug delivery systems, lipid- or polymer-based nanoparticles, can be designed to improve pharmacological and therapeutic properties of drugs [39]. A particular advantage of drug delivery systems is their ability to alter the pharmacokinetics and biodistribution of drugs.

Nanoparticles have several properties that can be used to improve drug delivery. Where larger particles would have been cleared from the body, cells preferentially incorporate nanosized particles. Various, complex drug delivery systems with targeting capability are under development that facilitate a drug nanocomplex's passage through the cell membrane and into the cell's cytoplasmic compartment and in some cases intracellular structures. Another capability of nanoparticles is the ability to optimize drug delivery by a triggered response to a particular cellular signal or location (endosome). This methodology controls activation. Furthermore, a drug with poor solubility will be enhanced by a drug delivery system with both a hydrophilic and hydrophobic component. By controlling drug entry and release, nanotechnology can significantly decrease the toxicity of many drugs.

Photodynamic therapy is a promising therapy. Using this technique, nanoparticles are placed within the body and illuminated with light from an outside source. The light is absorbed by the particles and if the particles are made of metal, energy from the light will heat the particles and its surrounding tissue. Light may also be used to produce high-energy oxygen radical molecules released from photosensitizers, which will chemically react with and destroy most adjacent organic molecules, including tumor tissue. Such applications are appealing for various reasons. The "toxic trail" of reactive molecules is avoided with "directed" light.

3.1.1 Liposomes

Liposomes are phospholipid vesicles (50–100 nm) with a bilayer membrane structure similar to that of biological membranes with an internal aqueous phase as illustrated in Fig. 9. Liposomes utilize the EPR effect in cancer neovasculature to increase drug concentration in tumor sites. Liposomes were the first type of nanoparticles widely used for clinical cancer treatment, shielding healthy cells from their toxicity and preventing their concentration in vulnerable tissues, such as the kidneys and liver. Liposome-encapsulated formulations of doxorubicin were approved 10 years ago for the treatment of Kaposi's sarcoma and are now used against breast cancer and refractory ovarian cancer. Liposomes continue to be refined and applied to more cancer indications [40–42]. With the help of liposomes, common side effects of cancer treatment such as nausea and hair loss are reduced. Their amphiphilic

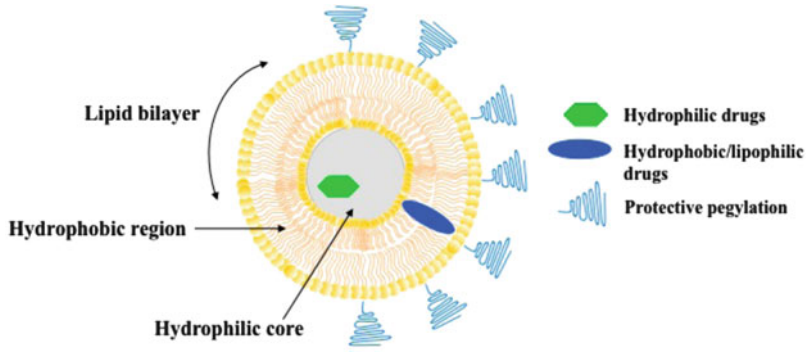

Fig. 9 Liposomal carrier for various types of drugs and compounds

nature enables liposomes to transport hydrophilic drugs entrapped within their aqueous interior and hydrophobic drugs dissolved into the membrane. Due to their physicochemical characteristics, liposomes show excellent circulation, penetration, and diffusion properties. Moreover, the liposome surface can be modified with ligands and/or polymers to improve drug delivery specificity [43]. To date, more than 200 clinical trials have investigated liposomal doxorubicin formulations. Many have demonstrated significantly reduced cardiotoxicity when compared to the free drug with preserved antitumor activities [44].

To overcome the barriers of the reticuloendothelial system (RES) and increase the half-life of liposomes in circulation, liposomes have been modified in various ways. A very promising design is grafting poly(ethylene glycol) (PEG), an inert and biocompatible polymer, to the liposome. Based on this modification a protective, hydrophilic surface layer on the liposome surface is formed, which prevents the recognition of liposomes by the RES system. Such PEGylated liposomes are often referred to as "sterically stabilized" or "stealth" liposomes. Doxorubicin lipid complex is an example of PEGylated liposomes [45]. PEGylation enables liposomes to extend their circulation times in the bloodstream, increasing their probability of accumulation and delivery of drug to the tumor. Further modifications of liposomes through tumor-specific molecules (i.e., folic acid [46, 47], transferrin [48], or various antibodies [49–51]) have shown to increase the drug's uptake by target cells while minimizing uptake by nontarget, healthy cells. Most of the commonly used anticancer anthracyclines (i.e., doxorubicin, daunorubicin, and epirubicin) have been formulated into liposomes. Nanoliposomal camptothecin (CPT-11), also widely known as irinotecan, belongs to the group of plant alkaloids and is known to inhibit topoisomerase 1. Combining CPT-11 with convection-enhanced delivery (CED) resulted in prolonged residence times for the drug within the brain tissue and substantially reduced toxicity [45].

3.1.2 Polymer Conjugates

Polymer conjugates are another form of nanocarriers, which are proving to be useful as anticancer nanomedicines. Several have already advanced into clinical trials and are being tested in phase I and II studies in humans [52]. Polymers like HPMA (N-(2-hydroxypropyl)methacrylamide copolymer), PEG, poly-L-glutamic acid, and dextran are examples, which have been conjugated to anticancer drugs or proteins. These types of polymer–anticancer drug conjugates are probably the most studied variant and are available in numerous chemical modifications. Doxorubicin, paclitaxel, platinates, and camptothecin have been combined with the abovementioned polymer representatives [53]. The mechanism of action and advantages of polymer–drug conjugates rely on the following characteristics [52]:

1. Conjugation of a drug to a polymeric delivery system decreases the exposure of normal tissues.
2. Improved tumor targeting is achieved through EPR effect.

3. Minimal immunogenicity is provided by the polymer.
4. Ligands can be attached which facilitates cell-specific and intracellular targeting.
5. Specific polymer–drug conjugates increase the renal elimination rate resulting in reduced toxicity, when compared to free drug.

The first polymer-based therapeutics used clinically was polymer–peptide/protein conjugates. Such conjugates are used as anticancer therapeutics, mainly as a combinatorial component to chemotherapy. An example of a clinically used polymer conjugate is SMANCS (styrene maleic anhydride neocarzinostatin), which has been used via intra-arterial administration in patients suffering renal or hepatic cancers [54, 55]. Another representative is pegaspargase, which received FDA approval in 1994 [56]. Phase I and II trials were conducted for acute lymphoblastic leukemia and non-Hodgkin's lymphoma, achieving partial responses while significantly decreasing hypersensitivity reactions, thus improving the treatment window [57]. Other examples include pegfilgrastim used for the prevention of severe anticancer chemotherapy-induced neutropenia [58] as well as peginterferon alfa-2a and peginterferon alfa-2b for the treatment of hepatitis C and renal cancer, respectively [59].

3.1.3 Polymeric Micelles

Polymeric micelles probably are arguably the most investigated and advanced nanotechnology application in clinical trials. They are structures that self-assemble from amphiphilic block copolymers either in aqueous solution or by dialysis of an organic solution against water and develop a size of 20–200 nm in diameter. Their various advantages include thermodynamic stability in physiological solution (i.e., bloodstream), narrow size distribution in the nanometer range, and a unique structure that facilitates the systemic delivery of water-insoluble drugs. These characteristics make them ideal candidates for intravenous administration. Their size helps them to avoid renal excretion and uptake by the reticuloendothelial system. These properties enhance their EPR effect. Recently, antitumor–antibody conjugated micelles, "immunomicelles," encapsulating the anticancer agent paclitaxel were introduced. They were able to recognize and bind to various cancer cells in vitro and successfully delivered higher drug concentrations to tumors in animal models [60]. Kataoka et al. are conducting human clinical trials using a micellar system that incorporates the anticancer drug doxorubicin and have documented complete tumor regression [61]. Paclitaxel, a drug stabilizing microtubules, represents an important class of drugs in the treatment of various cancers. The major limitation associated encountered during treatment with paclitaxel involves the development of a relatively common hypersensitivity reaction. The presence of Cremophor-EL in its formulation is thought to be responsible for those side effects and for alterations in the pharmacokinetics of paclitaxel. This limits the administration of higher doses that may be more advantageous therapeutically. These shortcomings have led to the development of several novel formulations of paclitaxel that are either free of Cr-EL such as ABI-007 (nanoparticle formulation of paclitaxel) and Genexol-PM or are formulated with smaller amounts of Cr-EL to improve safety and feasibility of shorter administration schedules such as BMS-184476. The modified paclitaxel agents are being used successfully in clinical trials [62, 63] and are illustrated in Fig. 10.

3.1.4 Nanogel

Bae et al. [64] have developed a virus-mimetic (VM) nanogel system for anticancer treatment that mimics viral properties more closely than any other delivery systems. Viral properties such as the infection of specific cells within host organisms, destruction of the cells, and the spreading from cell to cell similar to infectious cycles served as a model for designing this delivery vehicle, carrying toxic anticancer agents that otherwise exhibit severe side effects. This synthetic nanosized polymer vehicle

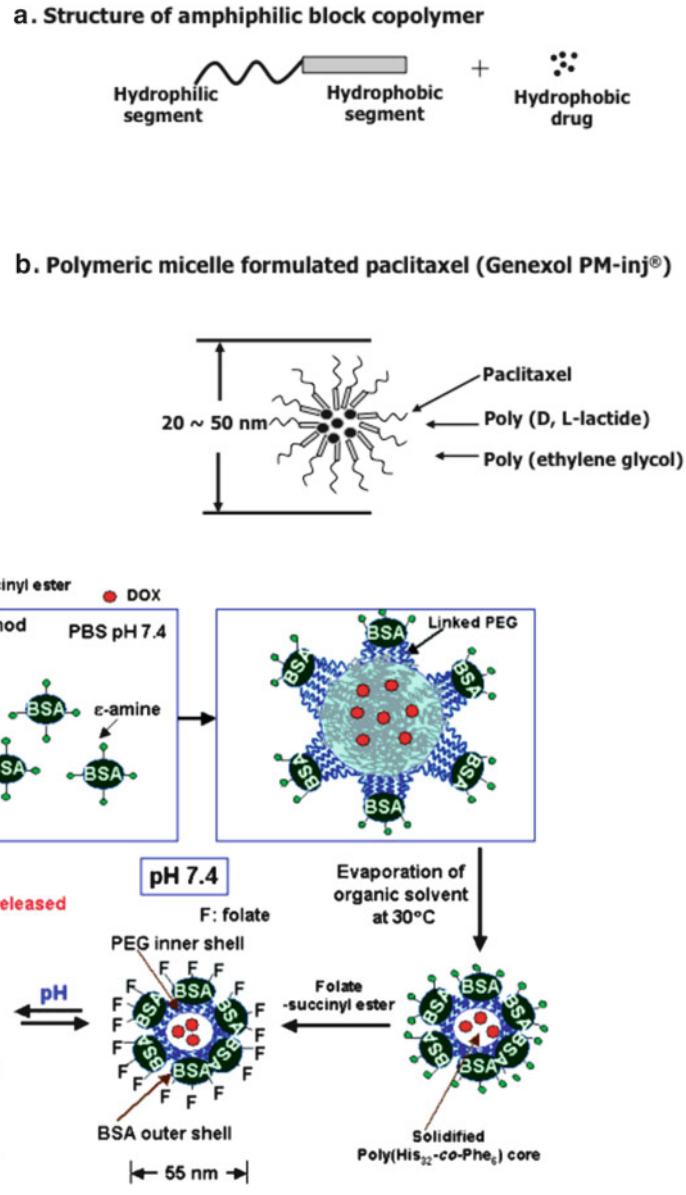

Fig. 10 Formulation of polymeric micelle loaded Genexol-PM. With kind permission from Samyang Pharmaceutical

Fig. 11 Virus-mimetic nanogel

that mimics viral properties consists of a hydrophobic polymer core, which can be loaded with anticancer drugs, and two layers of hydrophilic shells, which are designed to respond to pH changes throughout the body, and is shown in Fig. 11. Further, the outer shell is subsequently conjugated with ligands for specific recognition of receptors overexpressed on many tumors. This design contributes to the enhancement of the antitumor activity of VM nanogels by providing an additional active entry mechanism. At high pH, the VM-nanogel core is rigid. However, the core swells spontaneously when parts of the polymer are protonated at lower pH values found in the tumor microenvironment. VM nanogels incorporated into endosomes can be transferred into the cell cytosol (higher pH), where VM nanogels rapidly shrink back to their original size. Furthermore, the pH-induced reversible swelling/

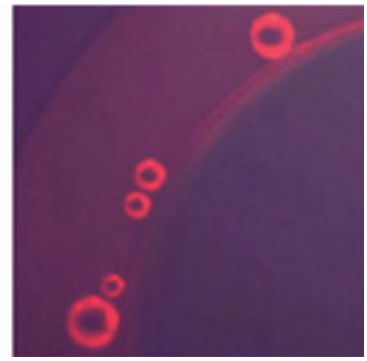

Fig. 12 Nanobubbles. With kind permission of Rapoport Group, University of Utah

deswelling of the core is closely linked to the release of incorporated anticancer drugs. The VM nanogel releases a significant amount of drug at the endosomal pH (e.g., pH 6.4) while reducing the drug release rate at cytosolic or extracellular pH (e.g., pH 6.8–7.4). This cycle repeats itself. Because the drug release induces apoptosis of tumor cells, the VM nanogels are then released from the dead cells for subsequent "infection" in neighboring cells.

3.1.5 Lipid Nanoparticles

Lipid nanoparticles can be derived from nanoemulsions by simply replacing the liquid lipid (oil) by a solid lipid. They are attractive because they combine advantages of various traditional carriers. Akin to polymeric nanoparticles, lipid nanoparticles possess a solid matrix that is protective for chemically labile agents and able to modulate drug release. Similar to nanoemulsions and liposomes, they are composed of well-tolerated, regulatory accepted lipids and can be produced easily on a large industrial scale. Lipid nanoparticles were first used in cosmetic products. Low-density lipid nanoparticles have also been used to enhance ultrasound imaging. Rapoport et al. use microbubbles as drug carriers, which allow for combined cost-effective ultrasound imaging and ultrasound-mediated therapy (Fig. 12). Ultrasound has a number of attractive features as a drug delivery modality. Targeted sonication of microbubble-infused tumor involves only transducer activation without any invasive procedures.

The ideal tumor-targeted microbubble-based drug carrier satisfies the following criteria: (1) stability in circulation, (2) prolonged circulation time to allow for effective accumulation in the targeted tissue, (3) size that allows extravasation through tumor microvasculature (100 nm), and (4) efficient release of drug locally in tumor tissue in response to an external stimulus such as tumor-directed ultrasound [65].

3.1.6 Dendrimer

Dendrimers are complex macromolecules, which retain significant expectations in the treatment of cancer. Dendrimers consist of many branches, which assemble around an inner core allowing other molecules to easily attach to their surface as illustrated in Fig. 13. A dendrimer can be prepared using multiple types of chemistry, the nature of which defines the dendrimer's solubility and biological activity. Dendrimers are used as tissue-repair scaffolds. Moreover, dendrimers are excellent drug and imaging agent carriers through the chemical modification of their multiple terminal groups [66]. Dendrimers have been modified to become sophisticated anticancer operatives carrying multiple tools—a molecule designed to bind to cancer cells, a second that fluoresces upon locating genetic

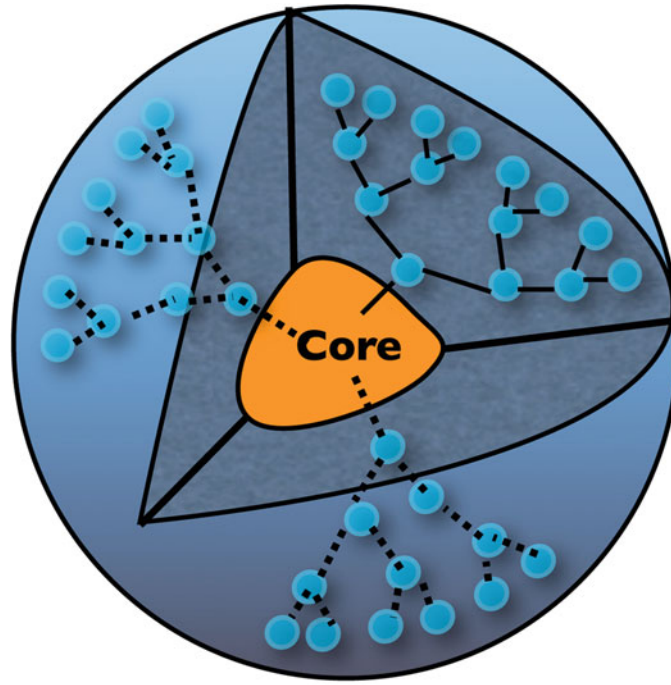

Fig. 13 Structure of dendrimer

mutations, a third to assist in imaging tumor shape, a fourth carrying drugs released on demand, and a fifth to send a signal when cancerous cells are destroyed [67]. University of Michigan scientists are investigating a more efficient and successful way of delivering anticancer drugs, which are less harmful to the surrounding tissue. Baker et al. have developed a nanotechnology that can locate and then eliminate cancerous cells. Using dendrimers equipped with over 100 hooks on the surface allows them to attach to cells in the body in numerous attachment schemes. Baker et al. attach folic acid to a number of the hooks. Cancer cells overexpress folic acid resulting in endocytosis by cancer cells. To the remaining hooks on the dendrimer surface, Baker places anticancer drugs which are delivered in the tendon with the folic acid-mediated endocytosis [68].

3.1.7 Others

Carbon nanotubes belong to the family of fullerenes and are formed of coaxial graphite sheets (<100 nm) rolled up into cylinders. These structures can be obtained either as single- (one graphite sheet) or multi-walled nanotubes (several concentric graphite sheets). They exhibit exceptional strength and electrical properties and are efficient heat conductors. Due to their metallic or semiconductor nature, nanotubes are often used as biosensors. Carbon nanotubes can be rendered as hydrophilic compounds by surface functionalization. Therefore, they are also used as drug carriers and tissue-repair scaffolds [69]. Nanotubes are hollow cylinders made of carbon atoms. They are not entirely novel, since their reputation of being 100 times stronger than steel has promoted their usage in several industrial sectors throughout the 1980s. Primarily, they have found new utilization for the transport of DNA inside of cells as well as for targeted thermal ablation therapy. Newer approaches for delivering small interfering RNA (siRNA) into cancer cells are described in the complexation of siRNA targeted with hypoxia-inducible factor 1 alpha (HIF-1alpha), exhibiting specific inhibition of cellular HIF-1alpha activity in a wide variety of cancers. Intratumoral administration of

nanotube-HIF-1alpha-siRNA complexes significantly inhibited the activity of tumor HIF-1alpha in animals with pancreatic cancer [70]. Elevated levels of HIF-1alpha are found in many human cancers and are associated with drug resistance and decreased patient survival. Carbon nanotubes/siRNA complexes, therefore, may prove advantageous as a next-generation agent.

Fullerenes are crystalline particles that are carbon atoms whose molecular architecture is arranged in a soccer ball-like configuration. Also known as "buckyballs," they were discovered in the 1980s. Unlike other molecules that have applications as cancer drug delivery vehicles, fullerenes do not break down in the body and are excreted intact. This trait can be important for some cancer treatment compounds that are dangerous to healthy cells. For example, fullerene drug delivery particles that contain radioactive atoms would allow for the complete removal of radiation from the body following treatment.

Fullerenes show promise as functional components in several emerging technologies. For biomedical applications, they have been used in gene- and drug-delivery systems, imaging agents, and photosensitizers for cancer therapy. Their major drawback for biomedical application is their insolubility in water. Fullerenes attached to viral nanoparticles have recently shown promise in medical applications. Confocal microscopy demonstrated internalization of these complexes. These results open the door for the development of novel therapeutic devices with potential applications in photoactivated tumor therapy [71].

Nanopores have diagnostic, as well as treatment, applications in cancer. Engineered into particles, they are holes that are so tiny that DNA molecules can pass through them one strand at a time, allowing for highly precise and efficient DNA sequencing. As a DNA strand moves through a nanopore, scientists can monitor each "letter" on it, deciphering coded information, including mutations associated with cancer. By engineering nanopores into the surface of a drug capsule that are only slightly larger than the medicine's molecular structure, drug manufacturers can also use nanopores to control the rate of a drug's diffusion in the body.

Paclitaxel is a widely used and well-known anticancer drug, which binds to tubulin and effects as its inhibitor. The binding of paclitaxel to an intermediate site within a nanopore, which then facilitates the drug moving to its binding site in the microtubule interior, suggests that derivatives of paclitaxel that may reverse the isotype specificity or lead to an alternate stabilizing hydrogen-bond interaction with tubulin; thus, increasing the rate of passage to the luminal binding site would offer a therapeutic advantage in paclitaxel-resistant cases [72].

3.2 Thermal Ablation

Moving away from conventional chemotherapeutic agents that activate normal molecular mechanisms to induce cell death, researchers are also exploring ways to physically destroy cancerous cells from within. Thus, nanoshell technology is being used in the laboratory and in clinical trials to thermally destroy tumors from the inside. Nanoshells can be designed to absorb light of different frequencies, generating heat (hyperthermia). Once the cancer cells take up the nanoshells (via active targeting), scientists apply near-infrared light that is absorbed by the nanoshells, creating an intense heat inside the tumor that selectively kills tumor cells. Similarly, newly targeted magnetic nanoparticles are in development that will both be visible through MRI and can also destroy cells by hyperthermia.

Nanoshells have a hollow core of silica and a metallic outer layer (i.e., gold). Because of their size and EPR, nanoshells will preferentially concentrate at tumor sites. Targeting occurs by decoration of the nanoshells with molecular conjugates that recognize surface-expressed antigens. This second degree of specificity preferentially links the nanoshells to the tumor and not to neighboring healthy cells.

In animal experiments, Halas' research team at Rice University directed infrared radiation through tissue and onto the shells, causing the gold to superheat and destroy tumor cells while leaving healthy ones intact. The amount of heat can be externally controlled by the thickness of the outer gold

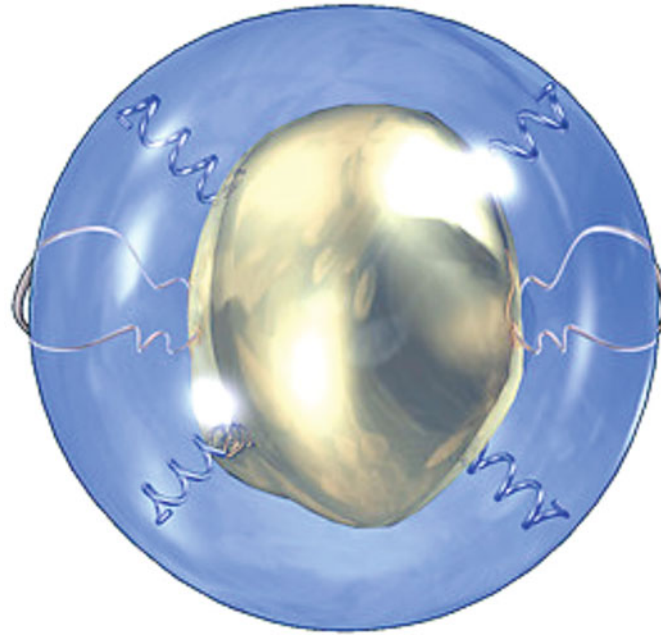

Fig. 14 Nanoshells. A drawing of CytImmune Sciences gold-based nanomedicine, Aurimune. Now in human clinical trials, Aurimune is a gold nanoparticle coated with an agent called tumor necrosis factor anticipated to weaken a tumor's blood vessels, making follow-up chemotherapy more effective [CytImmune Sciences]

layer and the type of laser used as external light source. Nanoshells could 1 day also be filled with drug-containing polymers. Heating them causes the polymers to release a controlled amount of the incorporated drug. Phase I studies with Aurimune (CYT-6091), consisting of recombinant human tumor necrosis factor alpha, a known tumor-killing agent, bound to the surface of PEGylated colloidal gold nanoparticles, have been conducted successfully in various solid tumors (Fig. 14). The external energy source can be mechanical, radio frequency, or optical—with identical therapeutic action. The result is greater efficacy of the therapeutic agent and a significantly reduced set of side effects. Others have also demonstrated the use of gold-coated nanoshells to treat tumors in mice [73].

3.3 Nucleic Acid Delivery

3.3.1 siRNA

A radically new mechanism for cells to control their protein production was discovered and described in a 1999 science paper discovered by plant geneticists Baulcombe and Hamilton. This process relies on small pieces of double-stranded RNA molecules to bind and neutralize specific messenger RNA molecules, resulting in the inhibition of translation. In 2002, Tuschl and his collaborators at the Max-Planck Institute for Biophysical Chemistry in Gottingen, Germany, showed that small interfering RNA (siRNA) molecules could also block protein production in mammalian cells.

Gene therapy based on RNA interference (RNAi) attracted broad attention due to its enormous potential for various clinical applications. RNAi, induced by double-stranded RNA, is a naturally occurring phenomenon in a cell. In this process, gene expression is regulated in a highly sequence-specific manner at a posttranscriptional level. siRNAs are composed of 21–25 base pairs, which can induce a gene-specific RNAi [74–76]. Several groups have screened a number of clinically active siRNA sequences. However, the use of siRNA in clinical settings is limited, due to its highly negatively charged backbone containing phosphodiester linkages. This backbone is responsible for a poor intracellular uptake through a negatively charged plasma membrane and a rapid degradation by

extracellular enzymes, respectively [77]. A number of delivery systems are being designed to overcome these drawbacks [78]. Delivery systems based on cationic polymers are popular choices for the delivery of synthetic siRNA since polymeric carriers are considered relatively safe for repeated administration and are non-immunogenic [77]. Nanoparticles have resolved this impediment and are proving to be ideal carriers for siRNA molecules. They have the ability to load large numbers of these molecules, allow them to travel through the body within a protected environment, target them to cancer cells, and release their RNA molecular load directly inside specific cells.

The marriage between nanoparticles and siRNA is proving to be auspicious with several companies pushing nanoparticle-delivered anticancer siRNA agents to human clinical trials.

A phase I study of Atu027, a liposomal siRNA formulation targeting protein kinase N3 (PKN3) to inhibit tumor-associated angiogenesis, is currently ongoing in patients with advanced solid tumors. Calando Pharmaceuticals and the National Cancer Institute (NCI) entered into a collaborative development program for a nanoparticle-based siRNA therapeutic aimed at treating neuroblastoma [79–85].

3.3.2 pDNA

Over the last decade, a number of nonviral gene delivery systems based on synthetic materials have been developed to replace complication-plagued virus-originated gene carriers [86, 87]. However, there is still room to improve transfection efficiency of the nonviral methods. Synthetic carriers should overcome a number of extracellular and intracellular barriers to the delivery of plasmid DNA (p-DNA) to the cell nucleus. Barriers to functional activity of p-DNA include the dissociation of p-DNA from a carrier complex, the transport of p-DNA to the perinuclear space in the cytoplasm, and the passage of p-DNA across the nuclear membrane. These rate-limiting steps in a polymer-based gene delivery carrier must be overcome [88, 89]. Recent studies showed that plasmids containing a specific sequence recognized by a nuclear protein (nuclear factor κB, NFκB) might improve transfection, possibly due to nuclear protein-guided intracellular trafficking and active nuclear translocation of the plasmid DNA [90–92]. In a study published by Kim et al., a plasmid DNA containing repeated NFκB binding sites, in conjunction with the reducible poly(amido ethylenimine) to improve the cytosolic release of plasmid DNA, was used to improve transfection efficiency (Fig. 15). It was hypothesized that the triggered release of the plasmid DNA with the NFκB-binding sites in the cytoplasm would facilitate nuclear translocation, which could lead to enhancement of transfection efficiency. Besides the intracellular delivery, additional functionalities, including improved extracellular stability and tissue specificity, are to be incorporated in this system to produce a gene delivery system that eventually mimics the virus infection machinery.

Positively charged liposomes (cationic liposomes) with or without modifications are often used for therapeutic gene delivery because their positive charge can retain negatively charged genes [93]. Moreover, cationic liposomal carriers are capable of interacting with the plasma membrane of cells and facilitating their uptake [94, 95]. Liposomes are also preferentially routed by leaky tumor vasculature and therefore have substantial potential to increase the amount of cargo (i.e., siRNA) delivered to tumor [96]. Liposomal gene delivery systems still seem to be a safer alternative than viral-based gene delivery systems based on their low toxic immune responses and low risk of altering the host's genomic structure [97].

3.4 Image-Guided Therapy

Drug delivery aims to deliver drugs with cell precision. Implementing nanotechnology to improve targeted delivery of imaging contrast agents in addition to anticancer therapeutics is a logical extension. Self-assembled biocompatible nanodevices that will detect, evaluate, treat, and monitor patient responses are expectations for the future of nanomedicine.

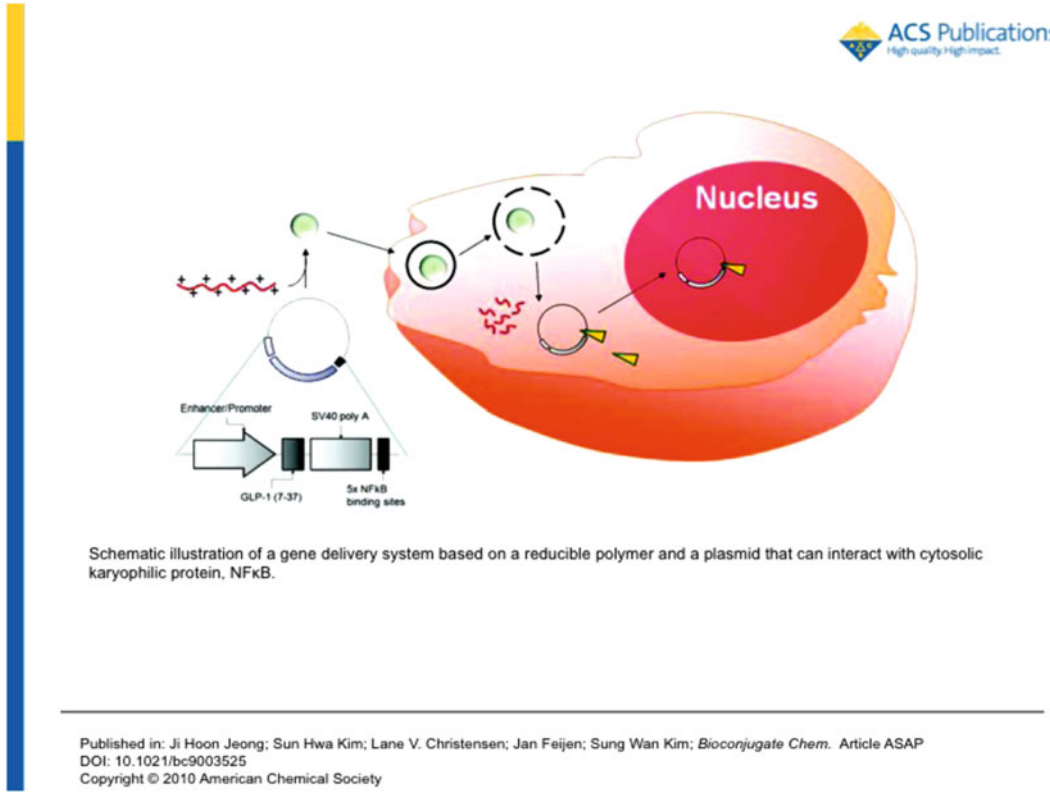

Fig. 15 Gene delivery system interacting with cytosolic karyophilic protein. NF B. American Chemical Society 2010, [115]

4 Safety Considerations

The NCI's Alliance for Nanotechnology in Cancer is working to ensure that nanotechnologies for cancer applications are being developed responsibly since it is a powerful tool for combating cancer. Even though our ability to manipulate objects at the nanoscale has developed in recent years, existing sources of nanoparticles, both natural and man-made, are as old as the Earth. Many nanoparticles are naturally occurring (e.g., volcanic ash or sea spray) or are derived from by-products of human activities (e.g., smoke and soot from fire as early as the Stone Age). Ambient incidental nanoparticles, which exist at order-of-magnitude higher levels than engineered particles, are one of the challenges of nanoparticle exposure studies.

The continuous safety monitoring of nanotechnology is required. The small size, high reactivity, and unique properties of nanomaterials have raised concerns regarding the environment, health, and safety. While the potential toxicity of carbon nanotubes being associated with tissue damage in animal studies remains to be elucidated, the majority of available data indicate that there is no class effect in terms of toxicity for nanoparticles. In fact, most engineered nanoparticles appear to be less toxic than household cleaning products or insecticides.

The potential risks of nanotechnology are being thoroughly evaluated by NCI's Nanotechnology Characterization Laboratory (NCL), and its services are available to the nanotech and cancer research communities. The NCL performs nanomaterial safety and toxicity testing in vitro and in vivo to

characterize nanomaterials that enter the bloodstream, regardless of their route of administration (intravenous, intraperitoneal, transdermal, oral, etc.). This evaluation is just one part of the NCL's cascade of tests to evaluate the physicochemical properties, biocompatibility, and efficacy of nanomaterials intended for cancer therapy and diagnosis. Working closely with the US Food and Drug Administration (FDA) and National Institutes of Standards and Technology (NIST), the NCL develops nanomaterial assays, validates these tests, and disseminates its methods to the research communities. Given the significant benefits of nanotechnology for cancer research, diagnostics, and therapy, the potential health risks associated with the manufacturing and usage of nanomaterials must be carefully evaluated in order to successfully and safely exploit this technology.

5 Nanotechnology Characterization Laboratory

Currently, several nanotechnology-enabled diagnostic and therapeutic agents developed by investigators funded through NCI's Alliance for Nanotechnology in Cancer are in clinical trials. A few examples of promising newly developed diagnostics and therapies based on nanotechnology are listed here, divided into the main areas of detection, diagnosis, treatment, as well as new technologies.

5.1 Detection and Diagnosis

5.1.1 MRI Contrast Agent

At the Siteman Center of Cancer Nanotechnology Excellence (Washington University CCNE), a nanoparticle magnetic resonance imaging (MRI) contrast agent has been developed that binds to the αvβ3-intregrin found on the surface of the newly developing blood vessels associated with early tumor development. This agent is being tested in phase I clinical trials aimed to assess its utility in the early detection of cancer [98].

5.1.2 Nanosensor for Biomarker Testing

A technology developed at the Nanomaterials for Cancer Diagnostic and Therapeutics Center (Northwestern University CCNE) has already received FDA approval for a nanosensor test for the drug coumadin. This same technology can be easily adapted to detect important cancer biomarkers, such as prostate-specific antigen (PSA), or to measure blood levels of anticancer agents. In fact, a joint project between Nanosphere, the Northwestern CCNE, and the Robert H. Lurie Comprehensive Cancer Center is conducting a clinical study using human tissue samples to monitor very low levels of PSA to determine if such measurements, which are well beyond the sensitivity of conventional PSA assays, can provide early warnings of disease recurrence [99].

5.1.3 Lymphotropic SPIOs

A clinical trial at the MIT-Harvard Center for Cancer Nanotechnology Excellence is being conducted to determine if newly developed lymphotropic superparamagnetic nanoparticles can be used to identify small and otherwise undetectable lymph node metastases [100].

5.1.4 Barcode Chip Measuring miRNA

The Integrated Blood Barcode (IBBC) chip, developed at the University of California Los Angeles CCNE, is currently undergoing validation tests to measure the levels of approximately 800 miRNAs from 21 melanoma patients before and after therapy [101].

5.1.5 Nanotube CT Scanner

Clinical trials are underway to evaluate a new type of CT scanner, developed at the Carolina Center of Cancer Nanotechnology Excellence (University of North Carolina CCNE), that uses carbon nanotubes as the X-ray source. This new scanner, developed through a joint venture with Siemens, contains 52 nanotube X-ray sources and detectors arranged in a ring, a configuration that eliminates the need to move the X-ray source and increases the precision and speed of CT scanning, which, in turn, could make CT scanning a preferred method for detecting small tumors [102].

5.1.6 Nanotubes as Imaging Agents

Carbon nanotubes to improve colorectal cancer imaging are being developed at the Center for Cancer Nanotechnology Excellence Focused on Therapy Response (Stanford University CCNE) [103].

5.2 Treatment

5.2.1 Adenovirus Nanoparticle for Lymph Node Injection

At the Center of Nanotechnology for Treatment, Understanding, and Monitoring of Cancer (NANOTUMOR) (University of California, San Diego CCNE), scientists are developing a chemically engineered adenovirus nanoparticle to deliver a molecule that stimulates the immune system. A phase I dose escalation study evaluated patients who received a direct intranodal injection of the chemically engineered adenovirus-CD154 (Ad-ISF35) in patients with chronic lymphocytic leukemia/small lymphocytic lymphoma (Clinical Trials.gov Identifier NCT00850057). Systemic clinical effects have been observed following a single intranodal injection with significant reductions in leukemia cell counts and reductions in the size of all lymph nodes and spleen. Injections were well tolerated with grade 2 or less toxicity, mostly subsiding within 48 h after the treatment has been administered. For one patient treated under the above-described regimen, complete remission could be achieved [104].

5.2.2 Nanoparticle for siRNA Delivery

Studies are being conducted with a cyclodextrin-based nanoparticle that safely encapsulates a siRNA agent (CALAA-01) that reduces expression of the M2 subunit of ribonucleotide reductase and subsequently inhibits tumor growth. The CALAA-01 siRNA is protected from nuclease degradation within a stabilized nanoparticle targeted to tumor cells. This open-label, dose-escalating trial tests the safety of this drug in patients who have become resistant to other chemotherapies (ClinicalTrials.gov Identifier NCT00689065).

5.2.3 Targeted Polymeric Nanoparticles

Targeted nanoparticles consisting of a polymer matrix, therapeutic payloads, functional surface moieties, and targeting ligands that allow for particle optimization (i.e., accumulation in target tissue, avoidance of being cleared by immune system, and delivery of drug with desired release profile) are expected to initiate their first clinical trials soon [105]. A recent study that evaluated CRLX101 (formerly IT-101), a nanopharmaceutical comprised of the chemotherapeutic camptothecin (CPT) conjugated to a linear cyclodextrin-based polymer, is designed to increase the exposure of tumor cells to CPT while minimizing side effects [106].

5.3 Combined Therapeutics and Diagnostics (Theranostics)

5.3.1 PET Imaging Agents Evaluating Drug Response

Researchers at the Nanosystems Biology Cancer Center (Caltech/UCLA CCNE) have developed a series known as the [18 F]-FAC family of positron emission tomography (PET) imaging agents, which are being tested for use in assigning patients for chemotherapy with drugs such as gemcitabine, cytarabine, fludarabine, and others that are used to treat cancers including metastatic breast, non-small-cell lung, ovarian, and pancreatic, as well as leukemia and lymphomas. Tumors that are responsive to these drugs show up as bright images in PET scans when patients are first dosed with [18 F]-FAC. So far, biodistribution studies have been conducted in eight healthy volunteers [107].

5.3.2 MRI Contrast Agent Crossing Blood–Brain Barrier as Treatment for Glioblastoma

A nanoparticle designed to cross the blood–brain barrier and specifically target glioblastomas is also nearing clinical trials. This nanoparticle agent can function as both an MRI contrast agent and a drug delivery device [108].

5.3.3 Lithography and Nanoparticle Manufacturing

A Pattern Replication In Non-wetting Templates technology called PRINT enables the design and manufacture of precisely engineered nanoparticles with respect to particle size, shape, modulus, chemical composition, and surface functionality [109].

6 Summary and Prospective

6.1 Advantages

Some of the major clinical advantages of nanomaterials are their ability to deliver therapeutic or imaging agents in a targeted and controlled fashion. This (1) protects the drug (i.e., agent) from degrading prematurely, (2) minimizes undesired interactions with the biological environment while travelling to its target site, (3) increases the drugs/agents absorption into its target tissue, (4) controls the

pharmacokinetic distribution profile, and (5) improves its penetration through intracellular compartments. The synergy among the therapeutic, diagnostic, and monitoring applications of nanotechnology has proven to be particularly potent. New imaging agents, diagnostic chips, and targeted therapies come together to facilitate a form of personalized medicine wherein early and more accurate detection will lead to the rapid initiation of treatments, followed by subsequent monitoring of treatment responses. This real-time therapeutic monitoring will facilitate the timely adjustment of therapy.

6.2 Challenges

One of the key challenges in creating effective nanoparticles is targeting them to the appropriate tissues and cells. Although biological targeting using antibodies on the surface of nanoparticles is one popular option, other researchers are beginning to exploit the physical characteristics of the particles to guide them to a desired location. Size, shape, physical properties, density, and charge all affect how particles travel through the body and whether or not they will cross biological membranes. Biological barriers such as the vascular wall limit the distribution of injected nanoparticles in the body. The optimization based on principles of engineering and physics requires thoughtful investigation.

Decreasing size to the nanoscale means that the surface area of such particles increases significantly. Expanded surface area/volume ratio allows many functional groups to potentially be attached to a nanoparticle, enabling it to detect and bind specifically to tumor cells. To maximize the benefit of this property, careful characterization and control will be required. This is particularly relevant since these novel, nano-based therapeutics/diagnostics will be circulating throughout the human body and will be exposed to a multitude of different body fluids (i.e., blood, plasma, urine, spinal fluid), components (proteins, immune cells, etc.), and tissue types [110].

Once nanoparticles reach their target site, despite their small size, they do not enter into biological systems, such as cells or organelles, easily. Therefore, it is essential to design strategies that enable nanoparticles to recognize the unique surface signatures of their target cells and subsequently enter the cells and access specific organelles. There are already several examples in the literature that illustrate different strategies for intracellular uptake and the efficient delivery of nanoparticles into target organelles, such as the endo- and lysosomes, mitochondria, and nucleus [111]. In this context, recent innovative imaging studies elucidated the process of transport and distribution of nanoparticles. Tada and coworkers analyzed the movement of quantum dots functionalized with tumor-targeting antibodies injected into mice from capillary vessels to the perinuclear region of cancer cells [112].

6.3 Current Limitations to the Efficacy of Nanoparticles

The ultimate in vivo application of nanoparticles will require a more extensive exploration of the physicochemical and physiological processes occurring in biological environments. For example, it is not yet possible to predict nanoparticle biodistribution according to individual physicochemical properties. Moreover, nanoparticle biodistribution can be affected by undesirable interactions with biological systems and molecules, such as proteins, in a process known as opsonization. Furthermore, the mononuclear phagocyte system, which consists of monocytes and macrophages, engulfs and metabolizes foreign molecules and particulates. These and other challenges require our foremost attention. In this specific context, poly(ethylene glycol) (PEG) coatings minimize unwanted recognition and increase nanoparticle circulation half-life [113, 114]. Additional solutions and opportunities are rapidly emerging in the quest for personalized nanomedicines.

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Imaging in Drug Development

Karen A. Kurdziel, Esther Mena, Stephen Adler, and Peter Choyke

Abstract The challenge of drug development in oncology is to achieve optimum delivery of active drug to the tumor with minimal exposure to normal tissue. Newer, highly specific targeted drugs require careful evaluation of these parameters as these agents often leave alternative signaling pathways unblocked, thus providing a mechanism for resistance. Determining these characteristics early in the drug development cycle can reduce the human and monetary costs. Imaging provides a potential means of approximating the fate of a drug throughout the body (whole body imaging) and over time (dynamic imaging) in a noninvasive manner. There are many promising imaging technologies that could be applied to drug development. Among these, radionuclide tagging provides the most sensitive method. This chapter will focus on positron emission tomography (PET), as the most sensitive and quantitative among existing imaging modalities.

Keywords Dynamic radionuclide imaging • Early drug development • Positron Emission Tomography (PET)

1 Background

The challenge of drug development in oncology is to achieve optimum delivery of active drug to the tumor with minimal exposure to normal tissue. Conventional chemotherapeutic agents have a narrow therapeutic index which requires the optimization of both the pharmacokinetic and pharmacodynamic properties critical to craft a clinically successful drug. Newer, highly specific targeted drugs require careful evaluation of these parameters as these agents often leave alternative signaling pathways unblocked, thus providing a mechanism for resistance. Determining these characteristics early in the drug development cycle can reduce the human and monetary costs.

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Monitoring of drug pharmacology in terms of pharmacokinetics (absorption, distribution, metabolism, elimination, etc.) and pharmacodynamics (cell death, enzyme induction/inhibition receptor binding, tolerance, etc.) has been successfully assessed in clinical practice by analysis of blood and urine samples and in some cases using biopsy specimens of relevant tissues; however, this strategy does not provide a complete picture and may not be accurate for molecularly targeted agents.

Imaging provides a potential means of approximating the fate of a drug throughout the body (whole body imaging) and over time (dynamic imaging) in a noninvasive manner. The recent development of specialized imaging drugs enables monitoring of multiple physiological pathways in addition to the traditional strength of imaging, anatomic depiction. The inclusion of imaging in the drug development paradigm may increase the understanding of drug effects and tumor biology at the systems level.

There are many promising imaging technologies that could be applied to drug development. Among these, radionuclide tagging provides the most sensitive method. This chapter will focus on positron emission tomography (PET), as the most sensitive and quantitative among existing imaging modalities.

2 Positron Emission Tomography Imaging: Basics

By labeling compounds with positron-emitting radioisotopes such as oxygen-15, carbon-11, nitrogen-13, and fluorine-18, a highly sensitive PET camera can be used to image the distribution of this compound within the living body with little risk of toxicity due to the low doses, micrograms (μg), involved. The biodistribution of these radiotracers (also known as radiopharmaceuticals) parallels that of the unlabeled compound although it should be recognized that there may be a dose-dependent biodistribution.

PET radiotracers, with half-lives ranging from seconds to several days, decay by positron emission. The emitted positron travels a short distance (~up to 5 mm, dependent on the positron energy of the emitting isotope) before encountering an electron in the surrounding tissues. These two particles (matter and antimatter) "annihilate" each other resulting in the emission of two gamma rays of 511 keV each, traveling approximately 180° in opposite directions. These paired gamma rays can be detected by a ring of detectors around the patient, which sense "coincident" gamma rays from opposite sides of the ring. The sequential acquisition of multiple, nearly simultaneous detection of the two gamma rays at opposite sides of the detector ring or "coincidence detection" [1] allows a 3D volume image to be created. The intensity and distribution of the imaging signal (counts) are proportional to the amount of radiotracer accumulating at that location. With the specific activity (mCi/μg [MBq/μg]) of the administered radiotracer known, quantification of the imaging drug concentration in tissues is possible.

While static scans (2–5 min acquisition time/bed position) provide a single snapshot of the tracer distribution, dynamic imaging of a single bed position (~15–17 cm field of view obtained every 5–10 s) can be performed for any length of time and provides information on rates of uptake. PET can provide valuable information on drug pharmacokinetics in tumor and normal tissues by mathematical modeling of data. One drawback to PET imaging is poor spatial resolution in comparison to computed tomography (CT). The typical CT scan has a resolution of approximately 500 μm, whereas a PET scan typically has a resolution of 4.6–8 mm (dependent on positron energy). Therefore, to accurately measure all of the activity present, a uniform lesion of 1–2 cm in size is generally needed due to the "partial volume effect" which will result in the averaging of normal and abnormal tissue if the lesion diameter is not at least two times the resolution. Other limitations include variable image counts statistics, errors induced by scattering and attenuation correction algorithms, and the inability to distinguish between the parental radiotracer and its radiolabeled metabolites [2].

3 Estimating Kinetic Parameters with PET

Similar to micro-dosing studies, assuming a dose-independent biodistribution, PET radiotracer imaging can be used to predict drug concentration in the target tissue, route and extent of elimination, and concentration in nontarget tissues. After making corrections for attenuation, randoms, and scatter, the total number of counts in each pixel imaged (Bq/ml) can be converted to micrograms (μg) using the known specific activity of the administered imaging drug (MBq/μg). These should allow for predicting optimal dose, normal tissue toxicities, and the effects of reduced drug excretion.

Conventional compartmental modeling methods (which are discussed in detail in a separate chapter in this book) can be applied to dynamic PET image data to provide estimates of the kinetic parameters commonly used in drug developments. Receptor binding kinetics can also be approximated. Due to the inherent variability and complexity of obtaining image-derived data, abbreviated methods [i.e., Logan and Patlak graphical analyses, simplified kinetic analysis, area under the radioactivity–time curve (AUC), standard uptake value (SUV)] have been developed to provide more stable measurements that represent an amalgam of kinetic/binding parameters.

3.1 Compartmental Modeling

A basic two-compartment kinetic model (Fig. 1) simplifies the body into two tissue compartments. A single input function, C_p, is used to model the delivery of an exogenous agent (e.g., PET tracer) injection [3]. Applying this model to PET image data, the first compartment, C_1, can be considered to contain free and nonspecifically bound drug, while the second compartment, C_2, consists of specifically bound, nonspecifically bound, and free tracer. Each compartment also contains a fraction of the blood pool which is assumed to be equal for both compartments. Following a bolus injection, free radiotracer initially enters both C_1 and C_2 from the blood. As the labeled drug becomes bound in C_2, more unbound drug enters from C_1 to maintain equilibrium. The rate constants for radiotracer entry into C_1 and C_2 are designated k_1 and k_3, respectively. As this is an open system, unbound radiotracer can return to the blood (rate constant k_2) as the blood radiolabeled concentration decreases (due to excretion). k_4 is the rate constant for "unbinding" from the target.

In this model, the total tissue concentration (C_T) can be expressed as

$$C_T = C_1 + C_2 + f_p C_p, \tag{1}$$

where f_p = the fraction of vascular space (blood pool) in the tissue. Assuming the nonspecific binding and vascular pool fraction are the same in both compartments, the rate of change in the unbound (free)

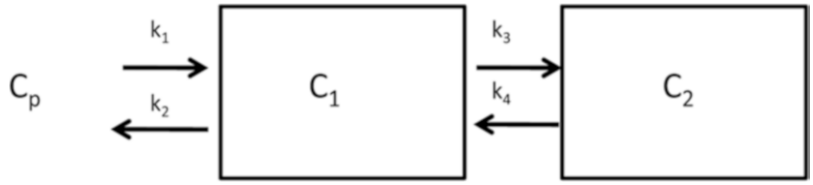

Fig. 1 Two-compartment kinetic model of PET tracer behavior: C_p = *input function* (*vascular blood pool*); C_1 = *unbound radiotracer + nonspecifically bound radiotracer + vascular blood pool fraction*; C_2 = *specifically bound radiotracer + nonspecifically bound radiotracer + vascular blood pool fraction*. The parameters k_1–k_4 are the kinetic rate constants describing the tracer transfer between the compartments

compartment C_1 is simply the difference between the amount of unbound tracer entering the compartment and the amount leaving it and can be described as

$$\frac{dC_1}{dt} = \left(k_1 C_p + k_4 C_2\right) - \left(k_2 C_1 + k_3 C_1\right). \tag{2}$$

The rate of change in the bound compartments, C_2, is dependent on the binding rate (k_3) and the rate of unbinding (k_4). This can be expressed as

$$\frac{dC_2}{dt} = k_3 C_1 - k_4 C_2. \tag{3}$$

For application in PET tumor imaging, C_p can be estimated from a blood pool input function, and C_2 can be derived from the tumor time–activity curve (C_2). The best-fitting kinetic parameters can then be estimated using a least squares method [4, 5].

Some simple but effective relationships can be formed from the kinetic flow parameters found in Eqs. (2) and (3) which can be related to the pharmacokinetics under study. To start, one can examine the compartment model system when the distribution reaches equilibrium and the rate of change of concentration between compartments is zero. Thus, you have the following relationships.

From Eq. (2) you have

$$k_3 C_1 = k_4 C_2 \tag{4}$$

$$\frac{k_3}{k_4} = \frac{C_2}{C_1} \tag{5}$$

And from there you can derive

$$\frac{C_2}{fC_p} = \frac{k_1 k_3}{k_2 k_4} \equiv V_d. \tag{6}$$

The constant, f, is the fraction of free radiotracer in plasma which is allowed to flow into compartment 2. V_d is the volume of distribution. It represents the volume that the total amount of tracer (bound and unbound) would occupy, if the concentration in that compartment was equal to that of the blood pool compartment.

By substitution into Eq. (2) and setting $dC_1/dt = 0$ to represent the condition of equilibrium, and from in vitro ligand binding analysis, in which one attempts to measure the maximum concentration of binding sites (B_{max}), one derives the following relationship:

$$\frac{B_{max}}{K_d} = \frac{C_B}{C_L} \equiv BP_{in\,vitro}, \tag{7}$$

where K_d is the ratio of the rate of unbinding to the rate of binding and C_B and C_L are the concentration of bound and unbound tracer, respectively, with BP, the binding potential, defined as C_B/C_L. It can be shown that using the equivalent definition of binding potential for in vivo studies, one has

$$BP_{in\,vivo} = \frac{C_2}{f_1 C_p} = \frac{k_1 k_3}{k_2 k_4} = V_d. \tag{8}$$

Equation (1) represents a reversible system, in which the radiotracer will bind to the target tissue under study, but eventually will unbind and eventually return to the plasma. The unbinding flow constants, k_2 and k_4, are usually assigned values greater than zero. One can also examine a system which is nonreversible, in which the tracer binds permanently to the target, or when k_4 is so small that the

rate of unbinding cannot be measured on the time scale of the scan. In this situation, one can set k_4 to zero and Eqs. (2) and (3) become

$$\frac{dC_1}{dt} = k_1 C_p - \left(k_2 + k_3\right) C_1 \tag{9}$$

$$\frac{dC_2}{dt} = k_3 C_1. \tag{10}$$

The solution to this set of differential equations is

$$C_1 = k_1 \int_0^T C_p \, e^{\left[-(k_2+k_3)(T-t)\right]} dt \tag{11}$$

$$C_2 = \frac{k_1 k_3}{k_2 + k_3} \int_0^T C_p(t) \left(1 - e^{\left[-(k_2+k_3)(T-t)\right]}\right) dt, \tag{12}$$

where $k_1k_3/(k_2+k_3)$ is referred to as K_{flux} and measures the rate at which the tracer is taken up into the bound compartment [4–6].

Due to the noise inherent in PET imaging and the errors induced by the measurement techniques themselves, estimations of the individual rate constants can be quite variable (particularly when they are small), and the combined parameters, notably V_d and K_{Flux}, tend to be more robust.

3.2 Blood Pool Input Function (C_p)

The "input function" (C_p) can be measured by serial arterial or venous blood draws or is "image-derived," estimated from a volume of interest (VOI) over a heart cavity, aorta, or other large vessel.

3.2.1 Arterial Input Function

The most accurate method of obtaining an input function is by rapid direct arterial blood sampling beginning at the time of injection. Since it is impossible to sample the artery that directly supplies the tumor, blood activity determination obtained through serial arterial sampling of a superficial artery (e.g., the radial artery) [3] immediately after radiotracer injection is used as a substitute. The concentration of the labeled drug in the blood can be determined using a gamma counter [7]. Extracting (by centrifugation) and counting the plasma radioactivity improves the accuracy of the input function for highly protein bound drugs. The timing of the blood samples needs to be matched to the PET scan times. Additional corrections are compulsory to accurately define the input curve such as the time delay and dispersion in the tubing, the contamination of the samples in the tubing, and the cross-calibration between sample detection setup and the PET scanner [8]. Alternate approaches for obtaining the arterial input function have been applied, including the insertion of a β-microprobe into the femoral artery in rats [9], the development of positron-probe system in humans [10], or running an arteriovenous shunt through an external gamma counter [11].

3.2.2 Venous Input Function

Arterial sampling is technically challenging, involves increased radiation exposure to staff, and causes discomfort and risks to the patient, such as infection, bleeding, and thrombosis. An alternative method

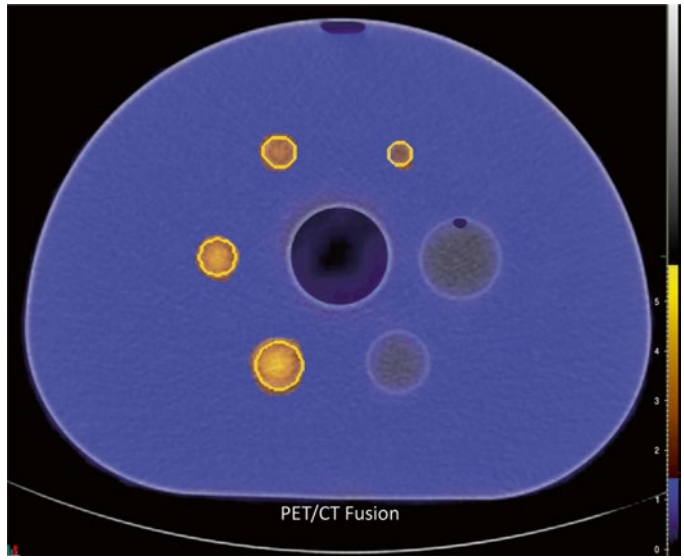

Fig. 2 PET/CT imaging of a phantom with the four smallest spheres is filled with tracer and the background with a lesser amount demonstrates the *partial volume effect*. The intensity of uptake in the smallest sphere is more difficult to identify despite containing the identical concentration of tracer as the other filled spheres. As the sphere to background ratio is known (4:1), volumetric regions of interest (VOIs) can be drawn along the actual borders of the spheres and in the background which can be used to calculate the measured fraction of activity (partial volume fraction). For this study, the ratios were 1.15, 1.45, 1.82, and 2.10, which equates to a fractional recovery of 28.7 %, 36.2 %, 45.6 %, and 52.6 % for the 10, 13, 17, and 22 ml spheres, respectively

for determining the input function is by venous sampling. This method does not require the initial rapid sampling as the radiotracer has already been dispersed by the time it reached the venous system. This, however, results in an initial underestimation and later overestimation of the actual arterial input function [3].

A method to obtain "arterialized" venous blood sampling, drawing blood samples from a venous line, from an extremity that has been warmed to 43–44 °C, thus increasing the regional blood flow, and minimizing the arteriovenous difference [3, 12].

3.2.3 Population-Based Input Functions

To avoid the difficulties encountered in measuring the input function in each patient, population-based input functions have been used [13–15]. A population-based arterial blood curve is generated by averaging the actual arterial blood curves obtained from samples of a group of similar subjects. This average population-based arterial input function is normalized to an individual subject's venous blood sample(s) to estimate the individual's input function. This method assumes that previous arterial blood curves for the specific radiotracer and route of administration are available.

3.2.4 Image-Derived Input Functions

The input function can also be estimated directly from the dynamic images. This can be done by deriving time–activity curves from the blood pool (i.e., VOI of heart chamber, aorta, or other large vessel) [16–24]. Due to the resolution limits of the PET camera, corrections for partial volume effects (Fig. 2) must be made to avoid underestimating actual activity [25–28] and/or spillover (Fig. 3, activity from adjacent organ "spills" into the blood pool VOI) [28, 29].

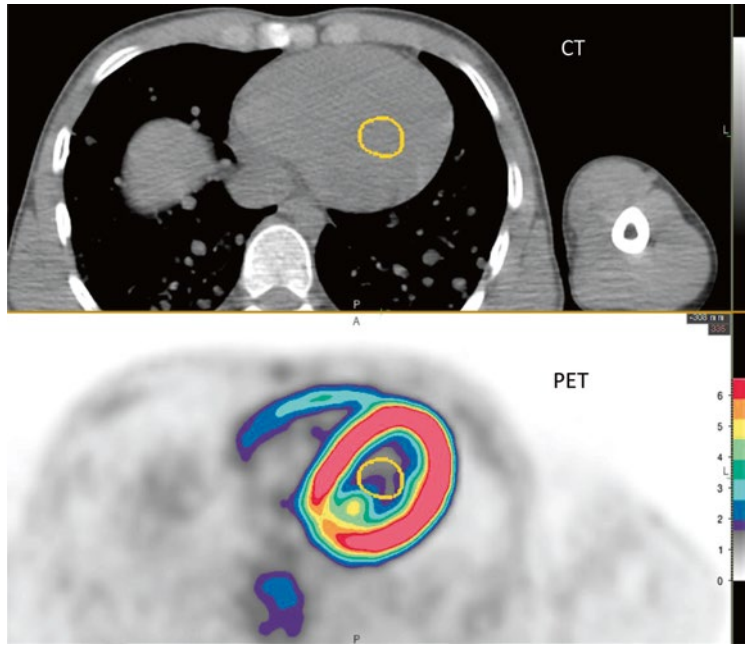

Fig. 3 These FDG PET/CT images demonstrate spillover effects. A volume of interest (VOI) drawn in the left ventricular cavity is adjacent to myocardium (which has higher activity) and therefore contains counts that have "spilled in" from the adjacent structure

These image-based approaches have the advantage of being noninvasive but always need to be initially validated for each individual tracer by comparing the input function derived from PET images with those derived from direct blood sampling from blood vessels or left ventricle [23, 30]. Similar to the individual-normalized population-based arterial input function, this involves combining image-derived input functions with a very limited number of blood samples [31–33].

3.3 Tissue TACs (C_1 and C_2)

C_1 and C_2 can be approximated using reference (background) tissue and tumor VOIs, respectively. Applying a VOI to the dynamic PET images yields a time–activity curve (TAC) in MBq/ml, which can be converted to μg/ml using the specific activity, thus providing a means of determining the drug concentration in the region over time. This is useful because, assuming a linear drug–dose relationship, it potentially provides an estimation of total administered drug needed to produce a therapeutic effect. Data derived from TACs in reference tissues can be used to delineate excretion rates and pathways and to predict dose-limiting toxicities. In the case of nonlinear pharmacokinetics, the varying doses of non-radiolabeled drug can be administered concurrently to examine these effects.

3.3.1 Metabolites

For some radiotracers, radiolabeled drug metabolites are a significant consideration. The activity in the tissues and blood includes both the unmetabolized (parent) imaging drug and any radiolabeled metabolites of the parent. In general, the fraction of radiolabeled metabolites can be determined by performing HPLC on plasma samples, and the resultant curve can be used to correct the blood and tissue data.

3.4 Graphical Analysis Techniques

Commonly employed graphical analysis methods include the Patlak [34, 35] and the Logan graphical analyses [36, 37].

3.4.1 Logan Graphical Analysis

By rewriting Eq. (2) as

$$\frac{dC_1}{dt} = \left(k_1 C_p + k_4 C_2\right) - \left(k_2 + k_3\right) C_1, \tag{13}$$

and using tumor TAC to approximate the target tissue concentration (C_2), Eqs. (1), (2), and (3) can be combined to yield

$$\frac{\int_0^t C_2(t)\,dt}{C_2(t)} = \left[\frac{k_1}{k_2}\left(1 + \frac{k_3}{k_4}\right) + V_p\right]\frac{\int_0^t C_p(t)\,dt}{C_2(t)} + constant, \tag{14}$$

which is in the form $y = mx + b$.

The plot of $\int_0^t C_2(t)dt/C_2(t)$ vs. $\int_0^t C_p(t)dt/C_2(t)$ becomes linear after reaching equilibrium, and the slope of this plot is the radiotracer-target V_d plus the fraction of vascular space within the tissue.

V_d is related to the number of binding sites and has been shown to be more robust than the individual rate constants [38].

The distribution volume ratio (DVR), another useful parameter, is the ratio of the bound and unbound distribution volumes:

$$DVR = \frac{V_2}{V_1}. \tag{15}$$

Assuming the ratio of k_1/k_2 is identical in both the bound (C_2) and unbound (C_1) compartments, the DVR can also be expressed as

$$DVR = \frac{k_3}{k_4} \approx \frac{B_{max}}{k_d} \approx BP, \tag{16}$$

where B_{max} is the free binding sites at equilibrium and k_d is the disassociation constant (equivalent to k_4). The DVR is affected by nonspecific binding and the fraction vascular volume; it is only an estimate of the binding potential [25, 37, 39, 40]:

$$BP^* = \frac{V_3}{V_2} = \frac{BP}{V_2} = \frac{B_{max}}{V_2 K_d}, \tag{17}$$

where BP* is the parameter measured by PET.

3.4.2 Patlak Graphical Analysis Method

The Patlak graphical [34, 35] analysis method is designed to measure the influx rate constant, K_i, which is the rate which a particular tracer accumulates in the target tissue assuming that the

radiolabeled drug is irreversibly bound ($k_4=0$). This corresponds to the K_{flux} in the compartmental analysis method. The equation used in the Patlak graphical method is

$$\frac{C_2(t)}{C_p(t)} = K_i \frac{\int_0^t C_p(t)\,dt}{C_p(t)} + \left(fV_1 + V_p\right). \tag{18}$$

where $C_2(t)$ is the concentration in the target tissue, $C_p(t)$ is the concentration in plasma, V_1 and V_p are the volumes of the tissue containing the reversible compartment and the volume of the plasma, respectively, and f is the fraction of the tracer which flows back into the plasma.

In order to measure K_i, which approximates the binding influx rate constant or uptake rate constant, one plots the quantities

$$\int_0^t \frac{C_p(t)\,dt}{C_p(t)} vs. \frac{C(t)}{C_p(t)}. \tag{19}$$

The resulting plot will become linear at times t when the system has had a chance to equilibrate and the slope of the linear portion will equal K_i.

3.4.3 Reference Regions

The requirement of drawing blood samples also makes estimation of the kinetic parameters impractical in high-throughput clinical environments. Several methods have been developed which allow one to use reference tissue TACs (C_{ref}) as a substitute for direct or indirect measurement of the radiotracer's concentration in blood (C_p).

To eliminate the need to measure the blood concentration in the Patlak plot, one can replace the blood concentration term $C_p(t)$ with a measured concentration of activity in an area which does not trap the tracer, $C_{\text{ref}}(t)$. Furthermore, this new measure of normalization activity can be expressed as

$$C_{ref}(t) = \left(V'_1 + V'_p\right) C_p(t), \tag{20}$$

where V'_1 is the volume of the reversible tissue and V'_p is the volume of plasma (vascular volume) within the reference tissue selected to replace the blood concentration. Substituting $C_{\text{ref}}(t)$ for C_p in Eq. (1) yields

$$\frac{C(t)}{C_{ref}(t)} = \frac{K}{V'_1 + V'_p} \frac{\int_0^t C_{ref}(t)\,dt}{C_{ref}(t)} + Const. \tag{21}$$

One can now plot the values of $\int_0^t C_{\text{ref}}(t)/C_{\text{ref}}(t)$ vs. $C_2(t)/C_{\text{ref}}(t)$, where C_2 again represents the radiolabeled concentration in the target tissue, and after time t, when the free radiotracer has equilibrated, the plot becomes linear. The slope of the linear component after this time t is then K_i normalized to the volume of distribution of the reference tissue.

The Logan graphical method can also be performed using a reference region in place of an input function and by substituting C_{ref} for C_p into Eq. (14). Assuming the ratio of k_1/k_2 is the same for both C_{ref} and C_2, Eq. (14) combined with Eq. (16) can be expressed as

$$\frac{\int_0^t C_t(t)\,dt}{C_t(t)} = DVR \times \frac{\int_0^t C_r(t)\,dt}{C_t(t)} + Const, \tag{22}$$

with $\int_0^t C_{\text{ref}}(t)dt$ equal to the integral of the reference tissue time–activity curve. The slope of the linear portion of the plot is DVR and the BP can be estimated as DVR −1 [37, 41].

Further modifications have been proposed to reduce bias [42–44]. The effect that various estimations and corrections may have on the utility of the resulting parameters has been investigated [2]. Parametric, pixel-by-pixel applications of kinetic models have also been used with both raw (i.e., as part of the image reconstruction) and image PET data [45]; however, these methods are beyond the scope of this chapter.

4 Imaging as a Biomarker

Imaging can be used for target identification (to differentiate between potential responders and nonresponder a priori), drug delivery and quantitation (estimate therapeutic doses and schedules), and therapy monitoring (identification of response to targeted therapy earlier than or in the absence of anatomic tumor response). The establishment of qualified imaging biomarkers may reduce the time and cost of translating promising therapeutics to clinical use.

4.1 Imaging Nonspecific Tumor Properties

4.1.1 ^{18}F-Fluorodeoxyglucose

Most PET studies in oncology use ^{18}F-Fluorodeoxyglucose (^{18}F-FDG), a radiotracer analogue of glucose. FDG uptake in cancer is based on the Warburg effect; malignant tumors generally exhibit increased glycolytic activity compared with normal tissue which derives its energy from oxidative phosphorylation [46]. Since glycolysis is significantly less efficient than oxidative phosphorylation, tumors differentially take up ^{18}F-FDG which is transported from the blood pool by GLUT1 transporters. Once inside the cell, it is phosphorylated by hexokinase to ^{18}F-FDG-6-phosphate, which is not recognized as a substrate for further glycolytic processing and therefore becomes trapped within the cells [3, 47]. ^{18}F-FDG uptake in tumors is dependent on a combination of factors including phosphorylation rate, degree of hypoxia, and levels of glucose transporters [48–50].

The initial work in modeling ^{18}F-FDG was based on work by Sokoloff et al. using ^{14}C-deoxyglucose autoradiography in rats [47] and was validated in the human brain [3, 51–54], heart [55–57], and tumors [58, 59]. This kinetic model is based on a biochemical model of reversible, transport-facilitated diffusion of FDG into the cell (k_1/k_2) and largely irreversible hexokinase-mediated phosphorylation (k_3/k_4 where $k_3 \gg k_4$; i.e., k_4 is negligible) of ^{18}F-FDG to $^{18\text{F-FDG-6}}$-phosphate (FDG-6-P), the terminal metabolite. The metabolic rate of glucose (MR_{gluc}) utilization can be estimated knowing the arterial glucose concentration and the relationship between glucose and ^{18}F-FDG transport and phosphorylation as follows:

$$MR_{glu} = \frac{C_{glu}}{LC} \times \frac{k_1 k_3}{k_2 + k_3}, \tag{23}$$

where C_{glu} is the circulating blood glucose concentration and LC is the lumped constant, a parameter that relates to the differential uptake and phosphorylation rates of ^{18}F-FDG and glucose. The units of MR_{gluc} are μmol/(min g).

As the contribution from k_4, the dephosphorylation rate, increases over time and may be significant in some tissues, notably the liver, an expansion on Sokoloff's estimation of glucose metabolism was made to account for dephosphorylation, by Phelps et al. [57].

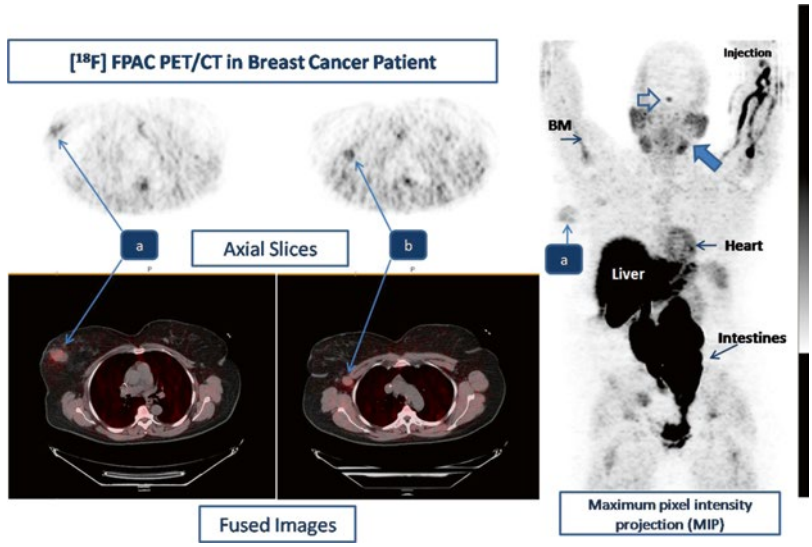

Fig. 4 PET/CT images acquired 80 min after the i.v. (left antecubital fossa) administration 6.1 mCi of [^{18}F]FPAC. The maximum SUV of the 4.4 cm right primary breast tumor (a) was 1.3. The SUV of the involved 2.5 cm right axillary node (b) was 1.6. The majority of the tracer is found in the excretory pathway (liver and intestines), with lesser uptake seen in the bone marrow (BM) and heart. Uptake is also seen in the pituitary (*open arrow*) and salivary (*solid arrow*) glands

To reduce the complexity of the data analysis, PET data is clinically recorded as a standardized uptake value (SUV). This is simply the activity within the VOI at a single time point normalized to the administered dose and patient's body size (most commonly the body weight; however, using either lean body mass [60] or body surface area [61] has been shown to improve accuracy) (Fig. 4).

In general, simplified kinetic estimates, such as SUV, have been shown to correlate with the glucose metabolic rate as determined by more complex kinetic and graphical methods [62, 63]. In the simplified kinetic analysis (SKA), the SUV of a target lesion at a single time point is normalized to the ^{18}F activity and blood glucose level obtained from a concurrent venous blood sample [64]. Variations on this model were also evaluated by Sundaram et al. [65], showing good correlation of simplified models with the Patlak plot; however, estimates using a single time point varied from the Patlak method by 15.1 %±3.9 %, while the use of a small number of dynamic image slope differed by only −1 %±1.4 %. Other techniques include normalizing the measured target VOI activity to the cumulative plasma concentration ($\int_0^t C_p$) and normalization to a background tissue (SUV_{tumor}:$SUV_{background}$). For ^{18}FDG, these basic methods are sufficient for clinical use [62].

During the last two decades, ^{18}F-FDG has become established as a clinical method of diagnosing tumors, identifying recurrences, and monitoring therapy. A remarkable early treatment response with ^{18}FDG was found in the treatment of gastrointestinal stromal tumors (GIST) with the tyrosine kinase inhibitor, imatinib mesylate (Gleevec; Novartis Pharmaceuticals Corp.). GIST is a rare subset of mesenchymal tumors specific to the gastrointestinal system, of which 90 % overexpress cKIT, a proto-oncogene responsible for the production of a growth factor receptor with tyrosine kinase activity. Protein kinase inhibitors such as imatinib downregulate tumor glucose metabolism [66]. Responders to imatinib show a marked and rapid decrease in ^{18}F-FDG uptake as early as 24 h after administration, clearly preceding the anatomic response [67–71].

While ^{18}F-FDG is not a radiolabeled therapeutic, and it does not target specific receptors, quantification of uptake can indicate therapeutic response and, therefore, be useful in drug development. Work is currently underway to qualify ^{18}F-FDG PET as an imaging biomarker of metabolic tumor activity.

4.2 Targeting Specific Tumor Receptors

4.2.1 ^{18}F-AH111585, an RGD PET Imaging Agent Targeting $\alpha_v\beta_3$-Receptors

$\alpha_v\beta_3$-integrin receptors are upregulated in the endothelium of angiogenic vessels. The integrin receptor type $\alpha_v\beta_3$ is preferentially expressed on proliferating endothelial cells associated with neovascularization in both malignant tumors and normal tissue, but is absent in quiescent blood vessels [72, 73]. $\alpha_v\beta_3$-integrin expression on endothelial cells modulates cell migration and cell survival during angiogenesis, while $\alpha_v\beta_3$-integrin expression on tumor cells potentiates metastasis by facilitating invasion and movement across blood vessels. Efficient tumor invasion requires partial degradation of the extracellular matrix (ECM) at the invasion front. The α_v-integrins bind specifically to the arginine–glycine–aspartic acid (RGD) domain of ECM proteins (fibronectin is a prime example). The $\alpha_v\beta_3$-integrins allow for metalloprotease-mediated degradation of the ECM and the subsequent invasion of tumor cells into the surrounding tissue [72, 74].

^{18}F-AH111585 ([^{18}F]fluciclatide) is a radiopharmaceutical developed for PET imaging, which targets $\alpha_v\beta_3$-receptors [75]. Fluciclatide is a small cyclic peptide containing a synthetic RGD tripeptide, which binds with high affinity to $\alpha_v\beta_3$-integrins. A phase 1 study designed to assess biodistribution, dosimetry, safety, pharmacokinetics, and preliminary efficacy in 18 tumors from seven subjects with late-stage metastatic breast cancer was performed [75]. As the fraction of parent radiotracer in the blood was ~74 % 1 h after injection, the analysis was performed by the modified Patlak graphical method [76] (assuming no receptor binding by the metabolites) and the classic Patlak graphical method (assuming the radiolabeled metabolites bind to $\alpha_v\beta_3$) [34, 35].

Tumor uptake of the radiotracer reached a plateau at ~40–60 min after injection. The uptake in the liver metastases was lower than that of adjacent normal liver uptake, suggesting ^{18}F-AH111585 has limited use in evaluating liver lesions. The high background activity in normal liver is consistent with metabolism of ^{18}F-AH111585 and with previous reports of hepatic accumulation of RGD ligands [77, 78]. Kinetic data indicated that the radiotracer was irreversibly trapped in tumors as shown by a linear phase on the Patlak plot during the first hour post injection. The slope of the Patlak plot was higher in the non-liver tumors than in the normal tissue, suggesting tumor-specific binding. The slope of the Patlak plot (K_i, the rate constant for irreversible retention of ^{18}F-AH111585) showed a statistically significant difference between non-liver tumors and normal tissues consistent with receptor binding. While further work is needed to validate that the specific uptake is due to $\alpha_v\beta_3$-binding, ^{18}F-AH111585 PET may have a role in imaging tumors to predict aggressiveness and for monitoring antiangiogenic treatment.

4.3 Radiolabeling Chemotherapeutic Agents

4.3.1 ^{18}F-Fluoropaclitaxel

Many tumors fail to respond to a wide variety of chemically unrelated chemotherapeutic agents, a condition known as multidrug resistance (MDR). Many of these tumors overexpress a membrane efflux pump called *P*-glycoprotein (Pgp) or other proteins that function similarly (i.e., MRP, LRP, BCRP) [79]. Paclitaxel is a neutral, tubulin-binding, and widely used chemotherapeutic agent whose success is often thwarted by MDR [80]. Its effectiveness and retention is dependent on tumor mitotic rate; however, MDR tumors that overexpress Pgp may actively pump paclitaxel out of the cell reducing its therapeutic value. The ability to image the biodistribution of paclitaxel in vivo has several proposed functions: the amount of tumor uptake can be quantified (tumors with low activity may be identified as MDR prior to treatment), nontarget uptake could be quantified thus providing insights into

anticipated tissue toxicity, and finally, the effectiveness of MDR modulators in restoring high concentrations of drug to target tissue could be assessed.

While ^{11}C paclitaxel has been evaluated [81, 82], the short half-life of ^{11}C (~20 min) and in vivo metabolism of paclitaxel limited its potential utility. ^{18}F-fluoropaclitaxel (FPAC) is a radiolabeled analogue of paclitaxel [83, 84] with in vitro cytotoxicity and a biodistribution similar to paclitaxel [84–86]. The 110 min half-life and limited metabolism (ensuring the measured tissue activity represents active drug) permitted FPAC to serve as an in vivo imaging surrogate for paclitaxel biodistribution (in both preclinical and clinical studies) [84–88]. Kinetic analysis of the 4D image data sets yields organ/tumor-specific parameters. With no a priori model knowledge or parameter estimations, the normal organ image data from dynamic PET studies performed in nonhuman primates was analyzed using Logan graphical analysis, total area under the tumor time–activity curve ($\int_0^t C_t(t)$/(injected dose/animal weight)), as well as simple normalization $C_t(t)$ normalized to injected dose/animal weight. All of the Logan plots became linear by 50 min [85] suggesting that, despite a presumed irreversible component (microtubule binding), the primary kinetics in normal organs is reversible. This may be due to the overall low mitotic rate of normal organs or high amount of active removal of drug by Pgp. This needs to be validated in ongoing studies in cancer patients.

5 Conclusion

This chapter focused on PET as a representative imaging modality which can provide in vivo systems-based quantitative functional data on drug targeting, determination of molecular pathways and drug effect on these pathways, and as a surrogate for treatment response. The primary advantage of imaging is that it is noninvasive. Additionally, it can provide data regarding physiological processes and can image the tumors within their natural microenvironment. There are significant limitations as well. The relatively low intrinsic resolution decreases the accuracy of measurements due to partial volume effects, the need to apply corrections for the limitations of physical systems can introduce measurement errors, and modeling assumptions introduce noise and bias. Meanwhile, radiolabeled drug metabolites decrease the specificity of the imaging signal.

An initiative to advance quantitative imaging and the use of imaging biomarkers [Quantitative Imaging Biomarkers Alliance (QIBA)] is currently working towards formally qualifying various imaging studies/protocols [dynamic contrast magnetic resonance (DCE MR) imaging, CT, FDG PET, etc.] for use in drug development. The future integration of imaging biomarkers into therapy development paradigms will hopefully result in improved cancer care.

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Exposure–Response Relationships of Anticancer Agents: Application in Drug Development and Drug Label

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Abstract The understanding of exposure–response relationship (ER) is critical in oncology drug development and drug approval. The ER relationship not only helps in selecting the optimum dose and regimen for testing in the pivotal clinical trial for approval, but allows recommending dose adjustment for patients with special conditions. The exposure variables are mostly area under the concentration–time profile (AUC) and/or plasma or serum concentration, while the response variable ranges from biomarkers to clinical endpoints. Application of ER assessment in oncology drug development is illustrated using approved cancer drugs, such as busulfan, zolandronic acid, imatinib, docetaxel, carboplatin, vandetanib, everolimus, and ipilimumab. The FDA guidance on ER relationships elaborates on the regulatory applications, study design, and data analysis aspects of the clinical trials that should be conducted during drug development. The results of the clinical trials exploring ER relationships are usually described in the "Clinical Pharmacology" section of the drug's package inserts; however, the impact and interpretation of the results of these trials reflecting in dose modifications, therapeutic drug monitoring, and safety are included in "Highlights," "Dosage and Administration," and "Warnings and Precautions" sections of the label.

Keywords Biomarkers • Exposure • Oncology • Pharmacokinetics • Pharmacodynamics • Response • Surrogates

1 Introduction

A clinical trial evaluating exposure–response relationship of a drug or a biological agent with an acceptable clinical endpoint or a surrogate can significantly contribute in substantiating evidence of effectiveness to support marketing approval of a product. A well-designed exposure–response study provides the rationale for dose selection for a pivotal registry trial and the basis for dose modifications in

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special populations (i.e., patients with organ dysfunctions or patients on concomitant medications) [1, 2]. The exposure–response evaluation is an assessment of the relationship between dose or exposure to a drug or a biological agent and the pharmacodynamic response directly or indirectly associated with the benefit and risks of the therapy.

In the past, pharmaceuticals developing cytotoxic agents for cancer treatment conducted limited investigation in understanding the exposure–response (ER) relationship of a drug during development. Cytotoxic agents (i.e., antimetabolites, anthracyclines, alkylating agents, and platinum drugs) were developed based on the maximum tolerated dose (MTD). The main objective of the clinical trial was to obtain a beneficial clinical response in a relatively healthy population at the expense of toxicity, which could be managed by supportive care, dose reduction, or delay of therapy. Registry trials did not investigate effects of a range of doses in the patient population and for this reason provided limited understanding of the impact of exposure variability on treatment outcome. As a result, inadequate ER analysis was included in the submissions for regulatory approval and subsequently in the product labeling once approved. The dose/exposure and response (efficacy or toxicity) relationships of these cytotoxic agents are very steep, and the therapeutic windows are usually narrow. In most of the investigations, ER assessment focused on exposure-associated toxicity evaluation [3–7]. In the package insert of these drugs, instructions regarding dose adjustment after initiation of the therapy are mostly based on toxicity (e.g., grades 3 and 4 hematologic or nonhematologic toxicities). Dose adjustment for an individual patient was mostly determined by the toxicity-based dose modifications specified in the clinical trial protocol. The therapeutic response or toxicity in certain population and nonresponse or absence of toxicity in other population could not be credited to potentially exposure-related differences. Therefore, the safe use of these agents involved complex factors that could tip the outcome of the therapy from the desired therapeutic benefit to life-threatening toxicity or lack of therapeutic benefit. The dose and dosing regimens of most of the drugs were modified and optimized postdrug approval. Clinical practice dictated dose adjustment for individual patient. Prescription labels of the drugs were updated based on postmarketing studies recommended by the agency or based on published literature and clinical practice.

The absence of adequate ER relationship information of a drug or a biological agent causes significant challenge of using the highly toxic agents safely in cancer treatment. A standard dose for all patients may not be appropriate for an individual patient. This approach reduces the likelihood of a patient to have optimized benefit from a tolerated dose that may be higher than the average dose or exposure prescribed for the overall population. Similarly, an average dose may be too toxic for an individual who may otherwise benefit from a lower yet effective dose without undesirable side effects. Therefore, absence of adequate ER relationship of cytotoxic agents prevents tailoring a dose for a particular patient and in turn an optimal benefit from the therapy.

Development of target-based therapy has been limited in the past with the exception of hormonal therapies. Hormonal therapies for breast cancer or prostate cancer involved drugs intended to interact with targets (e.g., receptors or enzymes) and produce the desired effects. These therapies have a different toxicity profile, and they are better tolerated than the cytotoxic agents [8–11]. The exposure–response profiles of these agents are shallow, and thus the therapeutic window is large. The optimum therapeutic dose or exposure is usually below the maximum tolerated dose or tolerated exposure. Dose selection of these agents is often based on maximum target interactions (e.g., enzyme inhibition, receptor occupancy, biomarker response). The treatments typically require chronic use to provide a long-term remission. Advances in science and technology have provided numerous molecular targets related with the pathogenesis of cancer. Overexpression or mutation of genes has been associated with various types of cancer and provided new targets for oncology treatment. The importance and value of understanding ER relationships during drug development of new drugs that interacts or modulates the targets is now well recognized [12–20].

In drug development, the dose–toxicity relationship of anticancer agents is usually evaluated in early clinical trials. However, the ER evaluations usually failed to demonstrate a relationship or developed a poor or unexplained relationship because of the following reasons:

1. Inadequate understandings of drug's mechanism of actions
2. Inadequate evaluation of the inter-patient and intra-patient pharmacokinetic variability
3. Lack of availability of response biomarkers
4. Selection of inappropriate biomarker or response endpoints
5. Selection and measurement of inappropriate exposure variable (inactive entity)
6. Selection of inappropriate sampling time (delayed response) for exposure and response measurements
7. Absence of evaluation of placebo or low-dose drug effect
8. Absence of range of doses studied in ER studies
9. Inadequate methodology used to measure the response variable

Therefore, to obtain reliable data from an exposure–response clinical trial that can guide dose selection for a confirmatory trial or dose selection for a particular subpopulation, we have to understand the pharmacokinetic variability of the drug, select appropriate response variables, and characterize appropriate entity associated with pharmacologic or adverse effect of the drug [21]. Also, trials should include treatment arms to evaluate placebo effect or at the very least a low-dose treatment effect on appropriate response variable.

2 Measures of Exposure and Response

The exposure variables are usually determined from the concentration–time profiles of the parent drug and/or any active or toxic metabolites. The measurement of these moieties in a biological matrix reliably with a reproducible, sensitive, selective, and precise assay is critical for the evaluation of ER relationship. In oncology, exposure variables [i.e., maximum concentration (C_{max}), total drug exposure (AUC), steady-state drug concentrations (C_{ss})] are better defined and well established compared to the response variables. Exposure variables of a drug may be associated with different response effects. C_{max} of anthracyclines is associated with cardiomyopathy, whereas AUC seems to be related to leucopenia [22]. Sometimes the moiety responsible for the pharmacologic effect of a drug may be different from the moiety associated with the toxicity of the drug. For example, active metabolites, fluorouridine triphosphate generated in the cells from 5-fluorouracil disrupts RNA synthesis, and fluorodeoxyuridine monophosphate inhibits the thymidylate synthase enzyme interrupting DNA synthesis of the proliferative cells and producing tumor cell death [23–25]. On the other hand, urinary catabolite, α-fluoro-β-alanine (FBAL) is likely associated with the neurotoxic effects of the drug [26–28]. Therefore, exposures to the two moieties influence the overall risk benefit associated with 5-fluorouracil therapy. In the assessment of the exposure variables, all the active moieties associated with either the pharmacologic effects or the toxic effects of the drug should be measured. Mostly, total drug concentration in a biological fluid is measured; however, when drugs are highly protein bound, and when protein binding varies significantly among patients, unbound drug concentration as an exposure variable may show a better correlation with drug's response [29].

The association of a response variable with disease progression and how a drug therapy modulates the response variable is the key in the evaluation of ER relationship determining the effectiveness of the drug and is rare in oncology. The importance of response variable measurements depends on the pharmacokinetics of the drug and the exposure range studied. When the pharmacokinetics of drugs is highly variable or a wide exposure range is studied in a patient population, the measured response variable may be able

Table 1 Measures of exposure and response in oncology drug development

Exposure variables	Response variables
Area under the curve: AUC_{0-t}, $AUC_{0-\infty}$, AUC_{ss}	*Biomarkers*: EGFR, VGEF, proteosome, CA-125, CD20, CD34, IgM, IgG, IgA, IL-6, C-reactive protein, calcitonin, serum amyloid protein, serum calcium level, bone alkaline phosphatase, telopeptides
Concentrations: C_{max}, C_{min}, C_{ss}, threshold concentrations	*Surrogate endpoints*: tumor response, time to tumor response, tumor response rate, hematologic response, cytogenetic response, hemoglobin levels
Other parameters: clearance	*Clinical endpoints:* survival, quality of life measures, duration of neutropenia

to identify a threshold exposure variable for a response. In oncology, the evaluation of ER relationship mostly correlates exposure with hematologic (e.g., neutropenia, leucopenia thrombocytopenia, hemoglobin) and/or non-hematologic (e.g., diarrhea, liver enzymes, edema, nausea, vomiting) toxicities [30–33]. Target-based cancer therapy development has progressed significantly over the last decade; however, understanding of the interaction of the drug or biological agent with the targets and subsequent response driving the therapeutic outcome is still investigational. As a result, development of ER relationship for these targeted molecules depends on traditional response variables (i.e., progression-free survival).

2.1 Exposure Variables

Exposure variables are usually assessed from the concentration–time profiles of drugs or biological agent in blood or plasma. Rarely, cerebrospinal fluid, urine, or other biological matrix may be sampled and drug concentrations measured. Exposure variables may be divided into two main categories: concentration measurements and systemic exposure measurements. The concentration measurements are usually maximum concentration (C_{max}), trough concentration (C_{min}), steady-state concentration (C_{ss}), or a threshold concentration associated with the safety or effectiveness of a drug or any of its active entity [34]. Cytotoxic agents in general are administered once every 2-, 3-, or 4-week cycle. In such dosing regimen, the exposure variable may be the maximum concentration measured from the concentration–time profile after the first dose in cycle 1 or in subsequent cycles. When treatment involves weekly treatment (daily × 5) with a rest period or daily dosing on a continuous basis or a long-term continuous infusion, the C_{ss} or the trough concentration at the steady state ($C_{ss,min}$) is usually the exposure variable that is correlated with the effectiveness or toxicity of a drug. In case of daily dosing, after the steady state is achieved, the blood sample taken prior to administering a dose assesses the steady-state concentration. This measurement represents the $C_{ss,min}$. When a drug is infused over a long period (48–96 h), C_{ss} is assessed towards the end of the infusion after the steady state is achieved. Drug concentrations may also be obtained in the second or subsequent cycles of therapy to understand the relationship between drug concentration and the long-term toxicity of a drug. Toxicity is sometimes associated with a threshold concentration that is maintained for a certain duration following drug administration. Neutropenia associated with paclitaxel therapy is related to the duration that plasma concentrations are at or above the threshold concentration of 0.05 μM value [34, 35].

The AUC measurement is a key pharmacokinetic parameter that represents an exposure variable in the assessment of exposure–response relationship of a drug. Carboplatin is dosed to achieve a target AUC based on a patient's renal function and desired platelet nadir for therapeutic benefit from the treatment [33, 36, 37]. The AUC measurements are usually considered for ER relationship for drugs that are taken on a chronic basis (i.e., hormonal agents, some targeted therapy, palliative therapy). There are usually three types of AUC measurements: AUC from time zero to last measured concentration time (AUC_{0-t}), AUC from time zero to infinity ($AUC_{0-\infty}$), and AUC at the steady state (AUC_{ss}). A list of exposure and response variables is presented in Table 1. Rarely, drug clearance values may be associated with the toxicity parameters in ER evaluation. The change in the clearance of a drug because of organ impairment is usually associated with the toxicity of the drug.

2.2 *Response Variables*

Response variables can be of two types: efficacy variables and safety variables. Hematologic measurements may represent both efficacy and safety variables depending on the purpose of the therapy and the degree of inhibition associated with the entity measured [38–41]. There are three major categories of response variables: biomarkers, surrogate markers, and clinical endpoints. A list of response variables in oncology is presented in Table 1. Sometimes more than one response variable may be assessed to evaluate the exposure–response relationship of a drug.

2.2.1 Biomarkers

A number of molecular and biological markers are currently assessed in various types of cancer to understand the role of these markers with diagnosis, disease progression, and drug response [42–46]. Biomarkers may be associated with the diagnosis of a disease. Chronic myeloid leukemia is characterized by a reciprocal translocation between chromosomes 9 and 22 yielding the BCR–ABL fusion protein. Progression of a disease may be associated with a biomarker. Prostate-specific antigen (PSA) is related to early detection and disease progression for prostate cancer [47–50]. Sometimes therapeutic intervention for prostate cancer patient is dictated by the PSA status, and modulation of the PSA level is considered as a treatment response. The overexpression of human epidermal growth factor (HER2-neu) is associated with therapeutic response to trastuzumab therapy in adjuvant and metastatic breast cancer and metastatic gastric cancer [51]. Although trastuzumab is an HER2-neu receptor antagonist, therapeutic intervention and treatment outcome is not associated with the HER2-neu receptor density and as such is not a response variable for exposure–response relationship. Some of the biomarkers (i.e., tyrosine kinases, vascular endothelial growth factors, epidermal growth factor receptors, cyclooxygenase) are related to the drug's mechanism of action with uncertain relationship to the clinical outcome. The CD4 cell count and human immunodeficiency virus (HIV) load is considered a reliable biomarker in the evaluation of ER relationship of anti-acquired immune deficiency syndrome (AIDS) drugs. However, in oncology such a reliable marker for drug evaluation is in development at this time. The CD20 antigen is expressed on the surface of normal and malignant B-lymphocytes. Rituximab, a monoclonal antibody directed against this antigen, is approved for the treatment of CD20-positive B-cell non-Hodgkin's lymphoma and rheumatoid arthritis [52]. Although depletion of B-cell receptor and apoptosis of CD20-positive cells are essential to induce a clinical response, CD20 receptor is not a biomarker for evaluating ER relationship of agents developed against this receptor.

2.2.2 Surrogate Markers

Tumor shrinkage and time to tumor progression are acceptable surrogates to predict clinical benefit for some types of solid tumors in cancer drug development. Hematologic and cytogenetic responses are also considered surrogates to predict clinical benefit for a number of hematologic malignancies. Cancer drugs receive accelerated approval (clinical response based on surrogates) for serious life-threatening illnesses with no approved therapy based on surrogate response. The regulatory process requires, at the time of approval, a commitment from the pharmaceutical company to conduct clinical trial(s) to establish clinical benefit of the agents, post-approval. Oxaliplatin with 5-fluorouracil for the treatment of first-line colorectal cancer patients received accelerated approval based on tumor response and time to tumor progression [53]. In the last decade, the initial approvals (first approved indication) of docetaxel, capecitabine, irinotecan, temozolomide, gemtuzumab ozogamicin, and imatinib were based on surrogate response. In the recent years, surrogate response was used for the approval of sunitinib, dasatinib, nilotinib, bevacizumab, bortezomib, pralatrexate, ofatumumab, lapatinib, everolimus,

brentuximab, crizotinib, and eculizumab. In cancer drug development, dose selection for efficacy trials used surrogate-associated ER relationship information. For example, selection of 400 mg dose of imatinib for treating chronic myeloid leukemia patients was based on 98 % complete hematologic response at or above 300 mg dose tested in the phase 1 trials [54]. Human immunodeficiency virus (HIV) viral load for the acquired immune deficiencies syndrome (AIDS) drugs or cholesterol levels and blood pressure measurements for the cardiovascular drugs are considered surrogates that have direct association with drug response and clinical benefit. Drugs have received Agency approval based on these surrogate endpoints. However, similar surrogate endpoints that directly correlate with the clinical benefit of a drug and allow for unconditional approval (requiring no follow-up clinical trial) are not present at this time in oncology.

2.2.3 Clinical Endpoints

In cancer, survival and "quality of life" assessment are usually considered the endpoints associated with the clinical benefit of a therapy. Survival is usually considered a credible endpoint for clinical benefit in both early and advanced stages of cancer. Approval of drugs for the first-line treatment of solid tumors is mostly based on improved survival. Gemcitabine approval for advanced or metastatic pancreatic cancer was based on clinical benefit response, survival, and time to disease progression [55]. Mitoxantrone was approved for patients with hormone-refractory prostate cancer based on palliative response associated with pain [56]. In rare situations, dose–response relationship using survival as the response variable has been conducted [57, 58].

3 Exposure–Response Relationship in Oncology Drug Development

Evaluation of ER relationship in drug development can facilitate selection of an average dose for a patient population and a selective dose for an individual patient. Prospectively developed ER relationships for oncology drugs are rare; however, recent drug development program included exploratory ER assessment focusing on correlation between exposure and toxicity. Several pharmacokinetic and pharmacodynamic factors including cellular efflux, topoisomerase I and II modulation, lactone stability, and alteration of metabolism influence the antitumor response and toxicity of camptothecins [59]. Intravenous busulfan is approved in combination with cyclophosphamide for bone marrow ablation prior to allogeneic stem cell transplantation for the CML patients. A target exposure to busulfan provides an optimal benefit with minimum toxicity from the treatment. Exposure (AUC) of <900 μM·min has been associated with failure of bone marrow ablation, whereas AUC range of 1,300–1,500 μM·min is associated with hepatic veno-occlusive disease [31]. Therefore, busulfan therapy is targeted to achieve an AUC within 900–1,300 μM·min for the optimum treatment benefit. Modeling and simulations of data obtained from a pediatric study indicated that only 60 % of the patients achieve a targeted AUC of 900–1,350 μM·min with the first dose of busulfan [60]. Therefore, based on the modeling of the exposure data from the pediatric study, dose adjustment and therapeutic drug monitoring scheme was derived to optimize the therapeutic benefit of the drug. After the first dose of busulfan, a dosing nomogram based on body weight to modify the subsequent doses of busulfan to achieve the target exposure is included in the label.

Zoledronic acid is indicted for the treatment of patients with multiple myeloma and patients with documented bone metastases from solid tumors [61]. The drug is also indicated for the treatment of hypercalcemia of malignancy. The pharmacokinetics of zoledronic acid and markers of bone metabolism were assessed in a phase 1 study of 59 advanced cancer patients with osteolytic bone metastases [10]. Various urinary markers of bone resorption (i.e., N-telopeptide, pyridinoline, deoxypyridinoline, hydroxyproline, calcium, and creatinine) were measured, and an attempt was made to correlate the

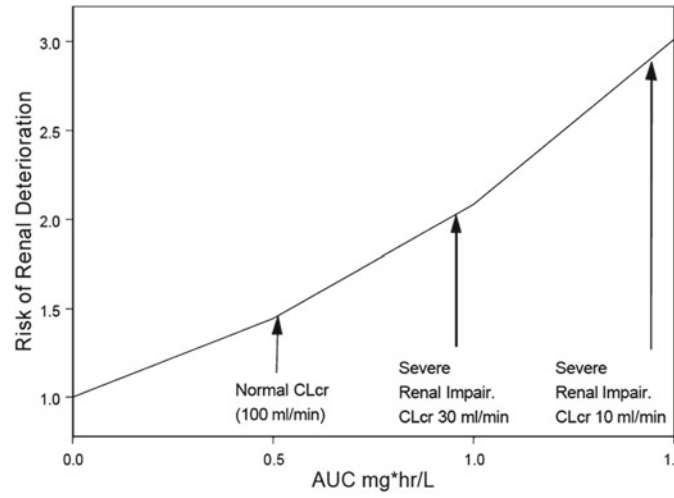

Fig. 1 The risk of renal deterioration versus zoledronic acid AUC. The *arrows* indicate risk of renal deterioration based on the baseline renal function determined by creatinine clearance. Extracted from the New Drug Application Review by FDA. NDA 021386. Zometa (zoledronic acid) Injection. EFOI. http://www.accessdata.fda.gov/drugsatfda_docs/nda/2002/21-386_Zometa_biopharmr.pdf

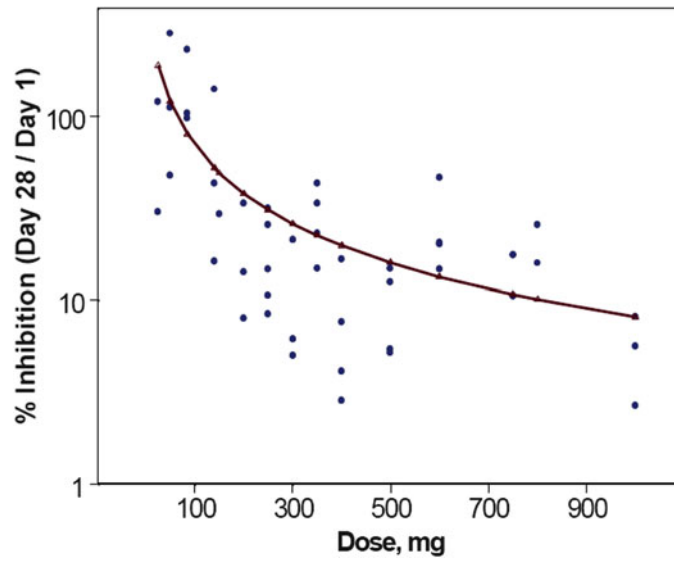

Fig. 2 Relationship between imatinib dose and inhibition of WBC count in CML patients. The plot shows dose-dependent decrease in the WBC count on day 28 normalized for the baseline WBC (day 1) count. Extracted from the New Drug Application Review by FDA. NDA 021335. Gleevec (imatinib mesylate) Capsules. EFOI. http://www.accessdata.fda.gov/drugsatfda_docs/nda/2001/21-335_Gleevec_biopharmr_P1.pdf

bone markers with the pharmacokinetics of the drug. The levels of urinary N-telopeptide, considered a biomarker, decreased 40–60 % in the 0.1–0.4 mg dose groups compared to 70–80 % reduction in the 0.8–4.0 mg dose groups [10]. Biomarkers pyridinoline and deoxypyridinoline levels decreased at doses greater than 1.5 mg dose. Evaluation of the relationship between dose and biomarkers helped in selecting the dose for testing in the phase 3 registry trials. Urinary excretion plays a major role in zoledronic acid disposition. Zoledronic acid clearance significantly correlated with the creatinine clearance [61]. The ER modeling predicting association between risk of renal deterioration and AUC is shown in Fig. 1. The analysis predicted increased risk of renal deterioration with exposure as well as the renal function of a patient and provided a dosing algorithm for patients with baseline creatinine clearance ≤60 mL/min [61]. Dose selection based on understanding of these relationships is expected to minimize risk of renal deterioration and obtain optimal benefit from the treatment.

Imatinib is indicated for the treatment of CML, ALL, and gastrointestinal stromal tumors. The drug is a potent BCR–ABL tyrosine kinase inhibitor. Imatinib administration decreased the white blood cell (WBC) counts over time in a dose-dependent manner as shown in Fig. 2 [62]. Exposure–toxicity

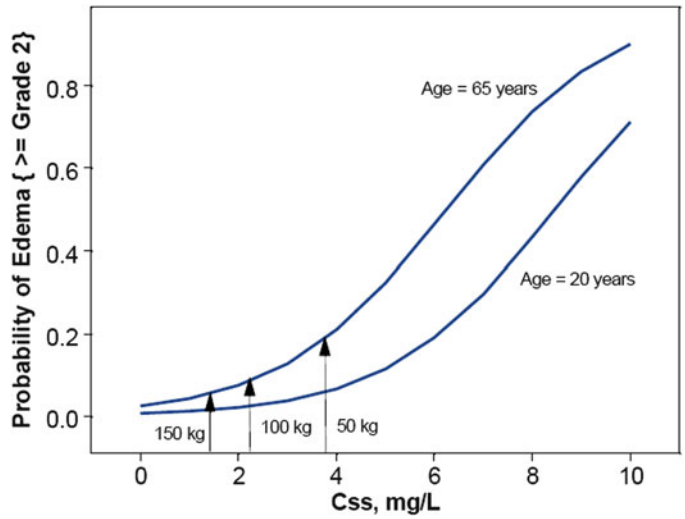

Fig. 3 The probability of grade 2 or greater edema occurrence in blast crisis CML patients as a function of steady-state drug concentration. The risk of edema increases with increased age. The *arrows* indicate the risk of edema for a 65-year-old patient increases with decreased body weight. Extracted from the New Drug Application Review by FDA. NDA 021335. Gleevec (imatinib mesylate) Capsules. EFOI. http://www.accessdata.fda.gov/drugsatfda_docs/nda/2001/21-335_Gleevec_biopharmr_P1.pdf

relationship analysis between the probability of grade 3 edema and steady-state drug concentration in blast crisis CML patients shown in Fig. 3 identified elderly population who are highly susceptible to grade 2 or higher edema compared to younger patients. The analysis also demonstrated that patients with lower body weight had a greater risk for edema because of higher exposure compared to patients with higher body weight.

Docetaxel (Taxotere) is indicated for the treatment of advanced or metastatic breast cancer, hormone-refractory prostate cancer, gastric cancer, head and neck carcinoma, and non-small cell lung cancer. Docetaxel clearance decreased and AUC increased in patients with mild to moderate liver function impairment (SGOT and/or SGPT > 1.5 times ULN). Exposure–toxicity relationship analysis demonstrated that patients with higher bilirubin, or SGOT and/or SGPT, or alkaline phosphatase are at increased risk for developing grade 4 neutropenia, febrile neutropenia, infections, severe thrombocytopenia, severe stomatitis, severe skin toxicity, and toxic death. The package insert of Taxotere recommends assessment of bilirubin, SGOT or SGPT, and alkaline phosphatase levels prior to each cycle of therapy and discontinuation of therapy in case of liver function deterioration [63].

Carboplatin is indicated in the treatment of advanced ovarian cancer. Dosing of this drug is based on the relationship between carboplatin exposure and hematologic toxicity. A target exposure based on AUC for single agent carboplatin in pretreated patients balances the risk of developing toxicity (thrombocytopenia and leukopenia) and achieving an effective treatment [37]. The dose of the drug is based on the targeted AUC and renal status of a patient.

Vandetanib is a kinase inhibitor indicated for the treatment of medullary thyroid cancer with unresectable locally advanced or metastatic disease [64]. Vandetanib can prolong the QT interval, and during drug development Torsade de pointes and sudden death were observed in clinical trials. ER analysis showed that vandetanib was associated with sustained plasma concentration-dependent QT prolongation (see Fig. 4). Modeling and simulation showed that average plasma concentrations after administration of 100, 200, and 300 mg will increase the QTcF (corrected by Fredericia method) from baseline by 21, 30, and 35 ms. In the clinical trial, 36 % of patients experienced greater than 60 ms increase in QTc and 4.3 % of patients had QTc greater than 500 ms [64].

Everolimus is indicated for the treatment of patients with progressive neuroendocrine tumors of pancreatic origin, advanced renal cell carcinoma, and subependymal giant cell astrocytoma (SEGA) [65]. ER analysis demonstrated an association between clinical responses measured by the reduction of SEGA tumor volume and increase in C_{ss} (Fig. 5). The analysis provided two significant dosing recommendations. The tumor response rates were 42 % for patients achieving plasma levels of

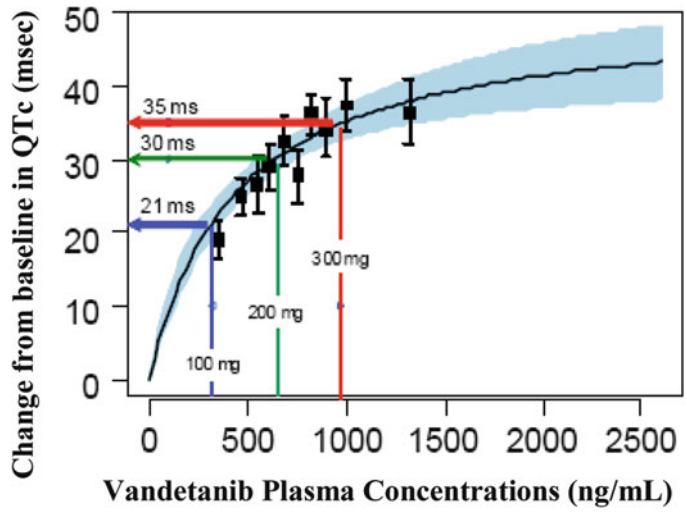

Fig. 4 Relationship between changes in QT and vandetanib concentrations. Based on log-linear relationship between concentration and QTcF (QT corrected by Fridericia method), the expected mean change in QTcF is 35, 30, and 21 ms for 300, 200, and 100 mg daily dosing of vandetanib. Extracted from the New Drug Application Review by FDA. NDA 022405. Caprelsa (vandetanib) Capsules. EFOI. http://www.accessdata.fda.gov/drugsatfda_docs/nda/2011/022405Orig1s000ClinPharmR.pdf

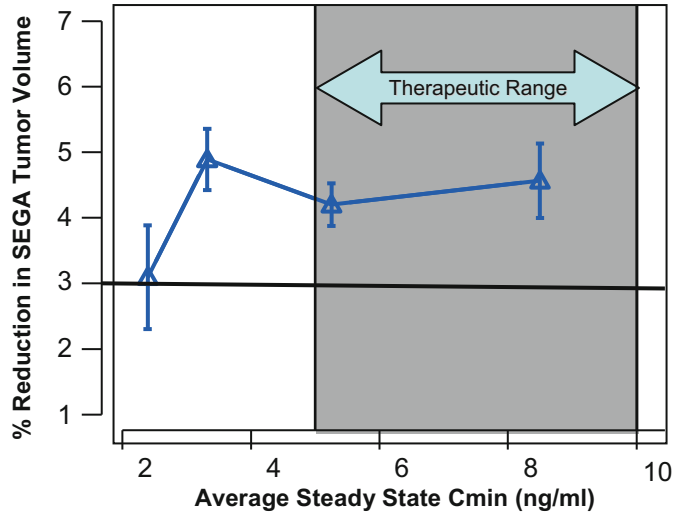

Fig. 5 Exposure–response relationship of everolimus. The plot shows the relationship between average steady-state minimum concentrations and the percent reduction in subependymal giant cell astrocytoma tumor volume at 6 months of treatment. The mean range of concentrations at each successive quartile was 1.8–2.8, 2.9–4.4, 4.6–5.7, and 6.2–11.0 ng/mL. Data are shown as mean ± SE. Extracted from the New Drug Application Review by FDA. NDA 022334. Afinitor (Everolimus) Tablets. EFOI. http://www.accessdata.fda.gov/drugsatfda_docs/nda/2009/022334s000_ClinPharmR.pdf

everolimus under 3 ng/mL and 90 % for patients achieving plasma levels of greater than 3 ng/mL. Based on this analysis dose titration is recommended to maintain plasma level of everolimus within 5–10 ng/mL to provide the optimal benefit from the treatment.

Ipilimumab is a human cytotoxic T-lymphocyte antigen 4 (CTLA-4) indicated for the treatment of unresectable or metastatic melanoma patients [66]. A time-to-event analysis for overall survival

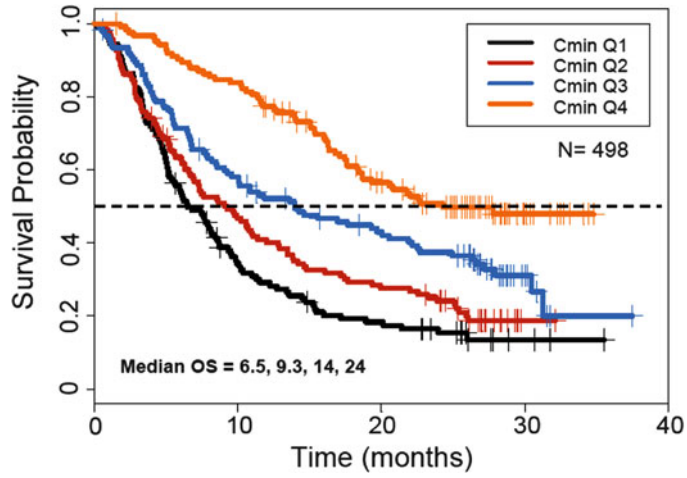

Fig. 6 Kaplan–Meier plot showing increase in overall survival with exposure in malignant melanoma patients receiving 0.3, 3, and 10 mg/kg doses of ipilimumab. The median survivals were 6.5, 9.3, 14, and 24 months in patients achieving minimum plasma concentrations of <19.5 (Q1), 19.5–43.7 (Q2), 44–65.3 (Q3), and >65.3 μg/mL (Q4), respectively. The database consisted of 498 patients; 98 % of the patients in the third and fourth quartile received 10 mg/kg dose of ipilimumab. Extracted from the New Drug Application Review by FDA. BLA 125377. Yervoy (Ipilimumab) injection for IV use. EFOI. http://www.accessdata.fda.gov/drugsatfda_docs/nda/2011/125377Orig1s000ClinPharmR.pdf

demonstrated an increase in survival with increasing exposures. The patients were grouped based on their steady-state minimum plasma concentration of ipilimumab. The median exposure from lowest to highest quartile was 8.5, 32, 55, and 82 μg/mL, and median survivals were 6.5, 9.3, 14, and 24 months [66]. A stepwise Cox hazard model identified minimum concentration as a significant independent predictor of overall survival. In addition, baseline level of lactate dehydrogenase (LDH) and ECOG status were identified as risk factor for overall survival (Fig. 6).

The discovery of various targets associated with the tumor growth and proliferation is expected to lead the pharmaceuticals to explore ER relationship of the new cancer agents prospectively in drug development. Since the utility of exposure–toxicity relationship in oncology is well established, research should focus on investigations on relationships between exposure and activity of an agent. The ER analysis will help in establishing the benefit risk associated with the treatment, selection of individualized dose for a particular patient, and selection of a subpopulation that may not benefit from a particular treatment. The establishment of reliable markers associated with the disease and disease progression will be helpful to allow ER evaluation.

4 Guidance on Exposure–Response Relationships

The FDA guidance on ER relationships elaborates on the regulatory applications, study design, and data analysis aspects of the clinical trials to be conducted during drug development to assess the ER relationship of a drug [1]. The purpose of the guidance is to encourage prospective collection of exposure–response data in the clinical trials to support the safety and efficacy evaluation of a drug. The guidance describes under what circumstances an exposure–response study provides (1) primary evidence of safety and effectiveness of a drug, (2) supportive evidence for the primary efficacy studies, and (3) evidence to support new target population or an altered dosage forms or an altered doses/

Table 2 Guidances providing general and specific recommendations on exposure–response relationship evaluation during drug development

1. Guidances providing general recommendation
Providing clinical evidence of effectiveness for human drugs and biological products
Guideline for the format and content of the clinical and statistical sections of an application
ICH E4, dose response information to support drug registration
ICH E5, ethnic factors in the acceptability of foreign clinical data
2. Guidances providing specific recommendation
ICH E7, studies in support of special populations: geriatrics
Study of evaluation of gender differences in the clinical evaluation of drugs
Pharmacokinetics in patients with impaired renal function: study design, data analysis, and impact on dosing and labeling
Pharmacokinetics in patients with impaired hepatic function: study design, data analysis, and impact on dosing and labeling
In vivo metabolism/drug interactions studies: study design, data analysis, and recommendations for dosing and labeling
Population pharmacokinetics

Source: FDA guidance, exposure-response relationships-study design, data analysis, and regulatory applications. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM072109.pdf

dosing regimens, or routes of administration. The guidance discusses integrating ER relationship assessment in all phases of drug development. The guidance also elaborates on the format and content for reports of ER studies in a regulatory submission. The ER study designs are described in the guidance with emphasis on prospective collection of information to support regulatory decisions. The guidance provides a pediatric decision tree integrating PK–PD in drug development for pediatric population [1].

The International Conference on Harmonisation (ICH) also published guidance, ICH E4 on dose–response information to support drug registration [2]. The guidance describes pharmacokinetics and pharmacodynamic studies and pharmacodynamic endpoints. The guidance describes the usefulness of dose or concentration–response relationship assessment from the global drug development perspectives. The ER information is expected to allow multiple regulatory agencies making approval decisions from a common database. The guidance highlights the strengths and limitations of various study designs and the limitations associated with conducting dose–response studies of drugs for life-threatening diseases like cancer. ICH has also published guidance on ethnic factors in the acceptability of foreign clinical data [67]. The ER relationship study using an established pharmacodynamic response variable that is related to a clinical endpoint may be acceptable as a bridging study. Various regulatory guidances describing the values of understanding ER relationship in drug development and drug use are presented in Table 2 [1]. These guidances are expected to promote evaluation of ER relationship during drug development and provide an understanding of regulatory decision making based on ER relationship of a drug.

5 Exposure–Response Information in Labeling

Although the ER relationship analysis data is included in the Clinical Pharmacology section of the package inserts, the interpretation of the data and instructions about therapeutic monitoring and dose adjustment to reduce toxicity or enhance benefit may be included in the "Highlights," "Dosage and Administration," and "Warnings and Precautions" sections. Information about the effects of antiestrogenic agents on estrogen suppression, on the levels of corticosteroids, and on other endocrine effects of these hormonal agents is included in the package inserts. The dose-related pharmacodynamic effect

of exemestane on estrogen suppression is included in the Clinical Pharmacology section of the package insert [68] as follows:

> *Pharmacodynamics*
> *Effect on Estrogens*: *Multiple doses of exemestane ranging from 0.5 to 600 mg/day were administered to postmenopausal women with advanced breast cancer. Plasma estrogen (estradiol, estrone, and estrone sulfate) suppression was seen starting at a 5-mg daily dose of exemestane, with a maximum suppression of at least 85 % to 95 % achieved at a 25-mg dose. Exemestane 25 mg daily reduced whole body aromatization (as measured by injecting radiolabeled androstenedione) by 98 % in postmenopausal women with breast cancer. After a single dose of exemestane 25 mg, the maximal suppression of circulating estrogens occurred 2 to 3 days after dosing and persisted for 4 to 5 days.*

Arimidex package insert includes information on dose-related serum estradiol suppression by anastrozole [69]. Similar information about the pharmacodynamic effects of letrozole is also included in the package insert of Femara [70].

In the Platinol label, a box warning [71] states, *"Cumulative renal toxicity with Platinol is severe. Other major dose-related toxicities are myelosuppression, nausea, and vomiting."* Although this statement is derived from clinical experience in absence of a thoroughly investigated exposure–response evaluation, the statement indicates the need for exposure–response evaluation for cytotoxic agents so that appropriate dose modifications can be recommended in the drug label. Similarly the Box warning section of Docetaxel label states an association between liver dysfunction and increased risk for the development of grade 4 neutropenia, febrile neutropenia, infections, severe thrombocytopenia, severe stomatitis, severe skin toxicity, and toxic death. This toxicity may partly be attributed to lower clearance and higher exposure to Taxotere found in patients with liver dysfunction. For zoledronic acid, the increased risk of renal deterioration with increased exposure was analyzed using PK–PD modeling [61]. The *"Special Populations"* section and the *"Renal Insufficiency"* subsection of Zometa include the following information:

> *Based on population PK/PD modeling, the risk of renal deterioration appears to increase with AUC, which is doubled at a creatinine clearance of 10 mL/min. Creatinine clearance is calculated by the Cockcroft–Gault formula:*
>
> *CrCl = [140-age (years) x weight (kg)]/[72 x serum creatinine (mg/dL)] {x 0.85 for female patients}*
>
> *Zometa systemic clearance in individual patients can be calculated from the population clearance of Zometa, CL (L/h) = 6.5(CLcr/90)$^{0.4}$. These formulae can be used to predict the Zometa AUC in patients, where CL = Dose/$AUC_{0\text{-}\infty}$. The average $AUC_{0\text{-}24}$ in patients with normal renal function was 0.42 mg.h/L and the calculated $AUC_{0\text{-}\infty}$ for a patient with creatinine clearance of 75 mL/min was 0.66 mg.h/L following a 4-mg dose of Zometa.*

The package insert of Xalkori (crizotinib) highlights the QTc interval prolongation potential of crizotinib. In the clinical trials, patients were found to have QTc prolongation greater than or equal to 500 ms and increase of baseline QTc greater than or equal to 60 ms. A pharmacokinetic/pharmacodynamic analysis suggested a concentration-dependent increase of QTc in non-small cell lung cancer patients [72]. The QT interval prolongation information is included in the "Warnings and Precautions" section, and appropriate recommendations are made to address this toxicity.

The package insert of everolimus recommends therapeutic drug monitoring for SEGA patients in the "Dosage and Administration" section based on exposure–response analysis [66]. The statement is as follows:

> *Therapeutic Drug Monitoring in Subependymal Giant Cell Astrocytoma*
>
> *Routine everolimus whole blood therapeutic drug concentration monitoring is recommended for all patients using a validated assay. Trough concentrations should be assessed approximately 2 weeks after the commencing treatment. Dosing should be titrated to attain trough concentrations of 5 to 10 ng/mL.*
>
> *There is limited safety experience with patients having a trough concentrations >10 ng/mL. If concentrations are between 10 to 15 ng/mL, and the patient has demonstrated adequate tolerability and tumor response, no dose reductions are needed. The dose of Afinitor should be reduced if trough concentrations >15 ng/mL are observed.*
>
> *If concentrations are <5 ng/mL, the daily dose may be increased by 2.5 mg every 2 weeks, subject to tolerability. Daily dose may be reduced by 2.5 mg every 2 weeks to attain a target to 5 to 10 ng/mL. If dose reduction is required for patients receiving 2.5 mg daily, alternate day dosing should be used.*

The recommendations are derived from ER information generated during drug development and analysis conducted by the FDA to optimize treatment benefit.

The exposure–response relationship constitutes the basis for dose modifications particularly in the special populations (i.e., geriatric and pediatric populations, different ethnic groups, renal or hepatic impaired patients, and patients on other concomitant medications). In oncology, dose modifications and therapeutic monitoring are recommended in the package insert based mostly on dose or exposure–toxicity relationship. In the recent years, exposure-based dosing recommendation is included in the package inserts of drugs for patients with genetic variations resulting in altered exposure [73, 74]. The target-based development of oncology drugs in the future is expected to explore ER relationship from efficacy perspectives during drug development, particularly design trials to assess the utility of the drug or a specific dose for subpopulations. This approach will help tailor a dose for an individual in the light of the safety and effectiveness of the drug for the indicated population.

6 Conclusions

Aggressive cancer therapy to obtain a cure has to be optimized with the knowledge of ER relationship of a drug and provide most favorable benefit of a treatment with minimal risks to an individual patient. In oncology, dose adjustment based on toxicity is a traditional practice, especially for cytotoxic agents. Under certain circumstances when severe toxicity is manifested, subsequent doses are reduced or cycles of therapy withheld until the toxicity is resolved. However, these adjustments are done empirically in absence of any ER information, and the therapy may not provide the best treatment outcome for a patient. New targets are discovered and drugs are developed against these targets. The understanding of the exposure-associated target response in early development allows for selection of optimum dose and/or dosing regimen for pivotal efficacy and safety studies. The ER information can tailor the treatment for individual patients in the clinical trial and improve the possibility in succeeding to demonstrate the safety and effectiveness of the drug for marketing approval. In the pivotal trials, ER assessment can validate the hypothesis generated in the early drug development and provide dosing algorithm for special populations. In the clinical trial protocols, dose modifications are proposed based on toxicity. The dose for the next cycle is reduced by a certain fraction or the treatment is withheld until the toxicity reduces to an acceptable level. The inherent assumption is that the toxicity at least in part is associated with the exposure to the drug. However, the relationship between the exposure levels from the modified dosing and the clinical response is not evaluated and understood from these trials.

The strict inclusion and exclusion criteria of a registration trial exclude patients who would otherwise be a candidate to receive the medication when the drug is approved. In clinical trials, the safety and efficacy of a drug is not established for patients with poor kidney or liver function, or on concomitant medications, or at the lower of upper end of the age groups or with poor performance status. The exposure–response relationship forms the basis on which rational dose adjustment can be made for these patient populations. Treatment-related toxicity is common in cancer; subsequent dose adjustment based on toxicity may be inadequate and expensive and may not provide the desired benefit from the therapy. Therapeutic drug monitoring helps to avoid toxicity and optimize drug therapy. The concept of therapeutic drug monitoring is mostly based on exposure–toxicity relationship.

From the regulatory perspective, exposure–response information not only helps in drug development but under certain circumstances may provide supportive evidence for the approval of a drug [1]. The Agency promotes evaluation of exposure–response relationship during drug development as well after a drug is approved [75, 76]. Although the ER evaluation of cancer drugs has been rare in the past, recognition of the utility of ER relationship in drug development and targeted therapy in oncology relying on various safety and efficacy markers are expected to promote exposure–response evaluation and optimal and individualized therapy in the future.

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The Role of Phase III Trials in Modern Drug Development

Janet E. Murphy, Lecia V. Sequist, and Bruce A. Chabner

Abstract While the backbone of cancer treatment remains cytotoxic chemotherapy, modern cancer research has increased our understanding of the molecular pathogenesis of cancer and, in some cases, identified driver oncogenes. The small molecule imatinib, for example, which specifically targets the BCR-ABL and CKIT kinases, has revolutionized the treatment of chronic myelogenous leukemia and gastrointestinal stromal tumors.

In this chapter, we review the traditional path to drug development and FDA approval. We discuss the many disease settings in which the long road from phase I to phase III clinical trials remains relevant. We then explore two case studies: lung cancer and melanoma, in which identification of oncogene-addicted pathways allowed for phase I trial design with cohorts enriched with patients who harbored translocations/mutations in *EML4-ALK* and *BRAF*, respectively, with rapid appreciation of antitumor activity and an abbreviated path to drug approval. Here, the traditional phase III model is less relevant. We explore the ethics of drug development in the targeted therapy era and offer several solutions for accelerating drug evaluation where oncogene-directed therapy holds great promise in selected patients.

Keywords Targeted therapies • Phase III trials • Genotyping of tumors • Ethical considerations in trial design

1 Introduction

We are in the midst of a molecular revolution in cancer drug development. Cytotoxic chemotherapy remains the backbone of cancer treatment, but interest in new cytotoxics has been eclipsed by small molecule targeted agents and antibody therapies that hone in on key oncogenic pathways. While many targeted drugs with strong preclinical rationale have failed to demonstrate clinical benefit, the drugs that

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Table 1 Drug development phases

	Typical number and type of patients	Common design	Objectives
Phase 0	Few	Pilot study looking for proof of concept with very low doses of drug (microdosing)	First-in-human: early proof of concept of drug activity in humans – study pharmacokinetics and pharmacodynamics of the drug
Phase I	20–100 Healthy volunteers or patients	Dose escalation (standard 3 + 3 with cohort expansion at MTD)	Define the maximum tolerated dose of drug for phase II trial. Complete a safety and toxicity evaluation. Study pharmacodynamics and pharmacokinetics of drug. Validate biomarkers for drug effect. (For targeted therapy, MTD may not be found, and phase I may seek evidence of target inhibition) [2]
Phase II	20–300 Patients with disease of interest	Single-arm series, commonly with phased enrollment (Simon two-stage) OR randomized trial	Define efficacy of drug – most commonly by demonstrating response rate; define progression-free survival relative to control group (RCT) or historical control (cohort study) [3]
Phase III	300+ Patients with disease or disease subgroup of interest	Prospective RCT	Definitive evidence of benefit over standard therapy – common endpoint is overall survival but can be powered to PFS/TTP in trials that allow crossing over at progression
Phase IV		Post-marketing surveillance trial	Long-term safety surveillance in all patient populations, including subsets of patients who would have been excluded from phase III (patients with brain metastases, etc.)

have succeeded, such as imatinib (Gleevec, Novartis), the BCR-ABL and CKIT tyrosine kinase inhibitor, have extended the lives of thousands of cancer patients. Small molecule tyrosine kinase inhibitors and antibodies targeted against both receptors and ligands have become standard therapies for an increasing number of malignancies. Designed with the intent of targeting pathways that the cancer cell depends on for survival, these drugs provide elegant, rational, and modestly toxic therapy with profound clinical benefit [1].

Prospective genotyping approaches identify a tumor's driving oncogene, and have allowed investigators to select patients for therapies with a higher likelihood of response in the earliest trials. Such studies have produced startling evidence of targeted drug activity in diseases in which there is a clear unmet need. If a drug shows substantial activity in the phase I setting, and there are no effective standard therapies, the traditional progression through phases II and III may no longer be tenable or appropriate (see Table 1). The initial results of the phase I trial the BRAF inhibitor vemurafenib (Zelboraf, Genentech) in patients with *BRAF*-mutated metastatic melanoma prompted public discussion of the need for a more flexible concept of drug development and earlier drug approval and have placed in question the need for, and indeed the ethical limitations of, phase III trials [4, 5]. In this chapter, we will review the role of phase III clinical trials in cancer therapy. We will discuss the methodological and ethical issues that emerge in the phase III setting when targeted therapies are evaluated and propose a vision of the new paradigm for rigorous drug evaluation in the current era of cancer drug development.

2 The Drug Development Process

Phase III trials represent the ultimate testing ground for new therapeutic interventions. In the traditional drug development paradigm, phase I trials commonly enroll patients with advanced, refractory disease to assess safety and toxicity endpoints and establish the maximum tolerated dose and drug

administration schedule to move forward to phase II [2]. In the past, limited attention was focused on the specific patients (and their cancers) accrued to phase I trials, other than acknowledging their fitness for the trial and lack of a standard treatment alternative. A broad array of cancer types was usually represented in phase I trials, and this distribution provided an early opportunity to look for signals of response. Most effective drugs that do target a kinase with some degree of specificity do show evidence of activity anecdotally, but this does not always translate into a positive signal in Phase II and/or Phase III. An example of this is the EGFR tyrosine kinase inhibitor gefitinib in unselected non-small cell lung cancer patients, which accelerated to an unsuccessful phase III based on promising early results [6].

In phase II, evidence of antitumor activity is sought in a particular malignancy by enrolling a defined cohort of patients. In cytotoxic drug development, phase II studies are commonly powered to detect a reasonable but modest response rate to therapy, the actual design depending on the tumor studied and alternative therapies available. Single-arm trials frequently compare a drug's antitumor efficacy to historical controls, though randomized phase II trials that contain a contemporaneous control group are becoming more common [3, 7].

Phase III clinical trials seek to provide the convincing evidence of the benefit of a novel therapy compared to standard alternative(s). To assess superiority of a novel therapy or combination, large numbers of patients are accrued at multiple sites of care. For purposes of drug approval, phase III trials are usually powered to demonstrate a survival benefit, although a delay in time to progression (TTP) and progression-free survival (PFS) are becoming acceptable endpoints, particularly when patients are allowed to crossover to the experimental drug at the time of standard treatment failure [8, 9]. Ancillary factors, such as toxicity, cost, and long-term quality of life may become pivotal outcomes in phase III trials, particularly in those trials that deal with comparative effectiveness [10]. Phase III trials provide a longer-term evaluation of drug toxicity, and may disclose toxicities not appreciated in phase I or II. Refinements of dose or schedule may result from these observations. For example, ponatinib (Inclusig, Ariad), a drug approved in 2012 for treatment of imatinib-resistance CML, was found to cause an excess of cardiovascular and thrombotic events in the initial phase of its trial as an up front treatment for CML, and the Phase III PACE trial study was suspended in this population [11]. In nondrug trials, phase III may be used to define the most effective treatment option, such as extensive surgery versus limited resection in an operable cancer. Because of their size and scope, phase III trials can offer the additional important opportunity to collect blood and tumor tissue samples for evaluation of secondary endpoints related to pharmacogenetics, somatic molecular determinants of response and resistance, or toxicity.

Phase III trials require an enormous expenditure of time, money, and professional effort. The average length of a phase III trial is 4.5 years, with 200 person-hours of work per study enrollee [12]. The total cost of developing a drug was estimated in 2006 to be $897 million (in year 2000 dollars). Of this cost, clinical testing was estimated at $175 million, of which 65–75 % is spent on phase III [13]. Walker and Newell conducted an analysis of 974 anticancer agents entering the clinical pipeline between 1995 and 2007 and found a clinical attrition rate of 82 % (indicating that 18 % of drugs were ultimately approved) [14]. The subset of kinase inhibitors studied had a lower than average attrition rate of 53 %. Improved success rates for targeted therapy were attributed to far lower attrition at the phase II to III transition, owing to prospective biomarker-driven patient selection and the rational design of drugs. The decision to "Go, or No Go" to phase III for a candidate molecule has been considered in detail by Roberts et al. and entails an analysis of likely benefit, toxicity, and the disease space for which the drug is being tested. The greater the efficacy, the lower the toxicity, and in the absence of effective competitors, the more likely that the drug will succeed in phase III [15].

2.1 Accelerated Approval and the Food and Drug Administration

Traditionally, Food and Drug Administration (FDA) approval has been based on favorable results demonstrated in one or two well-controlled trials, including at least one definitive phase III. However,

the demand for early access to experimental drugs for HIV prompted a revolution in drug approval two decades ago, initiating in 1992 the passage of legislation authorizing the accelerated approval of drugs for critical unmet needs by the Food and Drug Administration after phase II. In 2012, further legislation created a "breakthrough" category of drugs, for which the FDA was instructed to provide guidance and active support for marketing approval. This consideration has extended to oncology as well. While more than 100 agents, including more than 50 cancer drugs, have received early approval under this mechanism, concerns continue to be expressed that 50 percent such agents subsequently circumvent definitive trials because of lack of incentive to complete them once the drug is earning revenues [16].

Of all the agents that do undergo rigorous post-marketing study, the majority of drugs approved under the accelerated scheme are eventually granted full approval based on positive post-marketing trials. However, many drugs approved by this mechanism remain on the market without further definitive study. In an audit of the accelerated approval process by the General Accounting Office, it was noted that only 64 % of drugs were considered "closed," meaning that drug sponsors had met FDA's requirements for post-marketing approval studies or FDA determined that doing such studies was no longer needed or feasible [17]. Some drugs, however, such as (Iressa, Novartis) for unselected patients with non-small cell carcinoma of the lung, bevAcizumab (Avastin, Genentech) for advanced breast cancer, and gemtuzumab (Mylotarg, Wyeth) have seen their approval withdrawn because subsequent definitive study shows lack of efficacy—or, in the case of Mylotarg, a trend toward excess mortality [18]. Bevacizumab received accelerated approval in the treatment of advanced breast cancer after the ECOG 2100 trial demonstrated a greater than 5-month progression-free survival benefit of bevacizumab in combination with paclitaxel [19]. In November 2011, the FDA approval of bevacizumab was withdrawn because subsequent study failed to demonstrate a commensurate overall survival benefit [20, 21].

When a drug comes into the phase III setting with prior accelerated FDA approval, discussion with the Food and Drug Administration in the United States, or with other agencies in Europe or Asia, usually precedes formal initiation of a trial. The actual design of the trial will be influenced by the results of the earlier trials, the feasibility of identifying a reasonable control arm, the need to compare different doses or schedules, and the interval development of alternative agents and combinations. Whether or not accelerated approval has been granted after phase I/II, the phase III trial remains the standard for establishing the endpoint of survival. As such, it remains, as observed by Zia et al., "the cornerstone of evidence-based oncology" for both cytotoxics and targeted therapies [7]. A number of different phase III trial designs, illustrated with recent examples involving targeted therapies, are presented here.

2.2 Placebo Versus Targeted Agent

Large randomized phase III trials are critically important when measuring time-to-event endpoints that are less frequent—such as cancer recurrence after definitive treatment. Thus, phase III trials that enroll large numbers of patients in order to capture the minimum number of recurrence events in each arm are crucial for assessing the benefit of adjuvant therapy. In surgically resected gastrointestinal stromal tumors (GISTs), the recurrence rate after surgery is approximately 50 %, but cytotoxic chemotherapy is inactive [22]. This was the basis for the ACOSOG Intergroup Adjuvant GIST Study, which randomized patients with surgically resected tumors of 3 cm or larger to adjuvant imatinib 400 mg daily versus placebo. The study enrolled over 700 patients and demonstrated with great certainty the benefit of adjuvant imatinib—which improved recurrence free survival at 1 year from 83 % in the placebo group to 98 % with imatinib (HR 0.35, 95 % CI [0.22–0.53]) [23]. Notably, this study restricted enrollment to patients whose tumors expressed CD-117, or CKIT, the constitutively active kinase targeted by imatinib.

If there is no identified standard therapy for a given indication, placebo-controlled phase III trials may be ethically conducted. Hepatocellular carcinoma, for which there is no effective cytotoxic therapy, provided an opportunity for testing antiangiogenic drugs that appeared to stabilize disease, with few clear antitumor responses. It was in this setting that equipoise was maintained in the SHARP trial, a multicenter phase III double-blind, placebo-controlled trial of 602 patients with advanced hepatocellular carcinoma [24]. Patients were randomized to placebo versus the multi-target tyrosine kinase inhibitor sorafenib (Nexavar, Onyx). The median survival of patients in the sorafenib arm, 10.7 months, exceeded the placebo arm (7.9 months) and established sorafenib monotherapy as standard of care in this disease. FDA approval was granted in November 2007. It is not yet known which target(s) of sorafenib drove the effectiveness observed in this trial. Another example is found in progressive metastatic medullary thyroid carcinoma, in which the tyrosine kinase inhibitor cabozantinib (Cometriq, Exelixis) which has activity against MET, RET, and VEGFR2 was compared against placebo in a randomized, double-blind Phase III trial. It demonstrated a progression-free survival benefit at one year of 47.3 % for cabozantinib and 7.2 % for placebo [25].

2.3 Standard Therapy Versus Standard Therapy + Targeted Agent

Phase III trials have been crucial in assessing biologic therapies where the target, such as the vascular endothelial growth factor (VEGF), is ubiquitous, but there is no biomarker for response. Multiple trials have demonstrated an incremental benefit of these agents when added to standard treatment. Hurwitz et al. demonstrated a 4.7-month overall survival benefit incurred by adding bevacizumab to the IFL regimen in metastatic colorectal cancer—the largest survival benefit shown to date with bevacizumab therapy, with a degree of benefit not replicated in a subsequent Phase III trial with FOLFOX and XELOX [26, 27]. Similarly, bevacizumab established a new "ceiling" in lung cancer survival when the ECOG 4599 study adding bevacizumab to carboplatin and paclitaxel demonstrated a median survival of 12.5 months in the bevacizumab arm, the first randomized trial of advanced NSCLC ever to cross the "one-year" survival mark [28]. The FDA subsequently approved bevacizumab in combination with those specific chemotherapies for advanced colon cancer and lung cancer in 2006. No biomarkers for bevacizumab benefit have been definitively identified in either colon or lung cancer.

In 2009, the anti-HER2 antibody trastuzumab (Herceptin, Genentech), which has long been an established standard for treatment of HER2-positive breast cancer, was studied in the ToGA trial of advanced HER2-positive gastric cancer [29]. Patients were randomized to standard chemotherapy (infusional 5-FU or oral capecitabine plus cisplatin) with or without trastuzumab. Eligibility for enrollment was determined by *HER2* testing in the tumors of the enrolled patients, and 594 of 3,807 patients screened met criteria: 3+ for HER2 expression by immunohistochemistry (IHC) or amplification as demonstrated by fluorescence in situ hybridization (FISH). In *HER2* amplified or IHC-positive patients, median overall survival was significantly higher in the trastuzumab arm (13.8 versus 11.1 months), leading to FDA approval for this indication in October 2010, and HER2 screening has evolved into a standard procedure in the advanced gastric cancer setting. In these and many more cases in which the impact of targeted therapy is expected to be incremental, phase II trials may indicate a possible benefit of the experimental arm, but a large, randomized trial is needed to establish clear benefit in terms of overall- or progression-free survival.

2.4 Standard Therapy Versus Targeted Agent

The lack of a cytotoxic standard comparator arm in the SHARP trial in hepatocellular carcinoma represents an exception. For the majority of epithelial cancers, there is an established, if only modestly effective, cytotoxic standard therapy. When a targeted therapy demonstrates excellent single-agent activity in early trials, evaluating it head-to-head against cytotoxic chemotherapy in the phase III setting

may be essential to establish its benefit for progression-free or overall survival. This was the case in non-small cell lung cancers harboring activating EGFR mutations, a set of driver oncogene mutations that define a group of lung cancer patients exquisitely sensitive to EGFR-specific tyrosine kinase inhibitors (TKIs); (see discussion of "Oncogene Addiction" below). In the West Japan Oncology Group (WJOG) trial, chemotherapy-naive patients with stage IIIB/IV non-small cell lung cancer harboring EGFR mutations received either the EGFR TKI gefitinib or cisplatin/docetaxel, with a primary endpoint of progression-free survival [30]. Further treatment at the time of progression was at the physician's discretion, akin to crossing over to TKI treatment within the trial, making PFS the appropriate primary endpoint. The WJOG trial was the first to definitively establish the superiority of gefitinib over platinum-based chemotherapy in a prospectively defined cohort of patients with *EGFR* mutations, with a PFS of 9.2 months versus 6.3 months with standard therapy. This led to regulatory approval of gefitinib for *EGFR* mutation-positive patients in Europe in 2010, though not in the United States.

2.5 Multimodality Trials with Targeted Agents

Targeted therapy in combination with radiation therapy has been evaluated in the phase III setting—notably in the treatment of locoregionally advanced squamous cell cancers of the head and neck. In a multinational randomized trial among unselected patients, Bonner et al. compared high-dose radiotherapy with radiotherapy plus weekly administration of the anti-EGFR antibody cetuximab (Erbitux, Bristol Myers Squibb/Lilly) [31]. The primary endpoint was the control of locoregional disease, with secondary endpoints of overall survival, PFS, response rate, and safety. The addition of cetuximab to radiotherapy conferred a nearly 10-month locoregional control benefit and led to marketing approval for cetuximab as a standard treatment in this disease.

2.6 Conclusion

Overall, targeted therapies have fared better than their cytotoxic counterparts in the phase III setting. Of targeted therapies that make it through to phase III, Walker and Newell cite an 85 % probability of surviving the transition from phase III to registration (i.e., phase IV) [14]. Some agents, such as sorafenib in hepatocellular carcinoma and renal cell carcinoma, demonstrated definitive single-agent benefit [24, 32]. Other biologics add incremental benefit to standard therapy, such as the IFL-bevacizumab combination in colon cancer and the addition of trastuzumab to chemotherapy in *HER2* amplified gastric cancer. In an era of cost containment, the incremental benefit of some biologics when compared to their cost has come under scrutiny [33]. Incremental benefit, however, can be meaningful in extending survival. As noted by Sobrero and Bruzzi, when incremental benefits of successive treatments are additive, the net benefit can be large—the case in colorectal cancer, in which the incremental benefit of each of six therapies (oxaliplatin, irinotecan, bevacizumab, panitumumab, cetuximab, capecitabine) approved over the past 15 years was small, yet their net effect has been to increase the survival of patients from an average of 5 months with best supportive care alone to over 20 months with multiple lines of therapy [34]. Table 2 illustrates some key benefits of biologic therapies that have been demonstrated in the phase III setting as of 2012.

Phase III clinical trials remain the gold standard for assessment of efficacy of new drugs and new regimens and are the foundation of evidence-based oncology. Overall, this paradigm has not been undermined by the advent of the molecular era of drug development—indeed, many targeted agents have had successful phase III outcomes as discussed above. However, for agents that have

Table 2 Incremental PFS and OS benefit of targeted therapies in advanced solid tumors

Condition	Indication	No. of Patients in the Study	Design	PFS			OS		
				Median improvement over Control (months)	*P*	Hazard ratio	Median improvement over Control (months)	*P*	Hazard Ratio
Renal cell carcinoma									
Sorafenib [32]	First-line metastatic	769	Sorafenib *v* placebo	2.7	<0.001	0.44	NR[a]	NR	
Temsirolimus [35]	First-line metastatic with high-risk features	626	Temsirolimus *v* IFN alpha	2.4	<0.001	0.66	3.6[a]	<0.001	0.73
Sunitinib [36]	First-line metastatic	750	Sunitinib *v* IFN alpha	6.0	<0.000001	0.42	NR[a]	NR	
Bevacizumab [37]	First-line metastatic	649	IFN alpha + bevacizumab *v* IFN alpha + Placebo	4.8	0.0001	0.63	NR[a]	NR	
Breast cancer									
Trastuzumab [38]	First-line metastatic HER-2+	469	Doxorubicin + cyclophosphamide or paclitaxel plus or minus trastuzumab	2.8[a] (TTP, not PFS)	<.001	0.51	4.8	0.46	0.80
Bevacizumab [19]	First-line metastatic	722	Paclitaxel + bevacizumab *v* Paclitaxel	5.9[a]	<0.001	0.6	1.5	0.16	0.88
Lapatinib [39]	Refractory HER-2+	399	Capecitabine + lapatinib *v* capecitabine alone	1.9[a]	<0.001	0.57	NR	NR	
Colorectal cancer									
Bevacizumab [26]	First-line metastatic	813	IFL + bevacizumab *v* IFL	4.2	<0.001	0.54	4.7[a]	<0.001	0.66
Panitumumab [40]	Refractory	463	Panitumumab *v* best supportive care	0.15[a]	<0.0001	0.54	0.0	1	1.0
Non-small-cell lung cancer									
Erlotinib [41]	Second- and third-line metastatic	731	Erlotinib *v* placebo 2:1 randomization	0.4	<0.001	0.61	2.0[a]	<0.001	0.7
Bevacizumab [28]	First-line stage IIIB or IV	878	Paclitaxel, carboplatin, bevacizumab *v* paclitaxel and carboplatin	1.7	<0.001	0.66	2.0[a]	0.003	0.79

(continued)

Table 2 (continued)

Condition	Indication	No. of Patients in the Study	Design	PFS			OS		
				Median improvement over Control (months)	*P*	Hazard ratio	Median improvement over Control (months)	*P*	Hazard Ratio
GIST									
Sunitinib [42]	Second line	312	Sunitinib *v* placebo	4.8 (TTP, not PFS)[a]	<0.001	0.33	NR	NR	
Head and neck cancer									
Cetuximab [31]	Locally advanced	424	RT plus or minus cetuximab	9.5[a] (local control)	0.005	0.68	19.7	0.032	0.74
Pancreatic cancer									
Erlotinib [43]	First-line metastatic	569	Gemcitabine + erlotinib *v* gemcitabine	0.25	0.03	0.76	0.46[a]	0.025	0.81
Hepatocellular carcinoma									
Sorafenib [24]	Pretreated hepatocellular carcinoma	602	Sorafenib *v* placebro	2.7	<0.001	0.58	2.8[a]	<0.001	0.69

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NOTE: The registration trial data of imatinib in GIST (first line) are not included in the table because registration was based on the results of a phase II randomized trial comparing two drug doses in terms of frequency of objective responses, as compared with historical controls treated with chemotherapy.[19] The registration of cetuximab in advanced colorectal cancer is not included in the table because registration was based upon valuable responses reported n a randomized phase II trial of cetuximab and cetuximab plus irinotecan in irinotecan refractory patients.[20] Note that the registration of cetuximab plus RT in locally advanced head and neck cancer refers to a nonmetastatic phase

PFS progression-free survival, *OS* overall survival, *NR* not reported, *IFN* interferon, *TTP* time to progression, *IFL* irinotecan, fluorouracil, and leucovorin, *GIST* gastrointestinal stromal tumor, *RT* radiotherapy

[a]Primary end point of the study

demonstrated modest or no benefit in the phase III setting, it is unclear if the lack of benefit stems from a true drug inactivity or lack of identification of the right subset of patients who would derive substantial benefit. The latter was the case with the initial trials of the EGFR TKI agents, gefitinib and (Tarceva, Genentech), in NSCLC. Only after several phase III studies in unselected patients with both gefitinib and erlotinib were sensitizing EGFR mutations discovered. These mutations confer an oncogene-addicted biology and thus revealed a population that was highly sensitive to these drugs [44]. Erlotinib was subsequently approved by the FDA for this population, based on randomized trials comparing the targeted drug to chemotherapy.

3 Oncogene Addiction and Selective, High-Efficacy Targeted Therapy

While the need for phase III evidence is clear for most new therapies, the evolution of targeted drugs with suggestion of profound, rather than incremental, benefit in early phase trials has added new dimensions to the discussion of the appropriateness of phase III trials as the gold standard for drug approval. Indeed, had the EGFR activating mutation in lung cancer been known prospectively, the path to drug development may have proceeded quite differently. In the development of targeted drugs, investigators have the opportunity to select patients for the earliest trials based on the presence of a tumor "biomarker," often a mutated or amplified gene that confers addiction and represents the Achilles heel for the subset of disease in which it occurs. The concept of oncogene addiction was first offered by Bernard Weinstein and has since been demonstrated for a number of different tumors in which a driver mutation becomes essential for cell viability; inhibition of the addicting gene product leads to cell death in model systems, such as in cells addicted to BCR–ABL, or mutated *CKIT, EGFR, BRAF, EML4-ALK, ROS1*, and others [1, 45].

In phase I trials, if the population of patients with tumors expressing the target mutation is accrued at the inception of the trial, benefit may be demonstrated in the earliest stages of drug development. In other trial designs, the initial safety testing may proceed with unselected patients, but once a safe dose with modest toxicity is established, an expanded cohort of patients, selected for the presence of the molecular lesion in their tumor, may be entered in large numbers. This type of phase I trial may require the capacity to screen large numbers of patients in order to find adequate numbers with the biomarker of interest. Clear improvements in response rate may be readily detectable in a cohort 30–40 patients, with reasonable statistical certainty.

High-potency targeted therapies have begun to emerge from this approach, led by imatinib in CML and subsequently GIST, followed by gefitinib and erlotinib in *EGFR*-mutant lung cancer (identified retrospectively), and most recently, in *BRAF*-mutant melanoma and *ALK*-rearranged lung cancer. Therapies that provide high-affinity target inhibition of constitutively active, oncogene-addicted pathways have challenged our paradigms of drug development. In such settings, the need for Phase III trials prior to approval is not always apparent, and such trials may not be either ethical or feasible (see below).

3.1 Imatinib in CML and GIST

The experience of imatinib in chronic myelogenous leukemia (CML) had significant impact on thinking about drug development strategies, not only because of its early and dramatic efficacy and minimal toxicity in initial trials, but also because of its impact on trial design and expectations of success of targeted therapies. Patients with chronic phase CML who historically had suboptimal treatment options such as interferon therapy or stem cell transplantation (both with significant associated

toxicities and risks) were afforded the prospect of taking a daily pill. The majority achieved complete cytogenetic and even—with extended treatment—molecular remission. Imatinib marked the first success of the era of rational drug design—targeting the oncogenic pathway up on which cancer cell survival and proliferation is dependent and reaping sizable benefit by blocking its activity.

The brief history of imatinib development is instructive. The Philadelphia chromosome (t(9;22)) was first characterized in tumor cells isolated from patients with CML by Rowley in 1973 [46]. The translocation creates a fusion protein between the *BCR* transcription factor and the *C-ABL* nonreceptor tyrosine kinase creating a constitutively active tyrosine kinase that is the inciting molecular event in CML. Once the molecular lesion was identified, drug development ensued and random screening identified the class of 2-phenylaminopyrimidine derivatives, including imatinib, as potent substrates for the ATP-binding pocket of the fusion protein [47]. Druker et al. initiated a phase I trial of imatinib in 1998, and the study demonstrated dramatic responses. Enrolling 83 patients with chronic phase CML who had failed interferon alfa therapy, the dose escalation study demonstrated a 54 % rate of cytogenetic response to the drug in patients who received a dose of 300 mg daily or higher [48]. This finding prompted rapid expansion to a large phase II study that enrolled 532 patients with late chronic phase CML who had also failed interferon alpha. Patients received 400 mg of imatinib daily. Sixty percent of patients had a cytogenetic response and 41 % had a complete cytogenetic response. With median follow-up of 18 months, 95 % of patients were alive, with chronic phase disease present in 89 % [49].

Imatinib was brought to the phase III setting, post-initial approval, in a large multicenter trial which randomized patients up front (with no prior treatment for chronic phase CML) to imatinib versus interferon alfa and cytarabine, a combination with inferior historical response rates than seen with imatinib in phase II for relapsed patients [50]. The study offered crossover to the alternate group if stringent criteria for treatment failure or toxicity were met. Strikingly, 58 % of patients randomized to interferon alfa plus cytarabine ultimately crossed over to imatinib after failure due to disease progression or toxicity on upfront therapy. Delayed access to the new drug was well accepted by patients in the control arm because initial therapy in the control arm was known to produce a median of 4 years of control in this disease.

Imatinib had already received accelerated FDA approval for chronic phase CML refractory to interferon alfa, or accelerated phase CML, or blast crisis, in May 2001 [51]. Accelerated approval was provided with a commitment from Novartis for post-marketing follow-up to determine the duration of treatment response as well as median overall survival. Full approval was granted on December 5, 2003—9 months after the publication of the phase III results. Subsequently, new TKIs active against BCR-ABL kinase in patients with imatinib-resistant CML have been developed by Novartis (nilotinib), Pfizer (busotinib), now withdrawn due to arterial thrombotic events in the Phase III study, and by Bristol Myers Squibb (dasatinib). Phase III trials have shown that front line therapy with nilotinib produces superior molecular and cytogenetic response rates, and fewer relapses than does imatinib, leading to its approval for primary therapy of CML [52].

Imatinib also demonstrated affinity for the ATP-binding domains of PDGF-R and CKIT kinase, and it was posited that it would have activity in cancers constitutively driven by these pathways. Indeed, in 2001 striking benefit was reported in a patient with widely metastatic gastrointestinal stromal tumor (GIST) treated with imatinib, followed soon after by a randomized phase II trial in which GIST patients were, notably, randomized to imatinib 400 mg versus imatinib 600 mg daily, with no standard comparator arm [53, 54]. Again, accelerated approval for marketing was granted based on these early phase II results. Subsequent phase III trials conducted by EORTC and SWOG focused on dose, comparing 400 mg daily dosing to 800 mg daily dosing, with no standard comparator arm [55], showing no additional benefit with the higher dose. This design met regulatory standards because there were no other effective standard therapies for GIST.

CML and GIST are somewhat unique in that the targeted molecular lesions are virtually ubiquitous—the t(9;22) is the pathognomonic mutation of CML, and the *CKIT* mutation is present in nearly all GIST patients, and gene expression was a precondition for entry for GIST patients in the phase II trial [53, 56]. As such, these rare diseases represent obvious settings in which to demonstrate initial success with small molecule therapy in an entire histological category of disease. In other cancers,

specific molecular lesions are present in a much smaller subset of tumors, leading to the requirement for genotyping of tumors to identify appropriate candidates for treatment. We will profile two contemporary, important examples, and discuss the methodologic and ethical issues that currently surround their drug development.

3.2 Advanced Melanoma: The Vemurafenib Experience

Metastatic malignant melanoma represents one of the most difficult and refractory presentations of cancer. Prior to the advent of checkpoint inhibitors such as the CTLA-4 antibody ipilimumab (Yervoy, Bristol Myers Squibb), approved treatments, DTIC, interferon, and interleukin 2, benefitted a small minority of patients (fewer than 15 %) and caused significant toxicity [57, 58]. A new avenue to drug development was opened by the discovery that the V600E mutation in the gene encoding the serine–threonine kinase B-RAF is found in 60–70 % of malignant melanomas [59]. BRAF is a member of the MAP kinase pathway and when mutated, constitutively activates downstream signal transduction that promotes proliferation, stimulates the secretion of angiogenic and proliferative growth factors, and inhibits apoptosis. The Plexxikon drug PLX4032 (RG7204, vemurafenib) demonstrated preclinical inhibition of the V600E isoform of BRAF and was brought forward in a phase I study of patients with advanced melanoma and other solid tumors in a dose escalation format [4, 60]. While the V600E mutation was not a precondition for enrollment, it was overrepresented in the dose escalation phase because of the number of patients with advanced melanoma on study. Once the maximum tolerated dose was established, however, the phase I study continued with an expansion cohort comprised solely of patients with melanoma harboring the V600E mutation.

The results of the small Phase I trial were dramatic. Eighty-one percent (26 of 32) of patients with *BRAF*-mutated tumors had either complete (2 patients) or partial (24 patients) tumor regression—a seemingly dramatic improvement over the standard of care, dacarbazine. The median duration of response among those who responded to the drug was 8 months [61]. Figure 1 demonstrates the clinical response in patients with this disease. As the results were reported in the phase I setting, however, enthusiasm was countered with the argument from some that there was no "control" group and hence no definitive proof of improvement in progression-free or overall survival. This led to the multicenter randomized phase III BRIM3 trial in which patients were randomized to vemurafenib versus dacarbazine [62] (see Fig. 1 and Table 3). Of note, the trial, which was powered to detect a difference in overall survival, did not originally permit crossover of participants from dacarbazine to vemurafenib though it did permit the inverse crossover to dacarbazine at the time of tumor progression on vemurafenib. While the phase III trial was accruing patients, the drug was not available for compassionate use. However, the study's independent Data and Safety Monitoring Board (DSMB) reviewed interim data in January 2011 and determined that there was compelling evidence of vemurafenib's treatment benefit over dacarbazine to allow patients randomized to the DTIC arm to cross over to treatment with vemurafenib. FDA approval for the treatment of *BRAF*-mutated melanoma was granted in August 2011. The ethical issues surrounding the trial design and the limited access to drug in the initial stages of the Phase III trial will be addressed below.

3.3 Advanced Lung Cancer: The EML4-ALK Translocation

A similar magnitude of success was demonstrated with a new drug a small subset of patients with advanced lung cancer. Combination chemotherapy has improved the response rate, quality of life, and survival of patients with advanced non-small cell lung cancer, but only modestly, and at the cost of significant toxicity. The typical response rate to first-line combination chemotherapy is 25–35 %, the

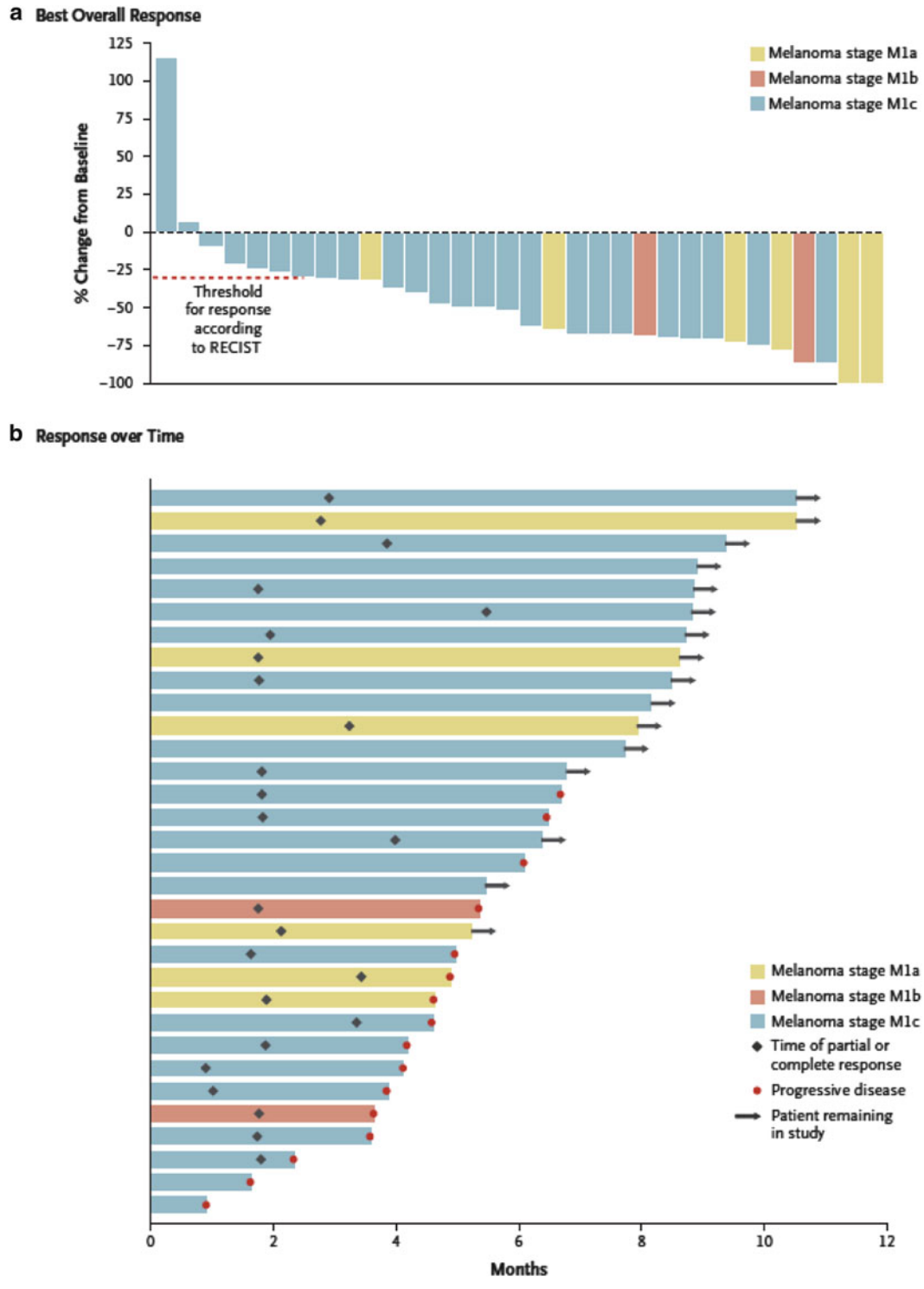

Fig. 1 Antitumor response in each of the 32 patients in the extension cohort. All 32 patients had melanoma tumors that carried the V600E mutation of the V-RAF murine sarcoma viral oncogene homologue B1 (B-RAF). Reprinted with permission from [62]

Table 3 Historical response rates of the comparators prior to the definitive Phase III trial of vemurafenib versus DTIC. PFS, and OS of agents in the BRIM3 randomized phase III trial at the time of study initiation

Drug	Trial	Number of patients receiving drug	Response rate (CR + PR)	Median PFS (months)	Median OS (months)
DTIC	Middleton et al. Randomized phase III study of temozolomide versus dacarbazine in the treatment of patients with advanced metastatic malignant melanoma, J Clin Oncol 2000 [63]	*N* = 149	12.1 %	1.5	6.4
vemurafenib	Flaherty KT et al. Inhibition of mutated, activated BRAF in metastatic melanoma. N Engl J Med. 2010 [4]	*N* = 32 (extension phase)	81 % (all patients had BRAF V600E mutation)	7+ (estimate)	Not reached

Shown are results in a phase III trial of DTIC and a phase I trial of the B-RAF inhibitor vemurafenib in a biomarker enriched population of B-RAF-mutant patients with advanced melanoma. Reprinted with permission from [62]

median time to progression is 4–5 months, and the median survival is 10–12 months [28, 64]. In advanced lung cancer, multiple oncogenic pathways have been implicated as containing driver mutations in subsets of patients, affecting *EGFR, KRAS, BRAF*, and *HER2* [65]. Activating fusions of the anaplastic lymphoma kinase gene, or *ALK*, with various partner genes had been previously observed in anaplastic large-cell lymphoma [66]. In 2007, a new aberrant fusion gene, *EML4-ALK*, was identified in NSCLC. It encodes a cytoplasmic protein with constitutive kinase activity [67]. Preclinical studies indicated that the fusion gene conferred addiction when transfected into normal cells, and its inhibition by experimental drugs led to apoptosis [68]. The ALK kinase bears close homology to *CMET*, another oncogene.

A first-in-man phase I trial of the CMET inhibitor crizotinib (Xalkori, Pfizer), which also inhibits ALK, was accruing patients at the time of the discovery of the *EML4-ALK* translocation in NSCLC. With this publication, patients with lung cancer were directed to this trial during the dose escalation phase, and several responded. Because the ALK rearrangement is not a highly prevalent lesion, approximately 1,000 patients were screened in order to enroll 82 patients with the specific mutation [69]. As screening continued, clinical characteristics of ALK-positive patients emerged, allowing some tailoring of the screening process to nonsmokers or light smokers [70]. The cohort of patients with tumors exhibiting the *EML4-ALK* translocation treated on the phase I crizotinib study demonstrated a 57 % objective response rate. An additional 33 % of patients had minor response or stable disease of at least 2 months duration (Fig. 2). The high response rates were likely attributable to patient selection through prospective tumor genotyping—if the study had not been enriched for patients harboring the target lesion, few significant responses would have been observed, since only 3–4 % of non-small cell lung cancers have this mutation. Armed with this information, the sponsor elected to go directly to a phase III trial in a genotype-specific population. The global, randomized PROFILE 1007 study enrolled patients with known *ALK*-rearranged NSCLC and compared PFS with second-line crizotinib versus standard second-line chemotherapy (either docetaxel or pemetrexed). Though the trial did not officially allow crossover, a companion phase II study was available to patients randomized to chemotherapy upon their progression [71]. The trial demonstrated a significant improvement in median PFS among crizotinib-treated patients over chemotherapy-treated patients (7.7 months versus 3.0 months). However, an interim analysis of overall survival showed no significant survival benefit from crizotinib (HR for death in the crizotinib group 1.02). Crizotinib received accelerated approval in August 2011 based on the results of the expanded Phase I cohort and a comparion Phase II trial in chemotherapy-resistant patients with ALK-positive NSCLC [72].

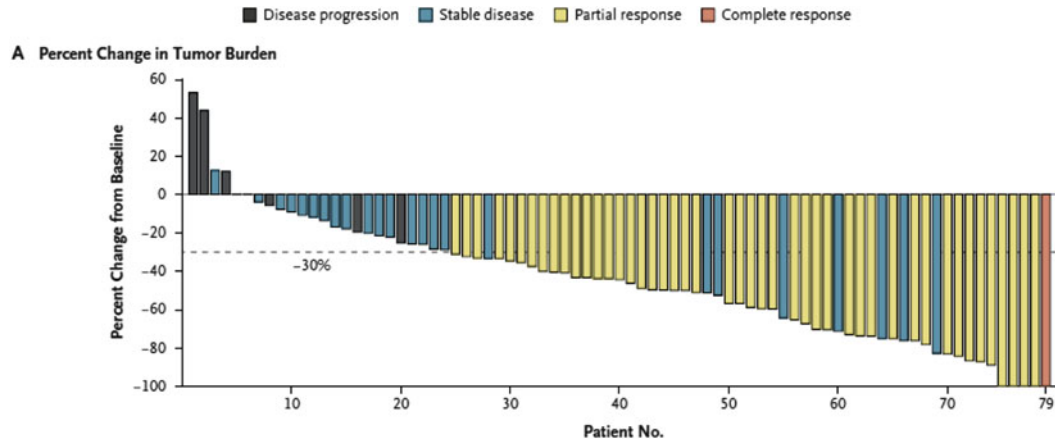

Fig. 2 The best response of patients with ALK-positive tumors who were treated with crizotinib, as compared with pretreatment baseline. Reprinted with permission from [69]

These recent examples in melanoma and non-small cell lung cancer powerful impact of patient selection in accelerating approvals of personalized therapy. In both cases, early phase development patient cohorts were enriched by up-front characterization of a molecular biomarker mutation known to be targeted by the study drug. Patients with mutant V600E BRAF melanoma and *ALK*-rearranged non-small cell lung cancers comprised the expansion cohort in phase I studies. In both trials, the drugs demonstrated great promise in lethal diseases in which there are no acceptable alternative therapies. For vemurafenib, an abbreviated Phase III trial led to approval, while for crizotinib, approval was granted while a Phase III trial had accrued patients and awaited analysis.

4 Methodologic Implications of Genotype-Directed Phase III Trials

Prospective genotyping and molecular enrichment of clinical trial populations represent a sea change from the traditional "all comers" design in phase I trials. Tumor genotyping streamlines drug development to some degree by enhancing the likelihood of observing, and even defining, level of benefit or failure in a relatively small but selected patient population during the initial trials. This has been referred to as the "fail early and fast" approach [2]. However, there are potential downsides to focusing drug development on a genotype-defined population. First, as noted by Susman, early success may breed downstream difficulty: when there is strong scientific rationale to a novel therapy, early demonstration of clinical response, and clear evidence of target inhibition (such as downregulation of pERK phosphorylation by a BRAF inhibitor), drugs might be granted accelerated FDA approval for an unmet need, but "once a pharmaceutical company has accelerated approval in hand, the incentive to seek full approval with a pivotal phase III trial diminishes" [73]. Additionally, if a drug does not proceed through to definitive testing in the Phase III setting, there will always remain a lack of certainty of survival benefit and an incomplete understanding of toxicity. For example, 15 % of patients in the dose escalation cohort and 31 % of patients in the expansion cohort of the BRAF inhibitor trial in melanoma developed cutaneous squamous cell carcinoma, all with features of keratoacanthoma [4]. The long-term morbidity of this toxicity is unknown (though it is unlikely to eclipse the benefits of treatment).

Another caveat to the enrichment design strategy is the loss of information about patients excluded from the trial who might derive benefit. Because of the "promiscuity" of targeted drugs, which hit multiple closely related targets, such as the ALK inhibitor crizotinib, which also inhibits CMET and the ROS1 kinase, it was not possible to evaluate effects on ALK-negative tumors in the earliest trials. Another example is offered by erlotinib, which inhibits both the mutated and nonmutated forms of *EGFR*, and has a demonstrated survival benefit compared to placebo in unselected patients (which are primarily wild-type *EGFR* genotype) [74]. Furthermore, while genotype-specific drug design is logical and appealing, and many additional drugs are likely to be developed under this paradigm, in reality only a handful of situations have arisen to date in which a clear biomarker is readily apparent early in development of a novel targeted drug. Hence, all-comer designs remain the best strategy when the molecular lesion is unclear or the putative mechanism of action is nonspecific. For example, the search for biomarkers to identify sensitive subpopulations continues with antiangiogenic drugs, but has been unsuccessful to date. Trials of targeted therapy in a molecularly heterogeneous population run the risk of not detecting meaningful benefits in a sensitive molecular subset of patients, and may lead to falsely negative conclusions about a drug's benefit. We cannot know if or how often this has happened already nor estimate how many drugs have been "killed" when a subset of ultrasensitive patients exists but was not adequately studied.

5 Ethical Implications of Genotype-Directed Phase III Trials

In cancers with high lethality and without acceptable standard options, it is ethically challenging to conduct a randomized phase III trial in which a cohort of patients harboring a biomarker associated with efficacy of a targeted agent in Phase I or Phase II are assigned to a control arm with limited efficacy. Not only are a group of patients subjected to therapy known to be minimally effective, but also the access of all other patients, including those ineligible for the trial or unable to join the trial for reasons of geography or convenience, is delayed for many months, or even years, until the phase III is completed. During the accrual phase of such a trial, it is not possible to open a compassionate access protocol for fear of compromising accrual. With new targeted therapies it may not be possible to conduct a standard phase III trial, powered to define a survival benefit, if the effect seen in phase I is dramatic and if there is no acceptable standard comparator.

The issue came to national attention in September 2010 when an article entitled "New Drugs Stir Debate on Rules of Clinical Trials" appeared in the New York Times [5]. The article detailed the parallel experiences of two cousins, one of whom was successfully treated with vemurafenib in its phase II trial and another who was assigned to the control arm of the phase III BRIM3 trial and died. To review, the phase III trial evaluated vemurafenib head-to-head against dacarbazine, and in its original design did not allow crossover to vemurafenib at time of progression. The trial was undertaken following the highly successful phase I trial, in which BRAF-mutant tumors showed an 81 % response rate in the expansion cohort and a median TTP of 7+ months [4]. Longer term follow-up of patients in the phase I expansion cohort on vemurafenib demonstrated emergence of drug-resistant disease at a median of 8 months on the drug among responders, and the BRIM3 trial was formulated with a randomization of vemurafenib against the standard therapy, DTIC. The trial schema addressed the question: while the drug clearly led to dramatic up-front responses, does this early effect correlate with an overall survival benefit? Oncologists interviewed for the newspaper article were divided in their endorsement of the trial. Study investigators defended the schema as necessary for a definitive understanding of the drug's benefits and risks. Other experts dissented, arguing that targeted therapy—with scientific rationale, minimal side effects, and dramatic response rates—need not be subjected to this degree of stringent testing before marketing. Dr. Richard Padzur, Director of the Office of Oncology Drug Products within the Center for Drug Evaluation and Research at FDA,

indicated that there may be some latitude on the subject of the necessity for phase III-level data for drug approval, stating "this is an unprecedented situation that will, hopefully, be increasingly common, and it may require a regulatory flexibility and an open public discussion" [5]. The study design was revised in January 2011 when the Data and Safety Monitoring Board (DSMB) reviewed the preliminary study data and found the data sufficiently compelling to modify the design to allow crossover.

6 Methods for Maintaining Equipoise

Given the paradigm established above regarding the new era of drug development for novel therapies active against defined subgroups of patients, how does one design a phase III study that can maintain equipoise—the credible expectation of equal efficacy in both arms? Equipoise is particularly difficult to guarantee when the investigational arm of a trial is based on striking phase I activity, and the control arm is a failed therapy in a lethal cancer (see Table 3). Several alternative trial designs offer options for bridging this dilemma and offer earlier, or initial, access to the experimental therapy to all patients on the study.

6.1 Early Stopping Rules Based on PFS

Phase III trials are under the strict scrutiny of an independent Data and Safety Monitoring Board (DSMB) which is comprised of a panel of independent oncologists and biostatisticians. Phase III study designs prospectively mandate interim data evaluations to ensure that one arm is not experiencing excess toxicity. These interim analyses are often powered to detect significant benefit if it is found in one of the treatment arms. By this mechanism, DSMBs are mandated to stop a study early and inform study investigators and participants of the results. Thus, as a Phase III study proceeds, patients randomized away from the superior therapy may be offered the opportunity to crossover and benefit from the study drug. Results are interpreted at the time of the interim analysis, regardless of whether the primary endpoint of the study was met. However, there is a disadvantage to interim analysis. Each interim look raises the statistical threshold for disproving the null hypothesis [75].

Recent trials illustrate the pro's and con's of employing early stopping rules to abbreviate highly positive trials. These rules were implemented in the National Cancer Institute of Canada Clinical Trial Group randomized controlled trial of letrozole (Femara, Novartis) versus placebo in early stage ER/PR positive tumors of the breast in postmenopausal women who had completed 5 years of adjuvant tamoxifen therapy [76]. The study enrolled 5,187 patients and the primary endpoint was disease-free survival (DFS). The DSMB interim analysis at 2.4 years median follow-up detected a statistically significant 4-year DFS benefit despite no statistically significant OS benefit, and it was recommended that the study be stopped and that women in the placebo arm be permitted to cross over. This decision came under intense scrutiny. Study investigators maintained that DFS was a meaningful endpoint, and the DSMB would have been unethical to continue randomization purely for the interest of demonstrating an overall survival benefit, as DFS is a meaningful and relatively accurate proxy [77]. Furthermore they maintained that improved quality of life associated with an absence of disease recurrence was in itself meaningful to the patient. Critics maintained that in the absence of continuing the study, overall survival, the gold standard endpoint, can only be extrapolated [78]. Had the study been allowed to continue and were the OS endpoint negative, the study would have been declared null. In addition, the toxicity of long-term aromatase inhibitor treatment might have added additional negative

considerations in the assessment of overall clinical benefit. Thus, stopping the study for a proxy endpoint could have thwarted the ability to ask highly important clinical questions. Subsequent analysis of the study cohort at 30 months demonstrated continued DFS benefit and an OS benefit, but only in the subset of patients with node-positive disease (HR = 0.61, 95 % CI = 0.38–0.98; $P = 0.04$) [79].

6.2 Compassionate Use Studies

In advanced cancers, promising agents awaiting a decision on whether FDA approval will be granted may be provided under compassionate use protocols. The sponsor may charge users for the costs of providing the drug. Compassionate use was first employed under the so-called Group C mechanism for cancer drugs, such as melanoma, and was later greatly expanded to accommodate the early release of AZT and other AIDS drugs prior to their marketing approval. Minimal data are collected by the sponsor, and the primary intent is to provide wider access during the preapproval process. Compassionate release has clear disadvantages for the sponsor, as well as for the conduct of clinical trials, in that this arrangement relieves the public pressure for FDA drug approval, yields no profit, and provides an alternative source of drug for patients not willing to join ongoing clinical trials. However, broad use of a targeted drug by the academic community under compassionate access programs may also yield novel observations not apparent in initial pharmaceutical-designed studies. When gefitinib was used in exactly this setting, it led to the discovery of *EGFR* mutations in NSCLC. As more and more light and never-smokers were exposed to the drug under compassionate access, and responded, the emerging pattern was recognized, and researchers tested responding patients to uncover their genetic predisposition for response.

6.3 Permitting Crossover at Progression

One answer to the dilemma of equipoise is to allow crossover of control patients to the experimental treatment at the time of progression. However, crossover may compromise the opportunity to assess a survival advantage, in that all patients may eventually receive the most effective drug. Such was the case in the phase III happened in the Phase III trial of crizotinib versus chemotherapy for *ALK*-mutated non small cell lung cancer. Crossover designs depend on time to progression (TTP) or progression-free survival (PFS) as their primary endpoint. These surrogate endpoints are clinically meaningful and acceptable for marketing approval in some cases, but inherently less well-defined than overall survival, dependent as they are on precise and timely imaging of disease. As discussed above, the crux of the ethical issue in the BRIM3 trial in advanced melanoma was the stringency of the original study schema—and lack of crossover at time of disease progression in the dacarbazine arm. Investigators maintained that crossover would have obscured the ability to demonstrate the primary outcome. However, if the benefit of a drug is profound, crossing over does not necessarily mitigate the differential survival. In the phase III imatinib trial in CML, 57 % of patients crossed over to imatinib at the time of progression or toxicity with interferon alfa and cytarabine, yet a survival benefit was still demonstrated [48]. However, in *EGFR* mutation-positive NSCLC, high rates of response to EGFR TKIs in the second and third-line settings likely obscured any survival benefit, even among *EGFR* mutants, to first-line gefitinib in the IPASS study, a large randomized trial comparing first-line gefitinib to carboplatin and paclitaxel [80]. In anticipation of difficulty showing a survival advantage in future crizotinib studies now that crizotinib is FDA approved, Shaw and colleagues did an extensive retrospective analysis of ALK-positive lung cancer patients receiving crizotinib

compared to similar ALK-positive patients who did not receive crizotinib before their death and showed a substantial survival benefit [81].

In conclusion, early stopping rules and crossover designs may speed the process of drug approval, but may sacrifice longer term evaluation of overall survival. Compassionate use programs similarly provide relief for patients with no other therapeutic options. These mechanisms grant earlier access to promising therapy within the context of a Phase III trial. These strategies, however, have potential negative impact on the outcome of the trial, compromising its accrual or realization of endpoints. Ultimately if investigational drugs show profound promise in an otherwise lethal and untreatable clinical setting, even these modifications in drug evaluation may not do enough to provide drug to patients. It may be necessary to rethink the position and necessity of phase III trials in the approval sequence for targeted therapies.

7 Redefining the New Paradigm of Drug Approval

The molecular revolution in cancer therapy has arrived. The human genome project, and specifically the full sequencing of the protein kinase complement, or "kinome," has brought us to the threshold of a new era of drug discovery [1]. It is estimated that there are currently 800 new therapies in the cancer pipeline. While ubiquitous targetable molecular lesions, present in every patient with a histological type of cancer, such as BCR-ABL in CML, may be the exception, sub-stratification of common cancers will likely reveal the presence of molecular driver oncogenes and will expand our repertoire of rational therapies provide early evidence of success in phase I trials, and alter drug development strategy. That said, we believe that phase III remains an important tool for demonstrating the longer benefits and toxicities of cancer therapies, but it best applies when true equipoise exists regarding the efficacy of alternative treatments. When dramatic effects are demonstrable in early trials, it may be either unethical or impossible to perform traditional phase III trials and deny patient access to the new agent.

The FDA has been forthcoming in offering accelerated approval to drugs that demonstrate great promise in diseases for which there are no effective alternatives and high likelihood of success in phase III. The path to full approval of imatinib in CML, in which crossing over in the clinical trials was permitted at the time of progression, demonstrates that survival benefit can still be demonstrated without a perfectly "clean" study if the benefit of the drug is significant to a broader population of patients pending final analysis of results, after accrual is complete and even before analysis of the results is final. Endpoints other than survival, such as PFS or TTP, may be acceptable alternatives in the event that crossover is a necessary feature of the Phase III trial. In sum, while phase III trials are required to prove benefit in many clinical settings, for drugs that demonstrate dramatic efficacy in lethal diseases, the cancer community should encourage and support drug development strategies that maximize patients' early access to highly active therapy.

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Clinical Trial Designs for Approval of New Anticancer Agents

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Abstract There is an imperative need for development of newer cancer therapeutics and their rapid availability for cancer patients. Better clinical trial designs may help cancer therapeutics become available for treatment sooner. This chapter focuses on our present knowledge about clinical trial design, discussions of successful trial designs, as well as proposals for novel strategies. An exhaustive review of methods used to select agents which are currently in use is included and followed by a summary table of all of the new agents considered by the Food and Drug Administration's Oncologic Drug Advisory Committee, including the type of trial performed, approved versus disapproved, and the primary endpoint used for approval. Novel clinical trial design suggestions have been included. We hope the present effort helps readers understand various clinical trial designs and get them enthusiastic about exploring new designs.

Keywords Antineoplastic agents • Drug therapy • Clinical trial • Drug approval • Drugs • Investigational • Drug evaluation

1 Introduction

The need for new treatments for cancer patients is great but progress has been way too slow. Major obstacles to new therapies have included (a) basic science that is non-predictive for the clinic, (b) clinical trials which do not really test the basic science findings, (c) administrative delays (year to 840 days) to activation of a new trial [1–4], (d) agents that proceeded to large randomized phase III trials despite less than sound results from phase II trials, and (e) recently identified minor protocol study matters [5] which cause great delay but which could have been handled by administrative review rather than a full board review, among many other problems.

It is highly likely our understanding of the science of tumor cells and their microenvironments and their supportive infrastructures is so primitive that it will be a while before our technologies improve to have widespread successes against this disease. However, we think if our clinical trial designs could be better, then there could be improvements for patients. This chapter is devoted to utilizing what we

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know, discussions of successful trial designs, as well as proposals for other designs to help decide whether or not to proceed with expending resources in large phase III trials. The reader will find (a) commentary on methods used to select agents which we use actively in the clinic; (b) a summary table of all of the new agents considered by the Food and Drug Administration's Oncologic Drug Advisory Committee, including the type of trial performed, approved versus disapproved, and the primary parameter (endpoint) used for approval; (c) special trial designs for testing particular types of anticancer agents (e.g., cytostatic agents); (d) trial designs that have been unusual for oncology agents but often are used in other therapeutic areas (e.g., randomized trials of different dose level); and (e) some more novel clinical trial designs which have not yet had regulatory acceptance but will hopefully get the readers enthusiastic about at least exploring these new designs.

2 Methods to Select Agents That Will Be Active in the Clinic

How does one select agents that would definitely work in the clinic with certainty? Good question! Ratain and others have reported that about 10 % of the agents brought into clinical trials actually make it to approval. It is obvious that we are doing something wrong. Most of our current animal models are just "furry test tubes." Additionally, we don't clearly understand the mechanism as to how the majority of our agents work or why it works in usually only a small percentage of patients.

Empirically it is becoming clear that for agents to have success (in terms of efficacy and use), the word is *targeted*. The selection of the target must be based on impeccable science. Recent examples of these targets included VEGF, proteasomes, PDGFR, c-kit, Her-2/neu, CD20, CD52, BCR-ABL translocation, B-Raf [6], and Enl-ALK [7]. It is likely that the list will soon include patched/smoothened, PARP, and others [8–11]. Even more recent experience teaches that if there is evidence that the tumor cells are "addicted" to the target, success is very likely. Likewise a mutation/translocation or amplification which is specific to cancer cells provides a higher likelihood of success. As a matter of fact, the next generation of molecules moving into phase I trials acts against targets specific for cancer cells owning to the fact that those agents have a higher likelihood of helping an individual patient. Because of this, in these authors' experience, there are more and more trials where responses are noted in even the first patient on the phase I trial [6–11].

In addition, there is hardly any team interested in investing in a new agent that does not have a well-defined final target and a well-defined target patient population.

Therefore, it is not only about the "methods to detect agents which will be active in the clinic" but also about selecting patients who will have the best chance of responding to the new agent. Certainly one way to help assure selection of an agent for the clinic with a fairly great degree of certainty that it will work is to select an analog of an already approved agent. Another method of selection to guarantee success is selecting an agent which is a newer delivery form of an already approved agent (e.g., a liposomal or an oral formulation of already approved drug). Design of trials for this type of agent(s) is discussed in some detail below. The major issue for analysis of new formulation is what type of trial design you will use for approval and how large will that trial design be. Proving similarity or noninferiority generally takes a very large number of patients.

The next promising area is going after targets in the microenvironments of the cancer. The success of bevacizumab in the area of antiangiogenesis started the field [12, 13]. Multiple new microenvironment targets which include hypoxia (HIF-1-alpha), SPARC, hyaluronidase, and others will likely be used to deliver agents as well as to "disrupt the lifestyle of the cancer cells" [14–16]. Of even more interest is some recent evidence that the microenvironment might be highly responsible for the resistance that develops in the cancer cells [17]. This resistance could even be mediated by cytokines such as IL-6 in the microenvironment [18, 19]. A final emerging area is targeting metabolic pathways in cancer cells versus in the normal cells [20, 21].

3 General Comments on Clinical Trial Design for Approval

To determine a high probability of success, some important aspects of clinical trial design include:

1. Try to select a clinical situation that closely mimics what was found in the preclinical data package. For example, if the new agent demonstrated only growth delays in an animal system, one should probably not design pivotal trials with response rate (e.g., tumor shrinkage) as a primary endpoint. Rather one should utilize median survival or progression-free survival (PFS), time to progression (TTP), or time to treatment failure (TTF) as primary endpoints. The TTP or TTF endpoints are usually acceptable to regulatory agencies only if the trial is double blinded. This is because clinicians caring for patients and patients themselves are most anxious to get off of a control arm and on to the new agent arm. This frequently will lead to a declaration that the control drug is not working in order for the patient to be crossed over to the new agent arm. Thus, double blinding is very helpful if it is at all possible. Another, more cumbersome method is when the treating clinician feels that the patient is progressing, and evidence for the progression is sent to an outside, independent, blinded review panel to determine whether or not the tumor progression has occurred.
2. Make sure the sample size is large enough to give the new agent a real chance. For example, a sample size that allows one to detect only a 50 % improvement in survival is too small of a sample size because that hurdle for any new agent is almost certainly too high and is a setup for failure. Sample size must be large enough to give the new agent a chance (e.g., a 25 % improvement).
3. It is clear that if you are expecting an agent to be used to change the upfront treatment for patients with a specific type of tumor, two well-controlled (and randomized) phase III trials will need to be performed. Normally two *well-controlled* trials do not necessarily mean they have to be randomized trials. For example, well controlled could mean a well-monitored study or a study in which patients serve as their own controls (see subheading Sect. 4.1). However, in the upfront situation, where the new agent is planned to change standard treatment, it is very likely that two *randomized* phase III trials will be a necessity. There may be one exception to the two well-controlled randomized phase III trial requirement. It might be possible to obtain approval for the new agent to be used in an "upfront" situation if the level of significance for the primary endpoint of the phase III trial is $p < 0.001$. As many experienced investigators can attest, a p-value of that magnitude is indeed unusual in most phase III trials.
4. It is frequently said that one must have an improvement in survival for a new agent to be approved. That is, of course, desirable. However, survival has not always been required. Table 1 details the new agents brought to FDA oncology advisory board for approval from January 1993 to January 2011, the type of study(ies) that led to approval, and the parameters used for approval. As can be seen in that table, there were 128 approvals and 14 disapprovals (note that some agents were brought multiple times for approval in different indications). As can also be seen in that table, there were 65 approvals based primarily on response, 27 on survival, 22 on TTP, on 25 DFS/PFS (some trials used multiple endpoints in consideration), and 14 based on other primary endpoints (incidence of hematuria, testosterone suppression, palliative response, salivary production and xerostomia score, skeletal-related events, development of cardiac events, need for transfusions, incidence and duration of mucositis, serum asparagines level depletion, and lowering of serum uric acid concentration). There were 111 based on phase III trials. One can also note from Table 1 that a variety of other endpoints have been used as primary parameters for approval (e.g., control of pleural effusion, reduction in dysplasia, requirements for transfusion, etc.). It is this investigator's personal experience that regulatory agencies will entertain endpoints other than survival if that new endpoint is discussed prospectively and in detail with the regulatory agencies.
5. As is noted above, the FDA and other world regulatory agencies have approved new agents based on response (as a surrogate marker or for benefit for the patient). The landmark publication that really codified response rate as a surrogate was the article by O'Shaughnessy and colleagues [23],

Table 1 Oncology drug considered for approval with indications and primary parameter for approval from September 1993 to November 13 2013[a]

Agent (disease)	Type of trial performed	Approval date	Primary parameter for approval
Abarelix (CRPC)	Phase III	11/25/03	Avoidance of testosterone surge and attainment and maintenance of medical castration
Abiraterone (metastatic CRPC, prior docetaxel use)	Accelerated (RCT)	4/28/11	OS
(mCRPC, no prior chemotherapy)	RCT		Radiographic PFS and overall survival (OS)
Abraxane (MBC)	Phase III	1/7/05	Target lesion RR
(adjuvant breast cancer)	Phase II	9/7/06	Disapproved
(NSCLC)	Phase III	10/11/12	ORR
(metastatic pancreatic adenocarcinoma in combination with gemcitabine)	Phase III	9/6/13	OS
Ado-transtuzumab (her2+ MBC)	RCT	2/22/2013	PFS and OS
Afatinib (metastatic NSCLC, EGFR mutated with exon 19 deletions or exon 21 (L858R) substitution mutations	open-label, randomized	7/12/2013	PFS
Alemtuzumab (B-CLL)	Accelerated (SAT)	5/7/01 (converted 9/19/07)	RR (converted for PFS)
Alitretinoin (AIDS-related Kaposi's sarcoma)	Phase II	2/2/99	Cutaneous KS tumor response rate by modified AIDS Clinical Trials Group (ACTG) response criteria
Altrasentan (CRPC)	Phase III	9/13/05	Disapproved
Anastrozole (breast cancer, second line)	Phase III	12/12/95	RR, TTP
(Breast cancer first line)	Phase III	9/1/00	RR, TTP
(ER + breast cancer adjuvant)	Accelerated (RCT)	9/5/02 (converted 9/16/05)	DFS
Arsenic trioxide (APL)	Phase II	9/25/00	CR; CR duration
Asparaginase (ALL)	–	2/1/94	–
Axitinib (second line advanced RCC)	Phase III	1/27/2012	PFS
Azacitidine (myelodysplastic syndrome)	Phase III	5/19/04	ORR, OS
Bendamustine (CLL)	Phase III	3/20/08	PFS, ORR
Bevacizumab (CRC)	Phase III	2/26/04	OS, PFS
(First-line MBC)	Accelerated (RCT)	2/22/08	PFS
(Glioblastoma)	Accelerated (SAT)	5/5/09	RR
(mCRC second line)	RCT	1/23/13	OS
Bexarotene (CTCL)	Phase II	12/29/99	Tumor response
Bicalutamide	Accelerated (RCT)	10/5/95 (converted 12/12/97)	TTP (converted for OS, TTP)
Bleomycin (malignant pleural effusions)	Phase III	6/6/96	Recurrence of effusion
Bortezomib (relapsed, refractory MM)	Accelerated (SAT)	5/13/03 (converted 3/25/05)	RR (converted for OS, TTP)
(Second-line MM)	Phase II	3/25/05	TTP, OS
(Second-line mantle cell lymphoma)	Phase II	12/8/06	ORR
(First-line MM)	Phase III	6/20/08	TTP
Bosutinib (Ph+ CML)	SAT	9/4/2012	major cytogenetic response (MCyR) at week 24 and the duration of MCyR
Brentuximab vedotin (refractory Hodgkin lymphoma)	Accelerated (SAT)	8/17/11	ORR
(Refractory systemic anaplastic large-cell lymphoma)	Accelerated (SAT)	8/17/11	ORR
Bropirimine (bladder cancer in situ)	Phase III	9/11/96	Disapproved

(continued)

Table 1 (continued)

Agent (disease)	Type of trial performed	Approval date	Primary parameter for approval
Cabazitaxel (prostate cancer)	Phase III	6/17/10	OS
Cabozantinib (medullary thyroid cancer)	RCT	11/29/12	PFS
Capecitabine (second-line MBC)	Phase II	4/30/98	RR
(First-line MBC, combination)	Phase III	4/30/01	DFS, OS, objective RR
(First-line CRC)	Phase III	4/30/01	Survival effect
(Adjuvant colon cancer)	Phase III	6/15/05	DFS
Carfilzomib (third line, multiple myeloma)	SAT	7/20/12	ORR
Carmustine	–	(3/7/77)	–
Carmustine wafer (malignant glioma, recurrent surgery)	Phase III	9/23/96	6-month survival
(Malignant glioma undergoing primary surgical resection)	Phase III	2/25/03	OS
Cetuximab (EGFR-expressing mCRC)	Accelerated (SAT)	2/12/04 (converted 10/02/07)	Objective RR (converted for OS)
Cisplatin-epinephrine gel (HNSCC)	Phase III	4/15/01	–
Cladribine (hairy cell leukemia)	Phase II	2/26/93	CR rate and duration
Clofarabine (ALL)	Phase II	12/28/04	Induction of CR
Crizotinib (locally advanced or metastatic ALK + NSCLC)	Accelerated (SAT)	8/26/11	ORR
Liposomal cytarabine (neoplastic meningitis)	Accelerated (RCT)	4/1/99 (converted 4/19/07)	CR rate (converted for RR)
Cytarabine foam	Phase II	12/18/97	Disapproved
Dabrafenib (unresectable or metastatic melanoma with B RAF V600E mutation)	RCT	5/29/13	PFS
Dasatinib (refractory CML, Ph + ALL)	Accelerated (SAT)	6/28/06 (converted 5/21/09)	Hematologic and cytogenetic response rates (converted for RR)
(CML newly diagnosed)	Phase III	10/28/10	Rate of confirmed complete cytogenetic response
Liposomal daunorubicin (KS)	Phase III	4/8/96	RR,TTP, and cosmesis
Decitabine (MDS)	Phase II and III	5/2/06	ORR
Degarelix (advanced prostate cancer)	Phase III	12/24/08	Testosterone suppression to castrate level
Denileukin diftitox (CTCL)	Accelerated (SAT)	2/5/99 (converted 10/15/08)	RR (converted for RR)
Docetaxel (breast cancer)	Phase II	12/18/94	Disapproved
(second-line breast cancer)	Accelerated (SAT)	5/14/96 (converted 6/22/98)	RR (converted for OS, TTP)
(Metastatic NSCLC second line)	Phase II and III	12/23/99	OS
(Metastatic NSCLC first line)	Phase III	11/27/02	OS (non-inferior to standard)
(mCRPC)	Phase III	5/19/04	OS
(Adjuvant node + breast cancer)	Phase III	8/18/04	DFS
(Gastric and GEJ cancer)	Phase III	10/17/06	OS
(Locally advanced HNSCC)	Phase III	9/28/07	PFS
Doxorubicin (adjuvant breast cancer)	Meta-analyses	5/8/03	DFS, OS
Liposomal doxorubicin(KS)	Accelerated (SAT)	11/17/95 (converted 6/10/08)	RR (converted for RR)
(ovarian cancer second line)	Accelerated (SAT)	6/28/99 (converted 1/28/05)	RR (converted for TTP)
(MM)	Phase III	5/17/07	TTP
(Breast)	Phase III	9/16/99	Disapproved
Enzalutamide (metastatic CRPC)	Phase III	8/31/12	OS
Epirubicin (adjuvant breast cancer)	Phase III	9/15/99	Relapse-free survival and OS
Eribulin mesylate (breast cancer)	Phase III	11/15/10	OS
Erlotinib hydrochloride (NSCLC)	Phase III	11/18/04	OS
(Metastatic pancreatic cancer, in combination)	Phase III	11/2/05	OS
(Maintenance NSCLC)	Phase III	4/6/10	PFS

(continued)

Table 1 (continued)

Agent (disease)	Type of trial performed	Approval date	Primary parameter for approval
Everolimus (advanced RCC)	Phase III	3/30/09	PFS
(Subependymal giant cell astrocytoma)	Phase II	10/29/10	Change in SEGA lesion
(Advanced pancreatic neuroendocrine tumors)	Phase III	5/5/11	PFS
(Hormone receptor positive and Her 2 negative metastatic breast cancer)	Phase III	7/20/12	PFS
(tuberous sclerosis complex (TSC) who have subependymal giant cell astrocytoma)	Phase III	8/29/12	Radiologic RR
Exemestane (breast cancer second line)	Phase III	10/21/99	RR and TTP
(ER + breast cancer adjuvant after tamoxifen)	Phase III	10/5/05	DFS
Fludarabine (Oral; B-cell CLL post-alkylating regimen)	Accelerated (SAT)	12/18/08	RR
Fulvestrant (ER + breast cancer)	Phase III	4/25/02	RR and TTP
Gefitinib (second-line NSCLC)	Accelerated (SAT)	5/5/03 (Not converted)	RR (failure to convert; restricted access)
Gemcitabine (pancreatic cancer)	Phase III	5/15/96	Clinical benefit response
(first-line NSCLC)	Phase III	8/25/98	OS, TTP, RR
(MBC)	Phase III	5/19/04	TTP, objective RR
(Ovarian cancer)	Phase III	7/14/06	PFS, ORR
Gemtuzumab ozogamicin (CD33 + AML)	Accelerated (SAT)	5/17/00 (not converted)	RR (failure to convert)
Oblimersen (melanoma)	Phase III	5/3/04	Disapproved
(Relapsed refractory CLL)	Phase III	9/6/06	Disapproved
Histamine dihydrochloride (melanoma)	Phase III	12/13/00	Disapproved
Ibritumomab (relapsed/refractory low-grade or follicular lymphoma)	Accelerated (RCT)	2/19/02 (Converted 9/3/09)	RR (converted for PFS)
Ibrutinib (second line mantle cell lymphoma)	Accelerated (single arm phase II)	11/13/13	ORR
Imatinib mesylate (refractory Ph + CML)	Accelerated (SAT)	5/10/01 (converted 12/8/03)	RR (converted for PFS)
(KIT + GIST)	Accelerated (SAT)	2/1/02 (converted 5/27/09)	DFS, RR (converted for PFS)
(first-line chronic phase CML)	Accelerated (RCT)	12/20/02 (converted 9/26/08)	PFS (converted for PFS)
(Adjuvant GIST)	Accelerated (RCT)	12/19/08	DFS
Ipilimumab (unresectable or metastatic melanoma)	Phase III	3/25/11	OS
Irinotecan (5-FU refractory mCRC)	Accelerated (SAT)	6/14/96 (converted 10/22/98)	RR (converted for survival)
(first-line mCRC)	Phase III	4/20/00	RR, TTP
Ixabepilone (breast cancer)	Phase III	10/16/07	TTP
Lapatinib (MBC with capecitabine)	Phase III	3/13/07	TTP
(MBC, with letrozole)	Accelerated (RCT)	1/29/10	PFS
Lenalidomide (myelodysplastic syndrome)	Phase II	12/27/05	Duration of transfusion independence
(Second-line MM)	Phase III	6/29/06	TTP
(Mantle Cell Lymphoma)	Phase III	6/5/13	ORR and duration of response
Letrozole (second-line breast cancer)	Phase III	7/25/97	Objective tumor response
(First-line breast cancer)	Phase III	1/10/01	TTP, objective tumor response
(Adjuvant breast cancer post-tamoxifen)	Accelerated (RCT)	10/29/04 (converted for 4/30/10)	DFS (converted for DFS)
(Adjuvant breast cancer)	Accelerated (RCT)	12/28/05 (converted 3/12/10)	DFS (converted for DFS)
Leuprolide depot (advanced prostate cancer)	Phase II	1/23/02	Testosterone suppression
Liarozole (prostate cancer)	Phase III	6/24/97	Disapproved
Mifamurtide (non-metastatic osteosarcoma)	Phase III	5/9/07	Disapproved

(continued)

Table 1 (continued)

Agent (disease)	Type of trial performed	Approval date	Primary parameter for approval
Mitoguazone (AIDS-related lymphoma)	Phase II	6/23/97	Disapproved
Mitoxantrone (CRPC)	Phase III	11/13/96	TTP
Nelarabine (T-ALL)	Accelerated (SAT)	10/28/05	Induction of CR
Nilotinib (refractory CML)	Accelerated (SAT)	10/29/07	RR
(Newly diagnosed Ph + CML)	Accelerated (RCT)	6/17/10	RR
Obinutuzumab (in combination with chlorambucil for previously untreated CLL)	Randomized open label	11/1/13	PFS
Ofatumumab (CLL)	Accelerated (SAT)	10/26/09	Objective RR
Omacetaxine mepesuccinate (chronic or accelerated CML)	Accelerated (2 SATs)	10/26/12	Major cytogenetic response (MCyR) and Major Hematologic Response (MaHR)
Satraplatin (CRPC)	Phase III	7/7/07	Disapproved
Oxaliplatin (second-line advanced CRC)	Accelerated (RCT)	8/9/02 (converted 1/9/04)	RR (converted For OS, TTP)
(First-line mCRC)	Phase III	1/9/04	OS, TTP
(Adjuvant CRC)	Phase III	11/4/04	3-year-DFS
Paclitaxel (second-line ovarian)	Phase III	12/29/92	ORR
(MBC)	Phase II	4/9/98	RR, TTP, OS
(First-line ovarian with cisplatin)	Phase III	4/9/98	RR, TTP, and OS
(First-line NSCLC with cisplatin)	Phase III	6/30/98	RR, TTP, and OS
(Adjuvant breast cancer)	Phase III	10/25/99	DFS, OS
Panitumumab (second-line EGFR-expressing colorectal cancer)	Accelerated (RCT)	9/27/06	PFS
Pazopanib (advanced RCC)	Phase III	10/19/09	PFS, RR
(advanced soft tissue sarcoma)	Phase III	4/26/12	PFS
Pegaspargase (ALL)	Phase III	2/1/94	Achievement of asparagine depletion to ≤ 1 μM
Pemetrexed (malignant pleural mesothelioma)	Phase III	2/4/04	Objective RR
(second-line metastatic NSCLC)	Accelerated (RCT)	8/19/04 (converted 7/2/09)	RR (converted for OS, PFS)
(first-line metastatic non-squamous NSCLC)	Accelerated (RCT)	9/26/08 (converted 7/2/09)	Based on non-squamous histology as ad hoc analysis (converted for OS, PFS)
Pertuzumab (first line Her 2+ metastatic breast cancer)	Phase III	6/8/12	PFS
(neoadjuvant therapy for Her 2+ breast cancer in combitantion with trastuzumab and docetaxel)	Accelerated (open label, randomized)	9/30/13	pathological complete response (pCR) rate defined as the absence of invasive cancer in the breast (ypT0/is)
Pomalidomide (relapsed refractory MM)	Open label RCT	2/8/13	ORR
Ponatinib (Ph+ CML and ALL)	Single arm study	12/14/12	Major Cytogenetic Response (MCyR) for patients with CP-CML and Major Hematologic Response (MaHR) for patients with AP-CML, BP-CML or Ph+ALL.
Porfimer sodium (NSCLC palliation of obstruction)	Phase III	1/9/98	ORR
Pralatrexate (refractory T-cell lymphoma)	Accelerated (SAT)	9/24/09	ORR
Regorafenib (Previously treated metastatic CRC)	Phase III	9/27/12	OS and PFS
(GIST)	Phase III	2/25/13	PFS

(continued)

Table 1 (continued)

Agent (disease)	Type of trial performed	Approval date	Primary parameter for approval
Rituximab (relapsed, refractory CD20+ B-cell NHL)	Phase II	11/26/97	ORR
(CLL)	Phase III	2/18/10	PFS
(Single, maintenance CD 20+ NHL)	Phase III	1/28//11	PFS
Romidepsin (cutaneous T-cell lymphoma)	Phase II	11/5/09	ORR
Ruxolitinib (intermediate and high risk myelofibrosis	Phase III (2 studies)	11/16/11	Proportion of patients with ≥35% reduction in spleen volume (by CAT scan or MRI) after 24 weeks (Study 1) or after 48 weeks of treatment (Study 2)
Satraplatin (CRPC)	Phase III	7/7/07	Disapproved
Sipuleucel-T (prostate cancer)[b] (vaccine)	Phase III	4/29/10	OS
Sorafenib tosylate (RCC)	Phase II and III	12/20/05	PFS
(Unresectable HCC)	Phase III	11/16/07	TTP
Sunitinib malate (GIST)	Phase III	1/26/06	TTP
(First-line advanced RCC)	Phase III	2/2/07	ORR
(Second-line advanced RCC)	Accelerated (SAT)	1/26/06 (converted 2/2/07)	RR (converted for PFS)
(Pancreatic neuroendocrine tumor)	Phase III	5/20/11	PFS
Tegafur and uracil (UFT)	Phase III	9/16/99	Disapproved
Temozolomide (refractory anaplastic astrocytoma)	Accelerated (SAT)	8/11/99 (converted 3/15/05)	RR (converted for survival)
(Melanoma)	Phase III	3/23/90	Disapproved
Temsirolimus (RCC)	Phase III	5/30/07	OS, TTP
Thalidomide (first-line MM)	Accelerated (RCT)	5/25/06	RR
Tipifarnib (first-line elderly AML)	Phase II	5/5/05	Disapproved
Tivozanib (renal cell carcinoma)	Phase II	5/2/13	Disapproved
Toremifene citrate (metastatic breast cancer)	Phase II	5/29/97	RR
Tositumomab (CD20-positive, follicular NHL)	Accelerated (SAT)	6/27/03	Durable objective response
Trametinib (unresectable or metastatic melanoma)	RCT	5/29/13	PFS
Trastuzumab (Her-2+ breast cancer)	Phase II	9/25/98	TTP, 1 year survival, ORR
Valrubicin (bladder cancer)	Phase II	6/11/98	Disapproved
Vandetanib (medullary thyroid cancer)	Phase III	4/6/11	PFS
Vectibix (CRC)	Phase III	9/27/06	PFS
Vemurafenib (unresectable or metastatic melanoma)	Phase III	8/17/11	OS, PFS
Vincristine sulfate liposome injection (Ph negative ALL)	SAT	8/8/12	Rate of complete remission (CR) plus the rate of complete remission with incomplete blood count recovery (CRi)
Vinorelbine (unresectable NSCLC)	Phase III	12/23/94	ORR
(breast)	Phase III	6/7/94	Disapproved
Vismodegib (basal cell carcinoma)	SAT	1/30/12	ORR
Vorinostat (cutaneous T-cell leukemia)	Phase II	10/6/06	Objective RR
Vorozole (breast)	Phase III	9/15/97	Disapproved
Ziv-aflibercept (second line mCRC; in combination with FOLFIRI)	Phase III	8/3/12	OS

The source for much of this information is http://www.fda.gov/Drugs/DevelopmentApprovalProcess/HowDrugsareDevelopedandApproved/DrugandBiologicApprovalReports/ucm121136.htm, Johnson et al. [22]

ALK anaplastic lymphoma kinase, *ALL* acute lymphocytic leukemia, *APL* acute promyelocytic leukemia, *CLL* chronic lymphocytic leukemia, *CML* chronic myeloid leukemia, *CR* complete response, *CRF* chronic renal failure, *CRPC* castration-resistant prostate cancer, *CTCL* cutaneous T-cell leukemia, *DB-RCT* double-blind randomized control trial, *DFS* disease-free survival, *GEJ* gastroesophageal junction, *GIST* gastrointestinal stromal tumor, *GVHD* graft-versus-host disease, *HCC* hepatocellular carcinoma, *HNSCC* head and neck squamous cell cancer, *ITP* idiopathic thrombocytopenic purpura, *KS* Kaposi's sarcoma, *MBC* metastatic breast cancer, *mCRC* metastatic colorectal cancer, *MM* multiple myeloma, *NHL* non-Hodgkin's lymphoma, *NSCLC* non-small-cell lung cancer, *ORR* overall response rate, *OS* overall survival, *RCC* renal cell cancer, *RCT* randomized controlled trial, *RR* response rate, *RT* radiation therapy, *SAT* single-arm trial, *TTP* time to progression

[a]Note: This table does not include chemoprotective and other supportive care agents. This table does not include all pediatric indications

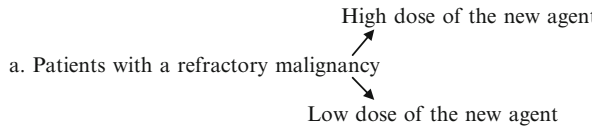
[b]Immunotherapy

in which the general guidelines were put forth for approval based on phase II results. There are many FDA observers who feel that response is no longer an approvable strategy, but Table 1 [24–26] does document that it still can be a strategy for approval under the right circumstances, including:

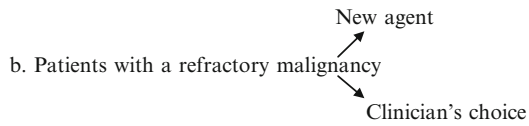
(a) A very high response rate or a substantial/complete response rate (where the responses are durable) which is something unexpected for a new agent. The best example of this is the high response rates noted with arsenic trioxide for patients with refractory acute promyelocytic leukemia and multiple others in many hematological malignancies.
(b) A lower response rate but a low incidence of side effects. An excellent example of this is the phase II experience with Herceptin for patients with refractory breast cancer (with response rate of 11 % but with no significant side effects).

A major area of controversy is the use of accelerated approval process [27–29]. The major issue is when an agent is approved by FDA under the accelerated approval mechanism and the approval is conditioned upon completion of a more definitive randomized trial, how often is that followed, and does that original approval stand? The good news is that the bulk of evidence indicates that accelerated approval based on nonrandomized trials generally is certified as safe and effective in more definitive studies [28, 29].

If you plan to use a phase II strategy for approval, in general it is better to utilize a phase II trial design with a reference arm. Otherwise there is a concern about patient selection (e.g., selection of long-term survivors regardless of treatment). Two possible strategies to give most reviewers confidence that is indeed your new agent that is making a difference include trial designs such as:



Endpoint: Response rate or TTP (if arms are blinded).



Endpoint: Response rate as it is more difficult to blind the trial.

(Note: This could be a 2:1 randomized of new agent vs. clinician's choice, but the design actually does require more patients even though many trials claim this is an incentive for a patient to participate in the clinical trial.)

One very recently approved new agent is the halichondrin derivative eribulin [30]. In the very well-designed trial, patients with 762 metastatic breast cancer refractory to at least two prior therapies were randomized to receive either eribulin or clinician's choice of best supportive care or a different single-agent therapy chosen by their oncologist. The median overall survival for patients receiving eribulin was 13.12 months compared with 10.65 months for those who received clinician's choice (hazard ratio, 0.81; $p = 0.041$). The 1-year survival rate was 53.9 % for eribulin-treated patients and 43.7 % for those given other drugs.

4 Special Trial Designs

4.1 Patients as Their Own Controls

This is a trial design that, until, recently as all but forgotten. There are at least two versions of this trial design, detailed in Fig. 1. As can be seen in Fig. 1, version 1, any patient who has a longer time on treatment on regimen B than on regimen A is considered a positive result (it is usually not an expected result for a patient to remain on treatment with a second-line regimen for a longer time than on a first-line regimen). This is certainly an inexact situation, as time on treatment is not the same as time to progression, but it is easier to measure when one does not have scans and X-ray films at regular intervals for the first regimen, as one usually has for the second regimen. Even though this is an inexact clinical trial situation, this trial design might offer some insight on whether or not the agent is having an effect on the natural history of the patient's disease. Figure 2 details how the data for such a comparison can be plotted. Based on the past experience [31], if ≥30 % of patients have a longer time on the new agent than on the regimen they received just prior to the new agent, then that is a promising result that should be pursued.

Version 2 of patients as their own control is also detailed in Fig. 1. To try this version of patients as their own controls, one must have some preclinical information demonstrating that there is some

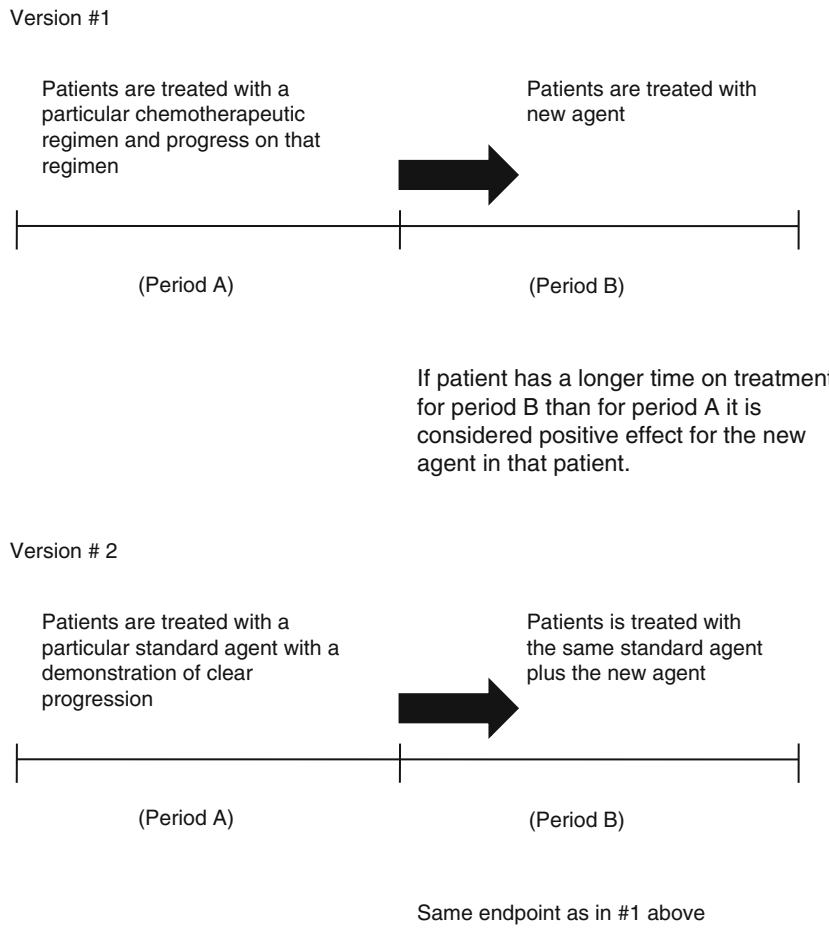


Fig. 1 Two versions of patients as their own control type of trial design [reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004.]

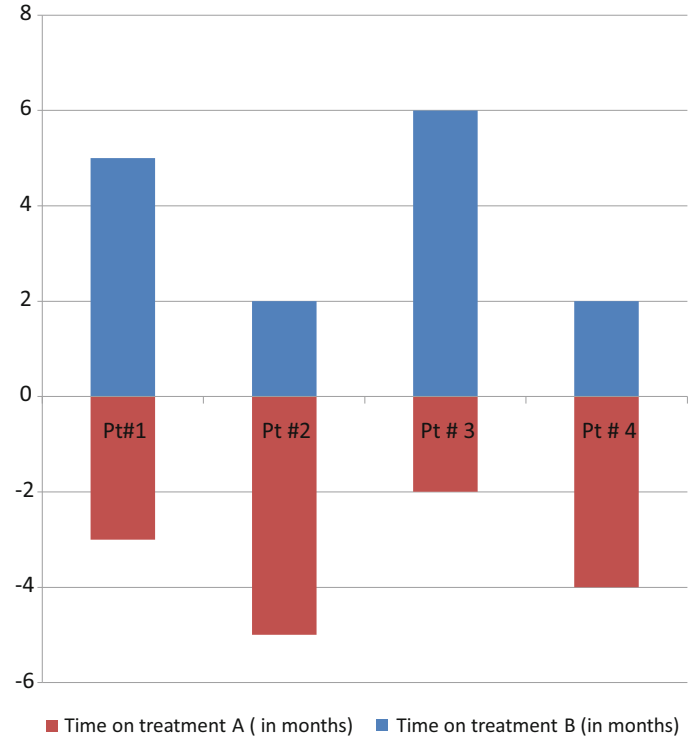


Fig. 2 Suggested manner for plotting time on treatment for period B versus time on treatment for period A. As an example, patient 1 was on treatment B for 5 months versus on treatment A for 3 months (which is a positive result). Patient 2 was on new agent B for 2 months but on the prior regimen A for 5 months—a negative result

synergy between the new cytostatic agent and the agent the patient is currently receiving. It is also critical in this design to make *very certain* that the patient is progressing on regimen A (best ascertained by an independent committee). Once again, the endpoint is the same as it is for version 1 and the data can also be plotted as noted in Fig. 2. It needs to be emphasized again that this type of trial is only an *exploratory trial*—but a trial that may again give hints of the agent changing the natural history of the disease. This trial design was utilized for an initial indication of the activity of anti-EGFR monoclonal antibody cetuximab in combination with irinotecan [32].

Of note in this patient as their own control has recently been used to examine the utility of molecular profiling for treating patients with refractory cancer [33]. The pros and cons of such an approach have been discussed by Dr. Doroshow [34]. The reader is also referred to an excellent analysis by Mick and colleagues [35] on the sequentially measured paired failure time trial design. That analysis details the important statistical considerations when one is evaluating the patients as their own controls approach.

4.2 The Randomized Phase II Trial

This type of trial design was mentioned above. With some clever additional variations, it can yield a great deal of information. Figure 3 details perhaps one very early and quite definite interesting randomized phase II trial, done by DeVore and colleagues [36] with a cytostatic agent. As noted in Fig. 3, the study was a three-arm study of chemotherapy versus chemotherapy plus a low dose of a mAb to VEGF versus chemotherapy plus a high dose of a mAb to VEGF.

As can be seen in Fig. 3, one of the endpoints for the study, in addition to toxicities, was the TTP. Once again, TTP can be a somewhat inexact endpoint and one that is not usually acceptable to a regulatory agency (except if the arms of the study are blinded—and this study was not). However, the above study design can provide information as to what sample sizes may be needed for a new drug

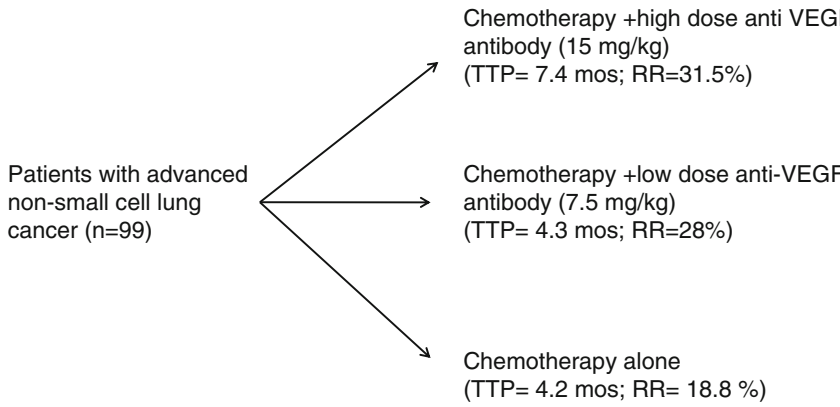


Fig. 3 Randomized phase II trial of a monoclonal antibody to VEGF [reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004.]

application (NDA)-directed study. Such a study design can also provide information as to whether patients will participate in such a study, accrual rates, and so forth. Such a study design also yields valuable information on the safety of the various arms of the study.

An important option for the study outlined in Fig. 3 is to continue with the study in a randomized fashion, selecting only one of the MAb-containing arms for comparison with the chemotherapy-alone arm. This approach can save significant activation time for a new protocol (e.g., continue the randomized phase II trial and power it up to be large enough for a phase III trial rather than writing and activating a whole new phase III trial).

Another randomized phase II trial that yielded very important information and which serves as an excellent model for solving drug development issues is a trial performed with the agent capecitabine. After results became available from the phase I clinical trials with several different schedules of the agent, there was uncertainty as to just which schedule was the best. Therefore, a randomized phase II trial was conducted to determine which schedule (and dose) of capecitabine would be best to take into expanded phase II and phase III clinical trials [37]. Patients were randomized to receive either (a) 1,331 mg/m^2 per day continually, (b) 2,510 mg/m^2 per day intermittently, or (c) 1,657 mg/m^2 per day plus leucovorin 60 mg/day p.o. intermittently. The specific aims were to evaluate the safety and efficacy of each schedule. Cleverly, one of the efficacy endpoints utilized (in addition to response rate) was TTP (in days). Utilizing TTP as a parameter of efficacy allowed a fine tuning because it allowed for a continuous assessment (in days) versus the dichotomous variable of response (response or no response). This clever randomized phase II design showed that schedule "b" was the best schedule in terms of toxicities and efficacy. That schedule was then taken on into successful phase II and phase III trials, which led to the very rapid approval of capecitabine.

4.3 Randomized Discontinuation Trial Designs

This is a unique trial design for the development of cytostatic agents that have several very desirable features [38–40], one of which was the original documentation of the activity of sorafenib. The trial design is as outlined in Fig. 4. The design is particularly well suited for a new cytostatic agent that patients want to receive (i.e., endostatin). As noted in Fig. 4, all eligible patients initially received the new agent. Those who progress before 4 months of treatment are completed are removed from the study. Those patients who do have a response continue therapy and those who have stable disease for 4 months are then randomized to continue the therapy or receive a placebo. The patients are carefully

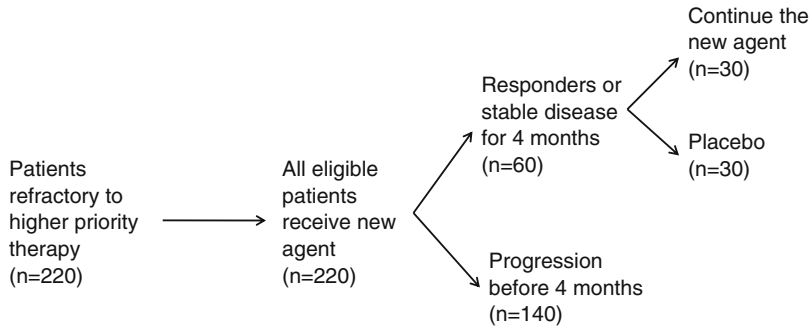


Fig. 4 Randomized discontinued design. Note: some versions of this design have all of the responders continue to receive the study drug (e.g., they are not randomized to continue the agent vs. to receive a placebo) [reproduced from Ref. W. D. Figg and H. L. McLeod. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Humana Press (edition 1), Totowa, N., 2004.]

observed, and if they have progressive disease (and are receiving placebo), they are placed back on the new agent. The endpoint for the study is the TTP for patients who continue on the therapy versus the TTP for patients who receive placebo.

The randomized discontinuation trial design has been used for testing new agents against the AIDS virus, but it is more commonly being used to evaluate new anticancer agents. Obviously when used in the situation with a new AIDS drug(s), one has viral titers to follow as compared to imaging techniques (e.g., CT scans) for oncologists to follow a patient's tumor. Viral titers are more sensitive than scans are. Also, some investigators question the ethics of randomizing patients who are responding to the new agent to continue or discontinue that therapy. This problem can be addressed by randomizing only the patients with stable disease (and not the responders). Very carefully administered informed consent is obviously a necessity. One other potential problem with the randomized discontinuation design is that there is a theoretical problem in comparing the patients continued on therapy versus those on placebo if there is a carryover effect of the agent.

Recently, there has been an interesting variation in the randomized discontinuation trial that has been proposed by Galsky and colleagues (see Fig. 5) [41]. In the target-specific, histology nonspecific, randomized discontinuation study design, during stage 0, patients with a diverse range of solid tumors are screened for the presence of target X. All eligible patients expressing target X are subsequently enrolled and treated with drug Y during stage 1. After 12 weeks of treatment, patients achieving an objective response continue treatment, patients with disease progression are discontinued for treatment, and patients with stable disease are randomized to continue treatment with drug Y versus placebo (stage 2). Patients subsequently progressing on placebo are offered crossover to open-label treatment with drug Y.

Regardless of its downsides, the randomized discontinuation trial design is one that should be considered for a new cytostatic agent. It does allow for a greater number of patients to have access to a potentially exciting new agent.

5 Unique Endpoints for Approval

5.1 Clinical Benefit

In the early development of the chain terminator gemcitabine, there were some patients with pancreatic cancer who demonstrated a decrease in their tumor-related pain and an increase in their appetite and

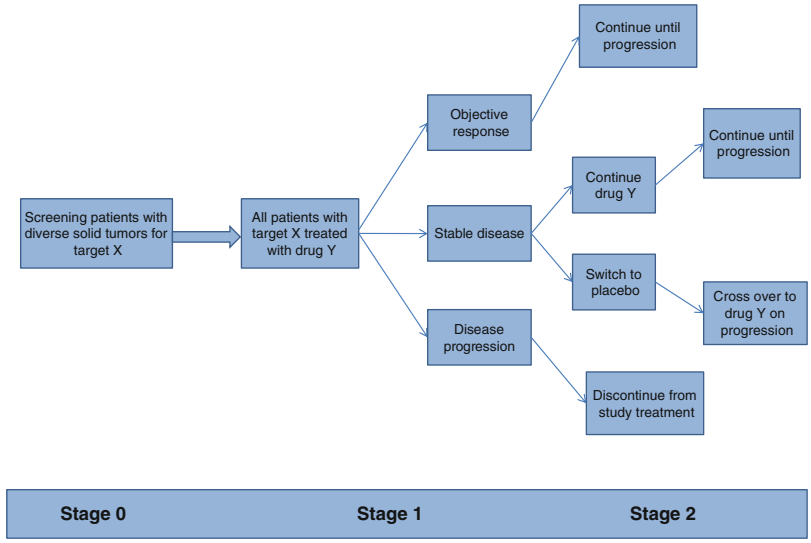
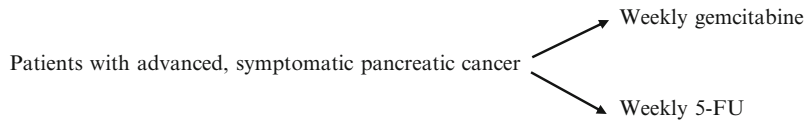


Fig. 5 Target-specific, histology nonspecific, randomized discontinuation trial design (Modified from [41] with permission)

weight [42]. Gemcitabine had a new mechanism of action. It did not cause regression of pancreatic cancer growing in nude mice but rather it caused a slowing of growth of the pancreatic cancer xenografts growing in nude mice (MIA PaCa, PANC-1, and PaCa-2) [43, 44]. Therefore, it was likely that one would not see a complete or partial response in patients. In conversations with Dr. Bob Temple at the FDA and Dr. Gregory Burke, our team was alerted to the fact that one endpoint they could accept in a trial was "fixing what bothers the patient." The term *clinical benefit* was derived from that conversation. Clinical benefit was not necessarily a quality of life parameter but it was an attempt to measure "fixing what bothers the patient." Because the three most common problems experienced by patients with pancreatic cancer included pain, weight loss, and deterioration in performance status, Dr. John Anderson at Eli Lilly devised an algorithm to measure clinical benefit [45]. This algorithm utilized pain (measured by the Memorial Pain Assessment Card) performance status (measured by the Karnofsky scale because it had a broader range of 0–100 in increments of 10 rather than the ECOG or SWOG scales which have a range of only 0–5) and a direct measurement of weight (with clear-cut definitions of what constituted weight gain or weight loss) [45]. The pivotal trial design for gemcitabine was as follows:



The primary endpoint for the study was an improvement in clinical benefit, with the secondary endpoints including response rate, median survival, and percentage of patients alive at 1 year [46]. Table 2 details the results of the study.

As can be seen in Table 2, the study was positive for clinical benefit as well as for the other parameters. Gemcitabine was approved for use for treatment of patients with locally advanced or metastatic pancreatic cancer by the Oncologic Drug Advisory Committee (ODAC) and by the FDA on the basis of this one study. In addition, there was a phase II trial that was uncontrolled but demonstrated a similar survival (and response) to that found in the randomized phase III study [47]. Gemcitabine was approved for treatment of patients with locally advanced or metastatic pancreatic cancer. Many observers who were present at the ODAC felt that gemcitabine would not have been approved if

Table 2 Results of randomized trial of gemcitabine versus 5-FU

Parameter	5-FU arm ($n=63$)	Gemcitabine arm ($n=63$)	p-value
Clinical benefit (% of patients)	4.8 %	23.8 %	0.0022
Response rate	0 %	5.4 %	
Time to progression (mo)	1.0	3.2	0.0002
Survival			
Median (months)	4.41	5.65	0.0025
1 year	2 %	18 %	

Data extracted from [46]

clinical benefit were the only parameter that was improved by gemcitabine. The other item of note is that gemcitabine was approved for frontline treatment of patients with advanced pancreatic cancer based on only one randomized trial. Observers at the ODAC felt that was because there were very few options for patients (and no prior controlled trials ever demonstrated an improvement in survival for any single agent) with advanced pancreatic cancer that gemcitabine was approved.

It is of note that no other attempts have been made to bring a new agent to the FDA using the clinical benefit parameter as the primary endpoint of the study. However, this investigator believes that with the proper algorithm it could be a solid primary endpoint for other pivotal trials with a new agent.

5.2 Puncture-Free Survival

The newest endpoint that has led to a European approval of a new agent is puncture-free survival (a new "PFS"). The agent studied was catumaxomab, a trifunctional bi-specific monoclonal antibody which has tumor-binding specificity to an epithelial cell adhesion molecule (EpCAM) and to the T-cell antigen CD3. Because it has an Fc fragment, catumaxomab additionally binds accessory cells including dendritic cells, macrophages, and natural killer cells. The drug was tested in patients with solid carcinoma (EpCAM-positive epithelial tumors) with refractory ascites with the agent instilled in the abdomen. The endpoint was the very innovative time between necessity for the withdrawal of malignant ascites performed for discomfort. The patients received either paracentesis plus catumaxomab or paracentesis alone. Patients treated with catumaxomab had a puncture-free survival of 46 days compared with 11 days in the control group ($p<0.0001$) [48]. The endpoint of puncture-free survival is innovative and highly patient oriented.

6 Special Challenges in Clinical Trial Designs for Approval

6.1 Analogs

Unfortunately in anticancer drug development, we are still in the sulfonamide era—meaning that it is probably more productive to find agents with new mechanisms of action (for greater progress) than to work on analogs. However, there have been many commercial successes with analogs, largely based on less (or different) toxicities rather than on improved efficacy [49–52]. The types of trials for approval for an analog program could be:

a. Patients whose tumors are progressing on the parent compound (with very clear documentation of that progression) → Patient is treated with the analog.

Endpoint: Response rate

Issues with this design include a very refractory patient population. However, if the analog has activity in that setting, it will certainly have a substantial chance for approval [53].

b. Treat patients with the new analog who have a tumor type that is not responsive to the parent compound → Patient is treated with the analog

Endpoint: Response rate

The issue here is that it is unlikely the analog will work in this situation. However, if it does work in this situation, it also will have an excellent chance for eventual approval.

c. Patients with a disease usually responsive to the parent compound → New analog / Parent compound

Endpoints: Survival, response rate, TTP, and toxicities

This is the best way to evaluate a new analog and the most likely way for an analog to be approved by regulatory agencies. Of course, superiority in one of the endpoints (not equivalence) is usually more convincing for approval.

7 A Personalized Medicine Approach to Approval

Drug development is clearly changing and the new challenge for drug development will be to make sure the right drug is made available to the patient who has the right target on signature to assure the patient has a maximum chance to respond. This is very critical and the technology to make that happen is clearly becoming available [33, 34, 54]. In order to make this happen, we will have to make sure patients' tumors are measured for the appropriate target (e.g., B-Raf, ALK, EGFR, c-kit, etc.). We propose that there are clearinghouses put in place so a tumor can be sent to a specific place and sent out for all of the appropriate testing. If an investigational agent is indicated a new methodology called "just-in-time" [53]. Utilizing that methodology, once a patient is identified with the specific genetic signature making them likely to respond to a new agent, the new agent can be given to the particular patient in the physician's practice if the physician's site is opened within a few days ('just in time').

8 Other Comments on Clinical Trials for Approval

Given the difficulty of treating patients with cancer, the present authors (as do many others in the field) believe that we should do everything we can to gain approval for new agents so patients have options. There are frequently numerous criticisms passing back and forth between investigators, regulators, educators, survivors, and others. At times their criticisms are valid—that perhaps we are asking for so much proof that an agent works (e.g., an improvement in survival) that it is discouraging to all involved and actually dampens any enthusiasm for development of new agents. It is our belief that the more these different constituencies communicate and work together (without assigning blame), the better chance we will have to develop innovative endpoints and trial designs for more rapid approval. Our job, together, is to obtain more options for clinical trial designs that allow development of new agents that work for our patients.

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Clinical Pharmacogenetics

Kamakshi Sachidanandam and Jill M. Kolesar

Abstract Pharmacogenetics in clinical medicine is an emerging field that has seen significant progress in the past decade, with the implementation of new clinical guidelines recommending routine testing of specific genes that have good predictive potential. This chapter describes the current understanding of clinical pharmacogenetics, some of the problems and challenges faced in development and execution, and the key requirements in order to make it an integral part of translational research. This section also lists the valid biomarkers associated with drugs used to treat solid tumors and hematological malignancies and describes their use in clinical practice. It illustrates the clinical impact of genetic polymorphisms on drug disposition and of drug target genes on the pharmacotherapy of patients with these conditions. Finally, this section outlines pharmacogenetic strategies for drug selection that aid in therapeutic optimization.

Keywords Targeted therapy • Biomarkers • EGFR KRAS • EML4-ALK

1 Introduction

Important advances in molecular biology, genetics, and biotechnology culminating in the human genome project bring us today to "genetic medicine" of which pharmacogenetics is an integral part. With the advance of genetic medicine into everyday practice, the importance of understanding and appropriately using pharmacogenetic information and communicating that information to patients is a priority. The main goal of pharmacogenetics in cancer therapy is to improve the efficacy of anticancer drugs by elucidating the impact and mechanisms of genetic polymorphisms that affect the pharmacokinetics and pharmacodynamics of these agents in each individual patient [1].

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2 Challenges in Translating Pharmacogenetics Research to Clinical Practice

The role of genetics in predicting and diagnosing disease is well established, while the field of clinical pharmacogenetics is still emerging. In most cases, the inherited disease predisposition genes are important in diagnosing a disease and as a potential target for gene therapy. However, a disease predisposition gene usually does not influence how an individual responds to a given medication.

Oncology is unique in two respects: first that there are both inherited genetic traits, such as the thiopurine methyltranferase (TPMT) deficiency associated with drug response and acquired genetic traits or mutations such as p53 mutations that influence response to therapy, and second, the disease-causing genetic traits and the drug response traits are exceedingly difficult to separate. In many cases, such as that with imatinib, or Gleevec, the genetic mutation that causes the disease is used to diagnose the disease and predicts who will respond to the therapy. Therefore the genetics of the disease as well as the therapy must be considered in the clinical use of pharmacogenetic information and in pharmacogenetic counseling.

Another challenge to translational research is the limited understanding of tumor biology. In spite of substantial progress in basic research in identifying potential targets for drug development, very few of these targets effectively translate to clinical practice. Most tumors are genetically heterogeneous and are comprised of multiple cell populations; thus many abnormally expressed or mutated genes may not be representative therapeutically because they may be specific to only a subset of the tumor cells [1].

3 Requirements to Translate to Clinical Practice

3.1 Defining Key Therapeutic Targets: Candidate Gene Versus Genome-Wide Approach

An obstacle to progress in translational research is the identification of the key molecular targets. Most tumors are genetically heterogeneous and many abnormally expressed or mutated genes may not represent good therapeutic targets because they are characteristic of only a subset of the tumor cells. Targets must be identified such that the treatment specifically inhibits the protein product of an oncogenic mutation, if delivered early enough, and be effective against all tumor cells, and nontoxic to normal cells [1]. Conventional strategies used for the identification of new targets mostly involved the candidate gene or single-nucleotide polymorphism approach that focused on determining whether there were differences between the case and the control groups in regard to a particular gene, gene variant, or a small group of genes. However, prior knowledge of the functionality of a genetic polymorphism is not always present or may not be reliable. Further, this approach focuses solely on variability in a specific point in the whole gene sequence, and more often than not, polymorphisms exist in multiple genes than in a single gene. The new approach for identifying potential targets involves whole genome association studies (WGAS). This technology enables us to study gene expression of thousands of genes in each sample, by means of a microarray assay. This has been possible due to the tremendous increase in overall knowledge of the human genome and genetic polymorphisms. Appropriate high-throughput analysis tools are required to collate the results of such complex pharmacogenetic studies and almost always require collaboration with statisticians to interpret the data [2].

3.2 Biomarker Development

Biomarkers are biological measurements that are reflective of the disease status and increase or decrease in correlation to the disease. Development of biomarkers is an important area in translational research. It is often seen that biomarker measurements are able to depict the effect of the treatment, but unable to predict prognosis. Establishing that the biomarker is a valid surrogate of clinical benefit is very difficult. It requires a series of randomized clinical trials demonstrating the concordance of treatment difference as measured with regard to the candidate biomarker. Such demonstration will have to be established for a specific type of cancer and for a specific drug or class of drugs. Very strong evidence establishing that a particular biomarker reflects and predicts the effect of the drug on the disease is often used as the basis of drug approval [1, 3].

3.3 Identification of Early Predictors

Identifying predictive biomarkers of disease are extremely important for making informed therapy decisions for the specific patient. In some cases, the drug has an unknown target or multiple targets, although it results in successful therapy. Using the reverse genomics approach, a specified set of tumor expression profiles is used to develop a predictive classifier of the tumor, which will enable us to differentiate from a responder versus a nonresponder. Sometimes it may be more appropriate to develop a predictive index that is based on combining expression levels of multiple genes, rather than a single biomarker [1].

3.4 Importance of Validation Studies

In the clinical development of a drug, diagnostic, or technology, it is required that these predictive biomarkers are properly validated. Merely focusing on the identification or new prognostic factors does not help; rather, studies should focus on validating and endorsing the factors published by other researchers. Such confirmatory studies often help in securing regulatory approval for drugs for their indications [1, 3].

3.5 Representative Study Subjects

Most cancers, apart from being comprised of several different cell types, have a heterogeneous population of patients. Utilizing heterogeneous sampling of patients in studies oftentimes results in diluting the results and providing false positives and negatives. For a study to successfully translate to the clinical setting, it is thus critical to define specific population sets and have very strict inclusion criteria for enrolment in clinical trials. For example, the Oncotype Dx is a routinely used prognostic biomarker for breast cancer patients who test positive for the estrogen receptor but are node negative. Because it was developed and validated for a clearly defined set of patients, it can be used to identify patients whose prognosis to tamoxifen therapy is favorable [1].

3.6 Robustness and Analytical Validation of Assays

Researchers often devise analytical research assays to detect biomarkers in the smaller sample sizes typically seen in basic science. Such assays should be capable of handling a large number of samples and must yield consistent results across batches. It is very important for successful translational research that basic scientists collaborate with diagnostic or device companies to develop robust assays that can provide analytical validity [1, 3].

3.7 Strong Partnerships

Several obstacles in translation research exist that revolve around the structural limitations of academic research, clinical practice, industry, funding agencies, health-care administrators, and regulatory authorities. For effective translational research, the focus of the departments involved must be to promote strong interdisciplinary collaboration [1].

4 Economic Opportunities and Challenges

Routine pharmacogenetic testing will enable us to predict how a patient responds to a treatment regimen, which could potentially assist to tailor therapy that is most likely to benefit them as well as spare patients unnecessary adverse effects. Screening every patient before making a treatment decision would increase the costs of overall health care; however, an important outcome of this practice is a significant reduction in healthcare costs associated with ineffective treatments. This will warrant good educational programs for clinicians, health-care administrators, and insurance providers about the cost effectiveness and improved patient care accompanying the routine use of clinical pharmacogenetics [4–6].

For example, the Kras protein is involved in the downstream signaling of EGFR, a target for colorectal cancer therapy. Screening for mutations in the *Kras* gene that occur in 30–50 % of all metastatic colorectal cancer patients helps identify patients who are likely to benefit from EGFR inhibitor treatment, and the cost and toxic effects of ineffective therapy can be avoided in those who would have adverse effects. An economic evaluation conducted by Mancl et al. suggests that the total health-care costs for screening patients for such mutations and subsequently administering successful therapy will be comparable to the costs of treatment without prior screening and management of adverse effects in patients who respond poorly to the medication of choice, followed by alternate treatment strategies [7]. Several more studies of this kind focusing on specific disease conditions and treatment options are required to endorse the utilization of pharmacogenetic testing in routine clinical practice.

In addition to decreased costs in therapy administration, pharmacogenetics is also expected to aid with the economics of drug development, providing clinical trial administrators with the opportunity to select patient populations that would respond to the treatment. This would make the process efficient with respect to both total cost and time to bring a product to the market. Given these potential benefits, it is also equally plausible that pharmacogenetic testing could add additional up-front costs and introduce complexity in administration and regulatory processes. However, with the promise of expediting and optimizing the drug development process, pharmacogenetics is most likely to become a routine tool both in patient care and in the pharmaceutical industry [5, 7].

5 Pharmacogenetics of Solid Tumors

The most common and deadliest solid tumors are lung, colon, and breast cancers. In spite of over three decades of intensive cancer research, the magnitude of cancer-related morbidity and mortality still remains very high. A major challenge in cancer therapy is the narrow therapeutic window of chemotherapeutic agents. Thus, selection of the right treatment regimen for the right patient is extremely important. Pharmacogenetic screening of cancer patients for specific biomarkers has enabled and will aid further treatment of patients without their experiencing adverse effects from failed therapies [8].

The characterization of genomic biomarkers is currently a routine part of research and development for new therapeutics. Biomarkers can be physiologic, pathologic, and anatomic measurements that relate to some aspect of health or disease. Such biomarkers in oncology are typically validated specifically for a specific type of cancer and for a specific drug or drug combination [8].

5.1 Breast Cancer

5.1.1 Targets and Drugs

Human Epidermal Growth Factor Receptor 2

Human epidermal growth factor receptor 2 (HER2), also known as ErbB2, belongs to the type 1 family of tyrosine kinase (TK) receptors. The other homologs of the TK receptor family are ErbB1 or epidermal growth factor receptor (EGFR), ErbB3, and ErbB4. These cell surface receptors contain an extracellular ligand-binding domain, a transmembrane domain, and intracellular TK domain. HER2 does not have a known ligand and functions as a co-receptor to the other Erb receptors. The remaining Erb receptors get activated upon ligand binding and recruit downstream signaling proteins. Deregulation of these molecular pathways by overexpression and/or mutations resulting in constitutively active receptors are linked to the initiation and spread of many human cancers [9, 10] (Table 1).

Prognosis

A major proportion of breast and ovarian cancers overexpress HER2, which is associated with a poor prognosis. This HER2 overexpression seems to trigger ligand-independent activation of the TK domain and autodimerization and, in some cases, added EGFR autocrine stimulation. This interaction between EGFR and HER2 may increase proliferation rates in tumor development. The crucial role of EGFR and HER2 in mediating cell proliferation, differentiation, and survival provides a strong rationale for their inhibition in treating tumors with abnormal EGFR and HER2 signaling. It also provides an opportunity for the utilization of pharmacogenetic screening for mutations in the *ErbB* genes [9–11].

HER2 Positivity: Establishment and Clinical Guidelines

The *HER2* gene has proved to be a significant prognostic and predictive biologic marker in breast cancer. Thus, the current standard of care is to test all patients with invasive breast cancer for *HER2* at the time of diagnosis. The earlier guidelines recommended performing immunohistochemistry (IHC) analysis on breast cancer tissue, and if these results were inconclusive in proving HER2 positivity, fluorescence in situ hybridization (FISH) be done. However, the most recent guidelines favor results from FISH testing over IHC, owing the former's accuracy, reproducibility, and precision across different testing laboratories. The consensus thus suggests FISH as the primary HER2 testing modality for breast cancer patients who are candidates HER2-targeted therapy [12].

Table 1 Clinically important polymorphisms in predicting efficacy or toxicity in cancer

Gene	Variant	Phenotype	Drug	Clinical use
EML4-ALK	Rearrangement	Increased sensitivity	Crizotinib	Routine screening to identify candidates for crizotinib
BRAF	V600E	Increased sensitivity	Vemurafenib	Routine screening. Individuals with mutation more likely to respond to vemurafenib
HER2	Overexpression	Unfavorable prognosis	Trastuzumab, Pertuzumab, lapatinib, Ado-trastuzumab	Routine screening. HER2+ receive ErbB inhibitors
KRAS	Mutation	Unfavorable prognosis	Cetuximab, panitumomab	Routine screening. Kras wild type receive EGFR inhibitors
EGFR	Mutation, amplification	Increased sensitivity	EGFR TKIs—erlotinib, afatinib	Screening performed. EGFR mutants with NSCLC may have better response to erlotinib or afatinib
CYP2D6	Several	Metabolic differences—PM, IM, EM, UM	Tamoxifen	Controversial
TPMT	*2, *3A, *3C	Increased toxicity	6-MP, pediatric ALL, Cisplatin	Routine screening. 90 % empiric dose reduction in 6MP for homozygotes associated with cisplatin ototoxicity, increased audiometric monitoring
DPD	*2A	Increased toxicity	5-FU, Capecitabine	Not clinically useful, low sensitivity
UGT-1A1	*28	Increased toxicity	Irinotecan	Dose reductions recommended in package insert, but not routinely done
TS	TSER*3	Increased toxicity	5-FU	Not clinically used
MTHFR	C677T	Severe myelosuppression	CMF[a] regimen	Under investigation
Bcr-Abl	Several	Decreased efficacy	Imatinib Dasatinib, Nilotinib	Routine diagnosis of CML and monitoring of drug therapy
15:17 translocation	PML-RARα		ATRA	Routine diagnosis of APL

[a]Cyclophosphamide, methotrexate, and 5-FU

Trastuzumab Ado-trastuzumab emtansine, Pertuzumab and Lapatinib

Trastuzumab is a humanized monoclonal antibody that targets HER2 by binding to the extracellular domain. By doing so, it possibly downregulates the HER2 and decreases cell signaling. Additionally, it may possess general antibody-related cytotoxic effects. Trastuzumab is indicated for breast cancers overexpressing HER2, in both the metastatic and adjuvant settings [8, 13]. Pertuzumab is a recombinant monoclonal antibody that targets the extracellular dimerization domain of HER2. It is used in combination with trastuzumab to enhance HER2 inhibition. Ado-trastuzumab emtansine is also HER2-targeted antibody but is conjugated to a microtubule inhibitor and is used as a single agent in patients with HER2 positive breast cancer.

Lapatinib is a small molecule, reversible inhibitor of both EGFR and HER2 TKs, and can be orally administered. It is approved for combination therapy with capecitabine, both in the treatment of advanced HER2-positive breast cancer. It is a quinazolone derivative with a large aniline head group

that confers benefit pharmacokinetic properties such as slow dissociation rate (half-life >5 h) causing prolonged inhibition of EGFR. Upon binding to the intracellular TK domain of EGFR and HER2, it inhibits autophosphorylation and blocks downstream signaling mechanisms resulting in either apoptosis or growth arrest. Lapatinib causes tumor inhibition only in cases of EGFR and HER2 overexpression. It has been approved for combination therapy with capecitabine in patients with HER2+ breast cancer that progressed with trastuzumab. Several ongoing and completed clinical studies also support the use of lapatinib as monotherapy or as an adjuvant with trastuzumab, although these have not received FDA approval [8, 9].

Selection of Therapy Using Gene Expression Signature

HER+ breast cancers in stages 1 and 2 are primarily treated by surgery and radiation, and in case of patients with a high risk of recurrence, adjuvant hormonal therapy is added. However, determining which patients are at high risk of recurrence is an important clinical question [14].

Oncotype Dx

Oncotype Dx is a 21-gene assay (or which 16 are cancer genes and 5 are reference genes) that is performed on RNA extracted from paraffin-embedded tumor tissue. A recurrence score is calculated which estimates the likelihood of recurrence of cancer in breast cancer patients who were HER+ and node negative and treated with adjuvant tamoxifen. Patients with a high recurrence score (above 35) benefited from adjuvant therapy, whereas those with low recurrence scores (below 18) showed little or no benefit from tamoxifen. Thus, patients with a high recurrence score are good candidates for adjuvant chemotherapy. An intermediate risk group with recurrence scores between 18 and 35 are being studied for therapy optimization [14].

Mamma Print

This is a 70-gene micro assay to determine risk of recurrence in younger patients (under 61 years) with early-stage breast cancer. Like the Oncotype Dx test, aggressive therapy can be considered in patients with a high recurrence score to prevent future recurrence. However, this test can be performed only on fresh biopsy samples so it is less preferred than the Oncotype Dx test [14].

Vascular Endothelial Growth Factor

Angiogenesis is the physiological process involving the growth of new blood vessels from preexisting vessels. The term “angiogenic switch” refers to a key step in malignancy whereby the tumor is able to recruit its own blood supply by shifting the balance between factors that promote and inhibit angiogenesis. Conditions affecting the tumor microenvironment, such as hypoxia, cytokines, and genetic factors, act like a trigger for angiogenesis. Vascular endothelial growth factor (VEGF) and its receptors play a pivotal role in angiogenesis by acting as a mitogen and survival factor for vascular endothelial cells and also stimulate enzymatic degradation of the extracellular matrix. VEGF is an established target in colon cancer therapy and plays a role in the angiogenic process in breast cancer [15].

Prognosis

Direct assessment of the growth of new blood vessels by counting, and indirectly measuring for angiogenic factors (VEGF), hypoxia inducing factor (HIF), fibroblast growth factor (FGF), and receptors (VEGF receptor 2, soluble VEGFs). The angiogenic factor content is another measure that is

assessed by IHC or from blood measurements. Intratumoral microvessel density (MVD) is a useful clinical indicator of relapse-free survival and overall survival in node-negative breast cancer, or in node-positive patients treated with adjuvant hormone or chemotherapy. Also, there is a positive correlation between HER2 amplification and MVD in primary breast cancer. VEGF expression has been used as an independent predictor for relapse rates and overall survival following adjuvant chemo-, endocrine, or radiation therapy. Further, high VEGF levels in the primary tumor have been shown to predict poor response to tamoxifen, especially in postmenopausal women. These findings suggest that the VEGF pathway could be important in predicting tamoxifen resistance. Although some studies suggest a strong link between VEGF gene polymorphisms and breast cancer, the results have been inconsistent and warrant large prospective trials to define VEGF testing as routine methodology.

Angiogenesis Inhibitors

Bevacizumab is an intravenously administered humanized monoclonal antibody directed to neutralize all VEGF-A isoforms. The ECOG 2100 trial demonstrated the benefits of adding bevacizumab to paclitaxel therapy in first-line therapy for metastatic breast cancer. Alternatively, small molecular tyrosine kinase inhibitors (TKIs) such as sorafenib and sunitinib act by binding the intracellular TK domain of the VEGF receptor and block downstream signaling. TKIs have the advantage that they can be orally administered, although their serum half-lives are shorter than that of bevacizumab. Further, these drugs may also have a nonspecific TKI effect and may help in "multitargeting" in anticancer therapy [15].

Estrogen Receptor and Progesterone Receptor

The estrogen receptor (ER) has been an important and one of the oldest targets in breast cancer therapy for several decades. Its inhibition through endocrine targeting (selective ER modulators) or by indirectly blocking the conversion of androgens to estrogen (aromatase inhibitors) remains a widely used therapy in breast cancer. ER positivity is seen in about 75 % of all breast cancer cases, where a higher percentage of these women are over 50 years.

The progesterone receptor (PR) is an estrogen-regulated gene and its expression is thus indicative of a functioning ER pathway. PR+ tumors comprise between 55 and 65 % of all breast cancer cases and are shown to have better prognosis than PR– tumors. Although ER expression is used as the main determinant of response to hormone therapy in breast cancer, combinatorial ER and PR expression analysis helps provide additional insights [16].

Prognosis

ER + women are more likely to respond to antiestrogen therapy. ER status has been used routinely in clinical management both as an indicator of endocrine responsiveness and as a prognostic factor for early recurrence. PR and ER expression are highly correlative, and if a patient is found PR– but ER+, it maybe a surrogate marker of aberrant growth factor signaling that could contribute to tamoxifen resistance. ER+/PR+ patients [double hormone receptor (HR) positive] show the best prognosis to hormone therapy, whereas double HR patients are least responsive and have a high recurrence rate and decreased overall survival [16].

Tamoxifen, Aromatase Inhibitors, and Progestins

Tamoxifen is an antiestrogen used as palliative therapy in postmenopausal and in some premenopausal ER+ metastatic breast cancer patients. It binds to the ER, thereby blocking the effect of endogenous estrogens. It is a prodrug that is metabolized by CYP2D6 to form the potent active

metabolite, endoxifen. *CYP2D6* genotype has been associated with the outcome of tamoxifen adjuvant therapy in postmenopausal breast cancer patients in some studies. Toremefine is a recent antiestrogen drug with fewer adverse effects as compared to tamoxifen. Alternatively, an aromatase inhibitor maybe used, which blocks the synthesis of estrogen. Formestane and exemestane are some steroidal aromatase inhibitors, whereas anastrozole, letrozole, and vorozole are some nonsteroidal therapy options. Progestins, such as megesterol and medroxyprogesterone, may be used in combination with antiestrogens. It has been shown that combination chemotherapy is better than using a single agent [16].

5.1.2 Drug Disposition

CYP450 2D6 and Tamoxifen

Several cytochrome P450 (CYP) microsomal enzymes cause primary and secondary metabolism of tamoxifen to active and inactive metabolites. In the primary metabolic pathway, the CYP3A enzyme is responsible for the oxidative metabolism of tamoxifen to produce an intermediary metabolite, N-desmethyltamoxifen. This metabolite is then converted to its active metabolite, 4-hydroxy-N-desmethyltamoxifen (endoxifen) by CYP2D6. Another minor metabolic pathway results in the conversion of tamoxifen to 4-hydroxytamoxifen (4-OH tamoxifen). Both endoxifen and 4-OH tamoxifen have a higher affinity to the estrogen receptor and are 30–100 times more active than tamoxifen.

The relationship between CYP2D6 poymorphisms and tamoxifen is controversial. Primarily retrospective trials suggested CYP2D6 poor metabolizers had decreased survival when treated with tamoxifen. Based on these trials, the labeling information for tamoxifen was changed to suggest CYP2D6 as a valid biomarker for tamoxifen response. However, a large prospective trial and a recent meta analysis suggest there is no relationship between CYP2D6 and outcome in patients treated with tamoxifen, and routine genotyping is not recommended [8, 17].

ABCB1 Polymorphisms: Paclitaxel, Docetaxel, and Irinotecan

P-glycoprotein (P-gp) is an important clinically significant transport protein in the plasma membrane encoded for by the adenosine triphosphate (ATP)-binding cassette geneB1 (*ABCB1*). P-gp is expressed in several organs in the body, and its physiological function is to protect cells from xenobiotics by acting as an efflux pump and by limiting their oral absorption, thus decreasing the oral availability and tissue concentrations of substrates.

ABCB1 polymorphisms may influence P-gp substrate specificity, drug efficacy, and toxicity. For example, the homozygous *CC* genotype in exon 26 is associated with twofold higher P-gp concentrations when compared to the *TT* genotype, and so individuals with the *CC* genotype will have a twofold decreased oral bioavailability. Therefore, *ABCB1* genotyping maybe important for individualized drug therapy.

Paclitaxel and docetaxel, both routinely used in breast, ovarian, and lung cancer therapy, are substrates of P-gp. In a polymorphism study in ovarian cancer patients treated with paclitaxel or carboplatin, homozygous *GG* carriers were significantly less likely to relapse after treatment. Although the mechanism for this difference is unclear, it is likely that patients with the *GG* genotype had increased drug clearance, resulting in decreased exposure and potentially poorer outcomes. At present, due to the lack of conclusive results from clinical trials, routine testing for *ABCB1* polymorphisms cannot be recommended.

Irinotecan, a topoisomerase 1 inhibitor that is commonly used in colon and lung cancer therapy, is also a substrate for P-gp. It was reported that colon cancer patients carrying the homozygous *TT* genotype had increased exposure to irinotecan and its active metabolite, SN-38. In another study with lung

cancer patients treated with irinotecan and cisplatin, *TT* carriers showed a significantly less area under the curve of SN-38, compared with *GG* or *CC* carriers. Routine monitoring for *ABCB1* mutations is not recommended in breast cancer therapy without results from additional studies [18].

5.2 Colon Cancer

5.2.1 Targets and Drugs

KRAS

KRAS is a G protein that is involved in downstream signaling in the activated EGFR pathway. Common point mutations in the *KRAS* gene lead to the replacement of an amino acid in either position 12, 13, or 61 which are critical to normal function. About 40 % of colorectal cancer patients and 25 % of non-small cell lung cancer (NSCLC) patients are reported to have *KRAS* mutations. This is associated with an unfavorable prognosis to EGFR inhibitors, as reported by four randomized clinical trials [19, 20].

Prognosis

Two independent phase III studies randomized patients with treatment-refractory metastatic colon cancer with panitumumab and cetuximab respectively. It was found that in patients receiving panitumumab and best supportive care, the median progression-free survival was markedly great in those with wild-type *KRAS* than in those with *KRAS* mutations. Similarly, treatment with cetuximab improved overall survival and progression-free survival in patients with wild-type *KRAS*. Among patients with mutant *KRAS* genes, there was no significant difference between cetuximab treatment and best supportive care alone, with respect to overall survival and progression-free survival. Further, in the group receiving best supportive care alone, there was no significant difference between the *KRAS* wild type and mutant tumors in overall survival. These results suggest that KRAS is an important predictor of response to therapy, but not a disease prognostic factor. However, KRAS testing is recommended by both the National Comprehensive Cancer Network (NCCN) and the American Society of Clinical Oncology (ASCO) [19, 21].

Cetuximab and Panitumumab

Cetuximab is a monoclonal antibody (moAb) that binds to the EGFR with high affinity and inhibits downstream signaling and KRAS. It was the first anti-EGFR moAb to be approved by the FDA in 2004 for use, either as monotherapy or in combination with irinotecan, in patients with EGFR-expressing colorectal cancer who are refractory to irinotecan- and oxaliplatin-based chemotherapy. It can also be used in combination with irinotecan for the treatment of EGFR-expressing mCRC refractory to irinotecan alone [19, 20, 22]. It is not recommended for use in patients with *KRAS* gene mutations in codons 12 or 13. Two clinical trials in metastatic colon cancer patients studied the effects of cetuximab in combination with folinic acid, 5-fluorouracil (5-FU), and either irinotecan (FOLFIRI) or oxaliplatin (FOLFOX) respectively. In both of these studies, it was found that patients with a KRAS mutation would not benefit from adding cetuximab to FOLFIRI or FOLFOX chemotherapy and might in fact do worse, because of potential toxicity from ineffective therapy. However, based on these studies, the NCCN recommends the addition of cetuximab with the two drug combinations as first-line therapy for metastatic colon cancer patients with wild-type *KRAS*. Cetuximab is also approved in the USA for treatment of locally advance squamous cell carcinoma of the head and

neck (SCCHN) in combination with radiation therapy in metastatic patients who have disease progression after platinum-based chemotherapy. Panitumumab is another humanized anti-EGFR monoclonal antibody and has similar clinical guidelines for the treatment of metastatic colon cancer as cetuximab. However, it is only approved for the treatment of metastatic colon cancer in patients with wild-type *KRAS* [8, 20].

5.2.2 Drug Disposition

Uridine Diphosphate-Glucuronyl Transferase 1A1 and Irinotecan

Irinotecan is converted to a more active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38), which must then be inactivated by Uridine Diphosphate-Glucuronyl Transferase 1A1 (UGT1A1). The UGT1A1 gene contains a TATA (thymine-adenine-thymine-adenine) promoter region. A 6-TA promoter is considered the normal allele, while 5, 7, or 8 repeats are also found. The (TA)7 polymorphism is common, with about 40 % Caucasians and African Americans carrying at least one copy of the allele. The (TA)7 homozygous genotype, also known as UGT1A1*28 or 7/7, is associated with mild hyperbilirubinemia or Gilbert's syndrome and a low plasma ratio of SN38G/SN38 compared with high-activity UGT1A1 expressors. Pre-dose plasma bilirubin concentrations have been used to guide dosing, in order to decrease adverse events [23].

Irinotecan, although effective in treating a variety of malignancies, can unpredictably produce severe diarrhea and neutropenia, both of which are limiters in therapy. Studies have shown an association of these side effects of irinotecan with the presence of the 7/7 genotype, although some other studies yielded conflicting results. A meta-analysis evaluating results from many clinical trials suggests that the UGT1A1 genotype can predict irinotecan-associated toxicity, but in a dose-dependent manner. Although not confirmed in prospective clinical trials, a one-level dose reduction of irinotecan is expected to provide similar outcomes in 7/7 genotype individuals as compared to those with the 6/6 genotype and with minimal toxic effects. This recommendation is now included in the package insert of irinotecan. However, UGT testing and the dose reduction have not been widely adopted due to the lack of clinical data validating these recommendations [24].

Capecitabine, 5-FU, and Dihydropyrimidine Dehydrogenase

Capecitabine is an orally administered inactive prodrug that is converted to 5-FU. 5-FU is a fluorinated pyrimidine that is a mainstay drug for adjuvant and metastatic treatment of colon and breast cancer and some other malignancies. Meta-analyses reveal that over 30 % of patients receiving 5-FU experience severe drug-related toxicity.

Dihydropyrimidine dehydrogenase (DPD) is the initial and rate-limiting enzyme in the three-step pathway for catabolizing pyrimidines such as thymine and uracil. It is widely expressed in both normal and tumor tissue, including the liver, GI mucosa, and peripheral blood mononuclear cells. DPD is critical in the catabolism of fluorinated pyrimidines and breaks down over 80 % of 5-FU. The remaining 5-FU gets cycled into cellular processes and is responsible in producing cytotoxic effects by inhibiting the enzyme thymidylate synthase (TS), which is necessary for the de novo synthesis of thymidylate (dTMP). 5-FU gets metabolized to 5-FdUMP and forms a stable complex with TS, thus limiting further enzyme activity of TS and impairing DNA and RNA synthesis and stability [25].

The gene encoding DPD, *DPYD*, is composed of 23 exons encompassing about 950 kb. Over 30 SNPs and deletion mutations have been identified within the *DPYD*, some of which have functional consequences on enzymatic activity. The most common variant, *DPYD*2A*, is reported in 40–50 % of people with partial or complete DPD deficiency. In addition, aberrant methylation of the *DPYD* promoter, resulting in gene silencing, may cause a partially deficient DPD phenotype.

Complete DPD deficiency is known to occur in less than 5 % of Caucasians but, owing to the wide therapeutic use of 5-FU and capecitabine, has clinical significance. African Americans have markedly lower DPD activity and a higher prevalence of partial DPD deficiency compared to Caucasians. About 12 % of African American women are reported to have DPD deficiency. In such cases, it has been reported that there is a marked alteration in 5-FU and capecitabine pharmacokinetics and severe toxic reactions after the administration of standard doses of the drugs, due to decreased drug catabolism and clearance and increased exposure. A commercially available screening method for DPD deficiency, screens for its most common variant, *DPYD*2A*. However, since this variant accounts to less than 50 % of all DPD deficiencies, its clinical value is still limited as patients who test negative may still have a DPD deficiency [26].

Thymidylate Synthase

TS is the intracellular target for several chemotherapeutic drugs, including 5-FU, capecitabine, and pemetrexed. Overexpression of TS is associated with resistance to TS-targeted chemotherapy, which is influenced by polymorphic tandem repeats located in the TS enhancer region (*TSER*). Three copies of tandem repeats (*TSER*3*) give a 2.6-fold greater in vitro expression of (*TSER*2*). Alleles containing four (*TSER*4*), five (*TSER*5*), and nine (*TSER*9*) copies of tandem repeats have also been identified, although the functional relevance of these genotypes have not been established. The homozygous (*TSER*3*) genotype is associated with poor survival after adjuvant 5-FU-based chemotherapy in colon cancer, and a poor response to neoadjuvant 5-FU in colorectal cancer. Preclinical and clinical data suggest that intratumoral expression of TS as measured by mRNA expression or protein levels predicts the sensitivity of cancer cells to 5-FU, i.e., high expression of TS corresponds to low risk of resistance to 5-FU. Further studies are required in order to endorse TS genotyping and expression analysis in the clinical setting [8, 27].

5.3 Lung Cancer

5.3.1 Targets and Drugs

Epidermal Growth Factor Receptor

EGFR, a member of the EGFR subfamily of receptors, is critical for the growth of many epithelial malignancies and is a promising target for anticancer therapy especially in lung and colon cancers. The two main ways of targeting this important molecule are with tyrosine kinase inhibitors (TKIs) such as erlotinib and gefitinib, which block the ATP-binding site in the cytoplasm, and with monoclonal antibodies (moAbs) against the EGFR. While the moAbs are mainly used in the treatment of colon cancer, the TKIs are widely used as chemotherapeutic agents in the treatment of lung cancers, especially non-small cell lung cancer (NSCLC) [28, 29].

Prognosis

EGFR protein expression by IHC is considered a valid biomarker by the FDA for patients with colon cancer being treated with cetuximab. However, none of the clinical trials could correlate EGFR staining intensity with tumor response and other clinical end points. The lack of usefulness of EGFR staining is mainly due to the low sensitivity and high variability of IHC techniques, in

addition to the lack of correlation between staining of the extracellular domain and the presence of a function TK domain.

The BR.21 trial studied the effects of the TKI, erlotinib versus placebo in previously treated patients with NSCLC, and was the trial that enabled licensing of erlotinib. Although EGFR positivity was not an inclusion criterion for this study, EGFR expression was assessed in available samples using a commercial FDA-approved IHC assay (EGFR pharmDx kit). A positive EGFR-expression status is defined as having at least 10 % of cells staining for EGFR. Erlotinib prolonged survival in the EGFR-positive group and part of the unmeasured subgroup, but not in the EGFR-negative group. However, there was a wide statistical overlap between groups and about 4 % EGFR-negative patients showed clinical response. FISH analysis was done for KRAS, EGFR, and *EGFR* copy number in a subset of patients in the BR.21 trial, and KRAS mutations, *EGFR* exon deletions or mutations, and high *EGFR* copy numbers were identified. These were defined as FISH positive and were prognostic of poorer and differential survival benefit from erlotinib. Despite these findings *EGFR* testing does not have a wide clinical utility due to the inconsistencies of IHC techniques and inadequate amounts of sample available to perform routine FISH testing [22].

Erlotinib and Afatinib

The current treatment of metastatic NSCLC is determined by tumor histology and mutation status. Individuals with squamous histology are unlikely to harbor mutations with known clinical significance and are treated with standard cytotoxic chemotherapy. Individuals with adenocarcinoma, large cell, or unspecified histology are typically tested for epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) mutations and treatment is determined based on mutation status. The first-line therapy option for patients with activating EGFR mutations is erlotinib or afatinib, targeted therapy for the EGFR receptor, as both erlotinib and afatanib have improved efficacy and decreased toxicity when compared to standard chemotherapy in patients with EGFR activating mutations. In addition, afatinib is active in patients with the T790M resistance mutation [29–32].

EGFR Gene Mutations

Function-gaining somatic mutations in exons 18–21 of the *EGFR* gene that code for the intracellular ATP-binding domain of the receptor and that may lead to dramatic clinical responses to EGFR TKIs have been identified in patients with NSCLC, receiving erlotinib and gefitinib. Three types of mutations have been described—missense mutations in exons 18–21, deletions in exon 19, and small in-frame insertions in exon 20. Experimental studies have shown that tumor cells with activating *EGFR* mutations have increased sensitivity to EGFR TKIs [30]. A resistance mutation in T790M in the EGFR receptor is also reported.

EML4-ALK Mutation

Recent work has identified a gene rearrangement involving the anaplastic lymphoma kinase (ALK) gene and the echinoderm microtubule-associated protein-like 4 (EML4) gene. The gene inversions have been found in adenocarcinomas, and predominantly among those with light or nonsmoking histories and younger age. The ELM4-ALK gene inversion has been detected in NSCLC samples independently of mutations in the EGFR, KRAS, and ERBB2 genes. EML4-ALK mutations have been found with frequencies of 2.4 to 13 % among NSCLC samples.

Crizotinib is an oral ATP-competitive selective inhibitor of the ALK and MET tyrosine kinases that inhibits tyrosine phosphorylation of activated ALK at nanomolar concentrations. Crizotinib was compared to standard chemotherapy in NSCLC patients with ALK-EML4 mutations, demonstrating improved survival and is recommended as first line treatment in patients with this mutation [33].

5.3.2 Drug Disposition

Excision Repair Cross-Complementing Gene 1, Cisplatin, and Gemcitabine

Cisplatin has been shown to provide survival and quality of life benefits for patients with advanced, unresectable NSCLC, but overall 2-year survival rates remain under 15 %. The cytotoxic effects of cisplatin is mainly due to the formation of large platinum–DNA adducts. Removal of these adducts from genomic DNA is mediated by the nucleotide excision repair pathway, by means of the Excision Repair Cross-Complementing Gene 1 (*ERCC1*) gene. DNA repair can be attenuated by blocking the interaction between ERCC1 protein and a dermal pigment protein XPA, and high *ERCC1* expression is associated with resistance to platinum-based therapy in human ovarian and gastric cancers. Cytotoxic synergism and a higher response rate have been reported with a gemcitabine and cisplatin combination than with the standard cisplatin and etoposide regimen in patients with advanced NSCLC. This synergism involves *ERCC1* and the nucleotide excision repair pathway in a way that low *ERCC1* expression correlates with prolonged survival after cisplatin plus gemcitabine chemotherapy in NSCLC. Patients with completely resected NSCLC and *ERCC1*-negative tumors benefit from adjuvant cisplatin-based chemotherapy, whereas patients with *ERCC1*-positive tumors do not [34].

Pemetrexed and Gemcitabine

Pemetrexed has wide-spectrum antitumor activity with fewer toxic effects than most therapy options for NSCLC. It is a folate inhibitor of thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyltransferase (GARFT). Gemcitabine, a deoxycytidine analog, is a prodrug that is transported into the cell by human equilibrative nucleoside transported 1 (hENT1) and undergoes further metabolism to its active form that causes DNA chain termination. The rate-limiting step in this activation is catalyzed by deoxycytidine kinase (dCK) and inhibited by 5′-nucleotidase (5′-NT) and cytidine deaminase (CDA). Preclinical and clinical studies suggest that combinations of pemetrexed with platinum compounds such as cisplatin, taxanes such as docetaxel, as well as gemcitabine produce additive or synergistic cytotoxicity. Preclinical data suggests that the mechanism of this synergy is by GARFT inhibition by pemetrexed, which can enhance dCK and hENT1 expression as a compensatory mechanism and enhance gemcitabine activity. Phase II clinical trials evaluating pemetrexed plus gemcitabine as frontline therapy for advanced NSCLC are ongoing. Pharmacogenetic profiling of these enzymes may contribute to the assessment of tumor-cell response to the gemcitabine and pemetrexed combination [35, 36].

6 Pharmacogenetics of Hematological Malignancies

6.1 Acute Lymphocytic Leukemia

Also known as acute lymphoblastic leukemia, it has a very high occurrence in the pediatric population and is also characterized by a high degree of therapeutic efficacy.

6.1.1 Thiopurine Methyltranferase and 6-Mercaptopurine

TPMT deficiency is inherited as an autosomal recessive trait, with 89–94 % of Caucasians having high activity, 6–11 % with intermediate activity, and 0.3 % with very low or no activity. This deficiency is largely explained by three polymorphisms in the TPMT gene (*2, *3A, *3C) which also have a profound influence on 6-MP (6-mercaptopurine) tolerance and dose intensity in children with acute lymphocytic leukemia (ALL). While these polymorphisms are rare, they are certainly important with cases of toxic deaths attributed to 6-MP dating back several decades [37, 38]. In a recent clinical trial, children who were homozygous for one of the alleles required 6-MP dose reductions of 91 %, while heterozygotes require a dose reduction of approximately 50 %. Children with dose reductions had equivalent overall survival when compared to children receiving full dose of 6-MP, suggesting that TPMT polymorphisms are important for drug metabolism and toxicity, but play no role in the pathogenesis of ALL. TPMT screening is recommended for children starting therapy with 6-MP with empiric dose reductions for those with genotypes associated with a deficiency [39].

6.1.2 Methylenetetrahydrofolate Reductase

A common polymorphism, occurring as a homozygous variant in up to 10 % of Caucasians, occurs as a C677T variant in the Methylenetetrahydrofolate Reductase (MHFR) gene. The normal function of MHFR is to regulate the intracellular folate pool used in DNA and protein synthesis. Individuals homozygous for the MHFR gene have only 35 % of normal enzyme capacity and accumulate 5,10-methylenetetra-hydrofolate (CH_2THF), in purine and pyrimidine synthesis. Toxicity, primarily myelosuppression, was reported in breast cancer patients with this polymorphism receiving CMF (cyclophosphamide, methotrexate, and 5-FU). The authors suggest that an excess of CH_2THF increases the ability of 5-FU to inhibit thymydilate synthetase and therefore increased myelosuppression. The relationship between this polymorphism and thymidylate synthetase appears convincing, as a recently reported phase 1 trial with the thymidylate synthetase inhibitor, raltitrexed, where individuals with the polymorphism had no toxicities associated with raltitrexed. While interesting, MHFR polymorphisms are not ready for routine clinical use [40].

6.2 Chronic Myelogenous Leukemia

It is a one-chromosomal translocation characterized by the replacement of the first exon of *c-Abl* with sequences from the *Bcr* gene, resulting in a *bcr-abl* fusion. The product of this gene is the BCR-ABL protein which shows enhanced TK activity and is absent in noncancerous cells, thus making it a good target for therapy. Imatinib can bind to the catalytic domain only when the BCR-ABL chimeric protein is in the closed conformation [41].

6.2.1 Imatinib

Imatinib, which was originally designed to inhibit platelet derived growth factor (PDGF) and ABL protein kinases, was also found to inhibit C-kit, a target in GI stromal tumors. The drug interacts competitively with ATP for the binding site of the *bcr-abl* catalytic domain. Over 50 mutations have been reported in the bcr-abl gene, the most significant of which result in the protein's P-loop alteration and cause the protein to switch to an open configuration, thus restricting the binding of imatinib. Dasatinib is another small molecule TK inhibitor that can bind to the activation site irrespective of the protein conformation, except in the case of *T315I* [41].

6.2.2 Management

Imatinib is not a cure, but helps in indefinitely delaying progression to advanced stages of chronic myelogenous leukemia (CML). Patients must be monitored every 3–6 months with bone-marrow biopsies or FISH or RT-PCR on peripheral blood samples. A cytogenetic remission is when mRNA of bcr-abl is undetectable, which around 87 % of patients achieve by 18 months of therapy. As resistance develops and patients no longer are in a cytogenetic remission, interventions such as dose escalations, mutation testing of the BCR–ABL protein, or trial with dasatinib followed by retesting with FISH or RT-PCR must be done [41].

6.3 Acute Promyelocytic Leukemia

6.3.1 All *Trans* Retinoic Acid

Acute promyelocytic leukemia (APL) is a distinct subtype of acute myeloid leukemia (AML) in which a balanced reciprocal translocation between chromosomes 15 and 17 results in a union of portions of the *pml* gene with the gene for retinoic receptor alpha. This chimeric gene encodes the PML-RAR-alpha fusion protein. Although about 80 % of patients with APL have a complete remission with standard chemotherapy, approximately 20 % die before or during chemotherapy due to bleeding attributable to disseminated intravascular coagulation, fibrinolysis, and proteolysis. All *trans* retinoic acid (ATRA) differentiates leukemic promyelocytes into mature cells. Phase 2 clinical trials have demonstrated that ATRA induces complete remission in most patients, with rapid resolution of coagulopathy and few deaths during induction therapy. The duration of complete remission is usually brief, and postremission chemotherapy is required to diminish the likelihood of relapse. It has been shown that in patients who are refractory to ATRA, histone deacetylase inhibitors have restored sensitivity to the antileukemic effects [42, 43].

7 Melanoma

7.1 BRAF mutations

Mutations in the BRAF protein kinase are present in more than half of the cases of metastatic melanoma and about 7–8 % of all cancers. There are over thirty distinct BRAF mutations with V600E being the most common variation in melanoma in which a normal valine is replaced by glutamic acid at amino acid position 600. BRAF is a serine/threonine protein kinase that activates the MEK/MAPK/ERK-signaling pathway that mediates cellular responses to growth signals. Once activated, BRAF is normally recruited to the cell membrane to undergo phosphorylation into a dimer to continue the signaling cascade and downstream activation. A mutation in BRAF results in bypassing the RAS activation, which is a requirement for downstream signaling and regulation in the MEK/MAPK/ERK pathway. When this RAF kinase is mutated there is abnormal activation and deregulation of the MEK/MAPK/ERK pathway, which leads to cell proliferation, invasion and survival [44].

7.2 Vemurafenib

Vemurafenib is an oral selective BRAF kinase inhibitor indicated for the treatment of patients with unresectable or metastatic melanoma with the $BRAF^{V600E}$ mutation as detected by an FDA-approved test. In patients with the BRAF mutation, vemurafenib is both more effective and less toxic than standard chemotherapy [45].

8 Conclusion

Over the past decade, pharmacogenetic testing for solid tumors has been routinely performed to analyze specific biomarkers that can predict therapeutic outcome. Several tests are in routine clinical practice, to help define parameters for selecting the most appropriate therapy for the individual patient. HER2 status as well as gene expression signatures are standard tests done in order to select therapy for breast cancer. KRAS mutation analysis in metastatic colon cancer will most likely become routine practice in the near future, to identify patients who will not respond to EGFR inhibitors. Pharmacogenetic testing is also being geared to predict pharmacokinetic parameters and prevent drug toxicity and is expected to be part of routine therapy in the current decade.

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