2 Molecular Challenges in Front-Loading Toxicity Testing of Anticancer Drugs in Drug Discovery

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2.1 INTRODUCTION

The oncology market is the fastest growing in the pharmaceutical industry, yet oncology still has one of the poorest records for success in clinical development, partly due to the less than optimal correlation between nonclinical models and the disease in humans [1]. Growth of the market is being fueled by the magnitude of the disease globally; currently, 25 million people are afflicted with cancer worldwide. The World Health Organization (WHO) predicts that cancer rates may increase by 50% from 2000 to

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2015. The Association for International Cancer Research (AICR) estimates that by 2020, there will be 10 million deaths from cancer per year. Even in developed nations, ~50% of patients diagnosed with cancer will eventually die of this disease. In the United States, cancer is the second leading cause of death by disease, one of every four deaths—and is exceeded only by heart disease. Approximately 570,000 Americans are expected to die of cancer each year, which equates to more than 1500 people a day. Nearly 1.4 million new cancer cases are diagnosed annually.

2.2 CYTOTOXICS

For several years, drug discovery efforts have focused on cytotoxics, compounds that block essential cellular functions and kill dividing cells [1,2]. These agents have included those designed to interfere with precisely defined physiological processes and those with pleiotrophic mechanisms. Examples of mechanisms in the first category include metabolite synthesis (e.g., methotrexate), microtubule polymerization (e.g., Taxol), and chromosome topology (e.g., irinotecan). Pleiotrophic molecules include those considered to be DNA-modifying (e.g., cisplatin). The therapeutic margin of these compounds is typically assessed as the differential between on-target effects in cancer cells and on-mechanism effects in normal cells. Dose response and distribution within tumors and normal tissues result in estimates of the therapeutic window (margin between efficacy and toxicity), and these estimates are studied in animal models and early clinical trials. Another class of anticancer drugs includes antihormonal agents such as estrogen receptor modulators (e.g., tamoxifen) and aromatase inhibitors (e.g., letrozole) [1].

2.3 TARGETED THERAPIES

There is a rapidly growing pipeline of targeted therapeutics designed to modulate single or multiple targets known to be important in cancer [2]. Accordingly, there has been a trend to move away from molecules with broad cytotoxic activity to more molecular targeted therapies [3,4], particularly in small-molecule research and development. Therapeutics of all types are now being developed in a targeted fashion, keying in specific molecular targets and specific signaling transduction pathways [2,3]. It is possible that broader therapeutic windows may be seen with these types of agents, particularly, if the specificity in targeting is limited to families of proteins prominent in tumors versus normal cells.

Biotherapeutics, another category of targeted anticancer drugs, represent a rapidly growing therapeutic modality. Biotherapeutics or biologicals are drug products where the active substance is produced or extracted from a biological source. These include recombinant hormones and proteins, monoclonal antibodies (mAbs), cytokines, growth factors, gene therapy products, vaccines, cell-based products, gene silencing therapies, tissue-engineered products, and stem cell therapies. A high proportion of biotherapeutic molecules under development and targeted for cancer therapy are mAbs, with most possessing IgG1 Fc designs with immunomodulatory function [5].

For targeted therapies including biotherapeutics, all effects attributed to the pharmacological mechanism, whether positive or negative, are considered to be related to

(i) binding or modulating a target (including unintended targets), (ii) the downstream effects from the modulation, (iii) targeting biological or signal transduction pathways, (iv) localized immune responses based on the design of the molecule, and (v) mechanistically based effects such as exaggerated pharmacology at the intended site of action or another tissue not intended as the primary site of action. Differences in molecules and effects also relate to the extent of target-related and nontarget elimination, receptor synthesis and turnover, molecule–receptor binding affinity, and molecule–receptor complex turnover [5].

Regardless of the type of therapeutic, the classic estimate of safety or therapeutic window in nonclinical mouse xenograft efficacy models, the ratio of tumor volumes in treated and control animals with the estimate of toxicity defined as *percent loss in body weight* (<10%) has carried on in the drug discovery field, even with newer targeted therapeutics [6]. Although this estimate has been used for decades, it fails to distinguish between on- and off-target effects or to differentiate between mechanism- and chemistry-based toxicities.

2.4 NONCLINICAL STUDIES: THE SHIFTING PARADIGM FOR TOXICITY TESTING

Nonclinical studies for anticancer drugs, which cover *in vitro* and *in vivo* studies, are designed to identify and quantify the pharmacological properties of the therapeutic, to understand the toxicological properties of the therapeutic including target organ(s) and dose (exposure)–response relationships, and to establish safe initial dose levels for the first exposures in humans [5]. First-in-human (FIH) guidance based on preclinical markers of safety and efficacy has been issued by the EMEA (European Medicines Agency) [7,8] and US Food and Drug Administration (US FDA) [9], covering both small molecules and biotherapeutics, and a new International Conference of Harmonization (ICH) guideline on nonclinical evaluation for anticancer therapies is pending [10]. These guidance documents can be interpreted as "limiting" the amount and extent of toxicology testing required to enter the first clinical trials, with the underlying thought that the best information for developing an anticancer therapeutic will come from experience in cancer patients. This is exemplified in the current concepts for FIH dose selection for biotherapeutics, which have evolved into a target-mechanism-based model (TMBM) utilizing exposure–response relationships from both *in vitro* and *in vivo* studies including (i) minimally effective exposure concentrations for biological activity (e.g., ED_{10}), (ii) pharmacologically active levels (e.g., ED_{50}) particularly important for FIH cancer trials, (iii) no observable adverse effect level (NOAEL) and/or toxicodynamic endpoint estimates from toxicology studies, and (iv) exposure- or concentration-related biomarker changes [5]. Ideally, these parameters are measured in the nonclinical studies, but in some cases, they are predicted via modeling or allometric extrapolation, such as in the case for estimated human doses and/or exposures. Dose escalation to a maximum tolerated dose (MTD) is a goal of phase 1 trials in order to select an optimal biological dose for phase 2, and most researchers are in agreement that nonclinical studies should be conducted, which allow such dose escalation to occur as rapidly as possible. In cancer patients, starting at a suboptimal or potentially subtherapeutic dose is not generally viewed as being acceptable

because there is an inherent desire to give each patient a potential therapeutic dose level [5].

2.5 EARLY TOXICOLOGY FOR DISCOVERY SUPPORT: HOW MUCH APPLIES TO ANTICANCER DRUGS

Several schemes for incorporating toxicity testing in the discovery process have been proposed with the objective of identifying potential side effects in humans and in animals used for pivotal toxicology assessments for regulatory purposes. Lead compounds or development candidates that pass through these "filters" increase the probability of success for a molecule. Screens, which include both *in vitro* and *in vivo* studies, are designed to be related to chemical reactivity or metabolic instability, unintended off-target effects possibly related to the unanticipated interaction with other targets or receptors, certain physicochemical properties of the parent compound and/or metabolites, and those properties that are related to the intended therapeutic mechanism of action, where unanticipated biological consequences or interaction with an unanticipated target takes place [11]. For the most part these are focused on understanding chemically related toxicity, where a correlation between chemical structure and potential toxicity is assessed [12]. Molecular mechanisms and the consequences of biological target modulation are typically reserved for efficacy and potency screening, particularly to attempt to understand the kinetics and dynamics of the compound at the target site [13]. Compounds or classes of compounds that are known to confer toxicity in a specific tissue will typically lead to an *in vitro* assay with results correlated with potential findings in either animal or human *in vivo* assessments. With a defined quantifiable endpoint and structural similarities in libraries, detailed quantitative structure–activity relationship (QSAR) modeling can be used as a predictive process for both efficacy and toxicity [11]. Therefore, early toxicity screening typically involves *in silico*, *in vitro*, and/or *in vivo* analyses and studies based on known toxicities that have either occurred in animals or resulted in slowing down or eliminating projects, where toxicities have occurred in clinical trials or where toxicities seen in patients after a drug reaches the market. The primary concern with screening assays is that they must be predictive, and the predictive endpoint must be human toxicity. If the goal of any program is to predict toxicity in rodents because rodents will be used in pivotal toxicology studies, then the toxicity induced in rodents must be relevant and predictive of the same effect in humans. Great effort is made in early screening on rank ordering compounds to filter out "bad actors" and increase the probability of selecting molecular structures that eliminate certain unwanted attributes. For the most part, these assays are done in human cell-based systems, which maintain genotype and phenotype [14], and even then there are several examples where the relevance of screening cannot be determined. This includes assays designed to understand the chemical interactions with the constitutive androstane receptor (CAR) and peroxisome-proliferator-activated receptor (PPAR) nuclear receptor families [15]. In addition, the difficulties of modeling and predicting toxicity from immunomodulatory drugs are well established [14,16]. One of the primary challenges in this field is that assays meant to be predictive are developed retrospectively based on experience, and as such, they tend to validate the previous finding and/or may be used to define a mechanism. Questions still remain as to the quality of some cell-based assays used prospectively for predicting potential toxicities with previously untested compounds.

2.5.1 Screening for Liver Toxicity

Hepatotoxicity is a major cause of drug failures both pre- and postapproval; however, the fact that several mechanisms can be involved and some liver toxicities are idiosyncratic (rare in occurrence) makes this a difficult task for accurate screening [11]. There are several systems in current use including primary cell cultures, immortalized cell lines, liver slices, and whole perfused livers [17,18]. The two most widely used methods use primary cells from freshly isolated hepatocytes from humans and animals used in preclinical assessments, and immortalized cells that over- or underexpress certain phase I CYP450 and conjugating enzymes allowing the assessment of maximal production of reactive intermediates. Although not as widely used, differences in metabolism may be assessed when polymorphisms affect metabolic rates in certain individuals [18]. Cytotoxic screening with newer technologies to assess multiple parameters such as nuclear morphology, cell proliferation, plasma membrane integrity, and mitochondrial function in a high content screening format are also being used [19]. An advantage of this type of screen is the ability to couple with assays measuring mechanistic endpoints such as oxidative stress, DNA damage, apoptosis, and cell cycle inhibition, again in a high content mode via multiplexed platforms [19]. It should be pointed out that these types of assays work the best when used to rank order compounds rather than as a predictive assay for an individual compound. Computational models for liver toxicity have also been developed [20,21]; however, there remains difficulty when several different mechanisms of liver toxicity are represented by compounds used to train computational models. Using cell-based assays in a predictive mode, prospectively, still carries a level of uncertainty because of differences seen between species and individual differences noted in freshly collected hepatocytes [18,19]. Array "omics" technologies have been utilized in virtually all these assay formats but have had limited success when used prospectively [14]. These technologies may be of greater utility in defining specific mechanisms rather than as predictive assays. An important limitation is the number of cells needed for assays and the fact that results are based on average changes from several cells [22]. Important work by Cohen and colleagues [23] noted differences in protein response of human cancer cells to drugs using central dogma (CD) tagging in human H1299 lung carcinoma cells and time-lapse fluorescence microscopy. These changes involved rapid translocation of proteins specific to the mechanism of the drug and slower, wide-ranging temporal waves of protein degradation and accumulation. They provide an understanding of how cells that seem identical show different responses to drugs [22,23]. Technologies that measure responses in individual cells rather than averages of several cells (with cell to cell variations) may offer a promising way to understand both efficacy and toxicity in the same assay.

2.5.2 Screening for Cardiotoxicity

Cardiotoxicity in humans is another type of toxicity seen with several classes of compounds including targeted therapeutics. Screening for QTc prolongation has been established for several years, and multiple screening approaches are being required and employed in most major industrial companies globally. Cardiotoxicities from targeted therapies (other than QTc prolongation) are more difficult to predict in preclinical studies, as these types of effects lead to defects in left ventricular function and congestive heart failure. Several screening approaches have been used in an attempt to

prospectively predict cardiotoxicity using *in vitro* models, which include cardiomyocyte cell lines or primary cells [24]. Primary cells are typically ventricular myocytes from neonatal or adult rats. The merits and limitations of these two approaches have been discussed and it has been concluded that cell lines are inferior to primary cells because the mitochondrial electron transport system is the dominant source of energy in cardiomyocytes, whereas cell lines rely on glucose for energy generation [24]. These systems are sensitive and may produce a high degree of false-positive results and will not detect toxicity from direct effects on the vasculature [24]. Several *in vivo* models have also been used, including echocardiography in mice, pharmacologic stressor probes in rodents, catheterization for contractile dysfunction, and transmission electron microscopy in rodents. Prospective use of these techniques as an effective predictive assay has not been reported to any extent [24].

Early screening programs are usually company specific based on toxicities seen in internal pipelines but tend to include structure–activity relationship (SAR) (alerts) by computational tools [11], identification of reactive moieties in chemical structures using both computational and capture assays [20], and screening in model systems based on suspected target organs as noted above. Early *in vivo* studies are typically designed to explore linearity of dose versus exposure, which may include assessing different vehicles and their effect on bioavailability, rising-dose studies to explore dose versus overt toxicity, and preliminary studies (7–10 days) to establish high and low dose levels for definitive toxicology studies [12].

2.6 NEWER MOLECULAR TARGETS AND CANCER THERAPEUTICS

New targets are continually being discovered by molecular genetic assessments of cancer biology. Molecular targeted therapies are composed of novel therapeutic agents that target biologically important processes central to the development and progression of cancer and generally fall into the following categories [25]:

- *Single-Target Signal Transduction Inhibitors:* These include inhibitors of any single target (primarily) such as EGFR (epidermal growth factor receptor), mTOR (mammalian target of rapamycin), PKC (protein kinase C), Bcr/Abl [e.g., imatinib (Gleevec; Novartis), trastuzumab (Herceptin; Genetech/Roche), and panitumumab (Vectibix; Amgen)]. Imatinib is generally considered to be a single-target agent but it also inhibits PDGFR (platelet-derived growth factor receptor) α/β and KIT to some degree.
- *Multitargeted Inhibitors:* These include any agent with more than one therapeutic target [e.g., sorafenib (Nexavar; Onyx), sunitinib (Sutent; Pfizer), dasatinib (Sprycel; BMS), and lapatinib (Tykerb; GSK)].
- *Angiogenesis Inhibitors:* These include inhibitors of VEGF/VEGFR (vascular endothelial growth factor/VEGF receptor), uPA, and integrin families [e.g., bevacizumab (Avastin; Genentech/Roche)].
- *Epigenetic Modulators:* These include the histone deacetylase (HDAC) inhibitors [e.g., vorinostat (Zolinza; Merck)].
- *Cell Cycle and Apoptosis Targeted Agents:* These include modulators of CDK, Bcl-2, TRAIL, and Chk proteins [e.g., bortezomib (Velcade; Millennium)].

- *Immunomodulatory and Immunoconjugated Therapeutics:* These include the cancer immunotherapies and radioimmunotherapies [e.g., rituximab (Rituxan; BiogenIdec/Genentech/Roche) and gemtuzumab ozogamicin (Mylotarg; Wyeth/Pfizer)].

Agents that target (i) cell division by newer mechanisms, such as the aurora kinase and cyclin-dependent kinase inhibitors, (ii) protein turnover (e.g., bortezomib), or (iii) chromatin modification (e.g., HDAC inhibitors) have been referred to as *neo-cytotoxics* [1].

A market report on molecular targeted therapies by Datamonitor in October 2007 [25] identified 329 different targeted therapeutics in the developmental pipeline. Of these, 44 (13%) were classified as angiogenesis inhibitors, 45 (14%) were single-target signal transduction inhibitors, 65 (20%) were multitargeted inhibitors, 95 (29%) were cell cycle or apoptosis targeted agents, 64 (19%) were immunomodulatory or immunoconjugated therapeutics, and 16 (5%) were epigenetic modulators. Datamonitor forecast these targeted therapy candidates to realize an aggregate seven major markets' sales potential of $6.03 billion by 2016. A report by Bioseeker in October 2009 [26] identified 409 protein kinase drugs (990 projects) either ongoing or recently stopped within the portfolio of 160 companies or investigators. The data included 133 identified targets, 200 drug target profiles, and 57 different cancer indications.

2.7 TOXICITIES OF TARGETED THERAPEUTICS

The increased interest in discovering and developing molecular targeted therapeutics has changed the focus of toxicities in patients away from traditional cytotoxic side effects seen with chemotherapeutics, which potentially changes the types of early toxicity screening programs that must be instituted in drug discovery and early development. It should be noted, however, that several targeted therapies do get approved as combinations with cytotoxic therapies. Early discovery-based screening in oncology drug discovery concentrates on chemical-based toxicity, where modifications of the structure of potential candidate drugs are accomplished during lead optimization, and mechanism-based toxicity, where a specific toxicity or tissue has been identified as being an issue to overcome during the lead discovery and development process. Various tiered approaches have been developed in industry, which include SAR structural alerts for toxicity (including genotoxicity), identification of reactive moieties in proposed chemical structures, and primary and secondary screening in model systems [11,12,17]. These systems have, among other endpoints, included novel approaches utilizing cytotoxicity as the defining type of toxic event [17,19,20].

Since many toxicities associated with molecular targeted therapies are associated with the actual mechanism for pharmacological targeting, these agents require a detailed understanding of the molecular aspects of the target, affected signaling pathways, and potential or unsuspected off-target toxicities that may be encountered. This raises the question of whether traditional toxicity screening programs and studies can or should be front loaded into discovery development programs of individual targeted therapies without a more complete understanding of how to monitor mechanistic toxicities where specific biomarkers of affected pathways may be needed. Realistically, the issue here is the lack of a clear understanding of the potential mechanisms and putative events

that trigger toxicities with molecular targeted therapies and the lack of defined and validated biomarkers. Biomarkers are defined as *objectively measured characteristics* that are useful as indicators of normal and/or pathological biological processes, or pharmacological responses to a therapeutic intervention. Biomarkers theoretically can help to determine whether the molecule reaches and modulates the desired target, whether it affects biological activity as intended, and whether this may lead to a desired or, in some cases, an undesired outcome [5]. Examples of the difficulties with mechanism-based biomarkers used for safety endpoints are seen with biomarkers of mTOR inhibition: phosphorylation of 4E-BP1 and S6K1. These biomarkers have been used effectively in animal studies and clinical trials showing dose-related modulation of downstream events from mTOR. However, adverse events in humans cannot be correlated per se with these mechanistic markers. Toxicities that occur in greater than 50% of patients receiving mTOR inhibitors (e.g., rapamycin) include mucositis, stomatitis, cold sores, pnuemonitis, hypersensitivity reactions, and skin rashes. The mechanisms for these types of reactions have not been determined, although all are usually managed adequately. Hyperglycemia can be associated with a disruption of insulin signaling [27], which is seen more commonly with inhibitors of targets upstream of mTOR, such as PI3K.

2.8 TOXICITIES EMERGING IN CLINICAL TRIALS OR POST MARKETING

Human adverse events from therapeutics are typically discovered during clinical trials or after market approval where exposure to the drug is expanded dramatically. One important type of analysis focuses on toxicities that have resulted in market withdrawal of the drug. Several authors have surveyed drug withdrawals and offered detailed analyses of potential mechanisms of individual drugs and the factors leading to human toxicity [28,29]. In these reports the reasons for market withdrawal have been classified as (A) based on the primary pharmacology (A1) of the drug and its intended target or (A2) secondary pharmacology due to nonselective activity against nonintended targets; (B) based on an immunological mechanism, which includes several idiosyncratic toxicities; (C) based on a chemical reaction with tissue macromolecules with a rapid ensuing response, typically occurring via bioactivation to a reactive chemical moiety; and (D) delayed responses from mechanisms similar to (B) and (C) such as carcinogenesis and teratogenesis. Type (A), (C), and (D) toxicities are for the most part thought to follow classic dose–response relationships, which defines how screening studies are designed, whereas type (B) toxicities do not [14,29]. MacDonald and Robertson [14] reviewed specific reasons for the withdrawal of drugs from the US, Japanese, and European markets during 1998–2008 after finding that the two major reasons were hepato- and cardiotoxicity. Other reasons included psychiatric problems and addiction, gastrointestinal toxicity, muscle toxicity, renal dysfunction, accelerated carcinogenicity or death, mutagenesis, drug–drug interactions, hypersensitivity, and pulmonary hypertension. Mechanisms and molecular events associated with liver toxicities included mitochondrial toxicity, cholestasis, cytotoxicity from reactive metabolite, and unknown mechanisms for rare idiosyncratic hepatotoxicity. Cardiotoxicity was a result of alterations in ion channel function leading to QTc prolongation, altered valvular function, and arrhythmias resulting in fatalities [14]. Not surprisingly, screening programs within

the industry key in on these findings, with the majority of programs screening for genotoxicity, cardiotoxicity, and hepatotoxicity [11]. This generally includes all small-molecule programs regardless of the intended indication, and results from screening can be used repeatedly to generate "safer" SAR libraries either virtually or synthetically.

The other important assessments are generated from clinical trial data when this information is accessible. Considering small-molecule and biologic targeted therapies, Roberts and colleagues [30] assessed issues emerging during phase I in cancer patients with data gathered from journal articles and abstracts submitted to annual meetings of the American Society of Clinical Oncology (ASCO) presented from 1991 through 2002. The database included 213 trials with 6474 patients, where 47% of the trials involved targeted small molecules and biologics (combined together by the authors). There were approximately 4% objective responses in all patients and 137 deaths (~2%) of which 35 were classified as treatment related. The overall toxic death rate was estimated to be ~0.5%. Targeted agents had a lower death rate than cytotoxics. The overall serious toxicity rate was ~10%, with major categories from small molecules being hepatic and neurological. During the early period of the study (1991–1994), the odds of a patient dying was 10 times that of the latest period (1999–2002), and over time, there was an increase in safety but a decline in response rates in phase 1 trials. The authors discussed several theories for these findings, which included the opinion that more targeted small molecules and biologics appeared in the later time periods, which supports the rationale that targeted therapies are better tolerated than cytotoxics. It has become apparent over the last 10 years that toxicities associated with targeted therapies in cancer patients are, in fact, different from those seen with standard chemotherapeutic agents and several can be predicted from effects of modulating a specific biological target or multiple targets.

2.9 IMMUNOMODULATORY THERAPEUTICS

Immunomodulatory therapies are widely used in a number of diseases including cancer, autoimmune disorders, multiple sclerosis, and organ transplantation, During the years 1995 to 2008, 174 biotherapeutics (excluding vaccines) were approved for marketing; 136 in the United States and 105 in the European Union, with 67 of these approved in both regions [31]. The majority of safety-related regulatory actions taken in the United States and European Union were related to immunomodulatory effects, which lead to an increase in the risk of secondary and opportunistic infections during long-term treatments. These types of reactions are primarily detected post approval and not well predicted from animal studies. As an example, patients on immunosuppressant therapies display an increased incidence and severity of community-acquired pneumonias and viral infections [16]. The type of infection depends on the underlying defect in the immune system. Cell-mediated immunity involves the activation of phagocytic components of the immune system, natural killer cells, antigen-specific cytotoxic T lymphocytes, and the release of cytokines. Abnormalities in cell-mediated immunity often result in infections and illness called *opportunistic infections*. Opportunistic infections are caused by microorganisms that are usually subclinical or latent in individuals with normal immune function. These types of infections are those normally seen in patients with HIV/AIDS and/or who have undergone organ and stem cell transplant [16].

2.10 MOLECULAR CHALLENGES IN SCREENING TARGETED THERAPIES

This raises the questions pertinent to the title of this chapter: are there molecular challenges to the standard practice of front-loading toxicity screening and testing in the discovery and development of targeted therapies? If so, what are the challenges? Three distinct development approaches, which affect "front loading," are currently used in the biopharmaceutical industry [12]. The major difference is when the decision is made to pursue full development of the lead compound or development candidate, which triggers full-scale process chemistry and development expenditures. In one scheme, full development starts when the development candidate is selected. This scheme is considered the fastest path to the market and follows the assumption that the drug will succeed. In other schemes, full development starts when all Investigational Drug Application (IND) work has been completed or at least when pivotal toxicology studies have been reported. This scheme imposes a risk-benefit decision based on preclinical data. With some exceptions, a hybrid of these two is the usual approach in the pharmaceutical industry, and this practice imposes the front loading of toxicity screening and testing in order to make the informed decision faster. In the third approach, full development starts when preliminary clinical information suggests an increased probability of success. This is the approach used by smaller companies in the biotechnology field, which then becomes the driving force behind additional funding or collaborative transactions with larger partners.

2.11 SIGNALING PATHWAYS IN CANCER RELATED TO TOXICITIES OF THERAPEUTICS

Because of the prominent role the EGF and VEGF pathways play in cancers and the continued information developing on toxicities seen with agents inhibiting these signaling pathways, these processes and drugs are highlighted in this chapter. Both targets and signaling pathways include small-molecule and biotherapeutic inhibitors representing both single- and multiple-target approaches. These signaling pathways are shown in Figs. 2.1 and 2.2.

2.11.1 Epidermal Growth Factor

EGFR is a transmembrane tyrosine kinase receptor, which maintains a pivotal role in regulating cell division and death. EGFR belongs to the HER family of receptors composed of four related proteins (EGFR(HER1/ErbB1), ERBB2(HER2), ERBB3(HER3), and ERBB4(HER4)). The HER receptors are known to be activated by binding to different ligands, including EGF, TGFA (transforming growth factor α), heparin-binding EGF-like growth factor, amphiregulin, betacellulin, and epiregulin. After a ligand binds to the extracellular domain of the receptor, the receptor forms functionally active dimers [EGFR–EGFR (homodimer) or EGFR–HER2, EGFR–HER3, EGFR–HER4 (heterodimer)]. Dimerization leads to the activation of the tyrosine kinase domain, which results in autophosphorylation of the receptor involving multiple tyrosine residues. This leads to recruitment of a range of adaptor proteins (such as Shc, GRB2) and activates a series of intracellular signaling

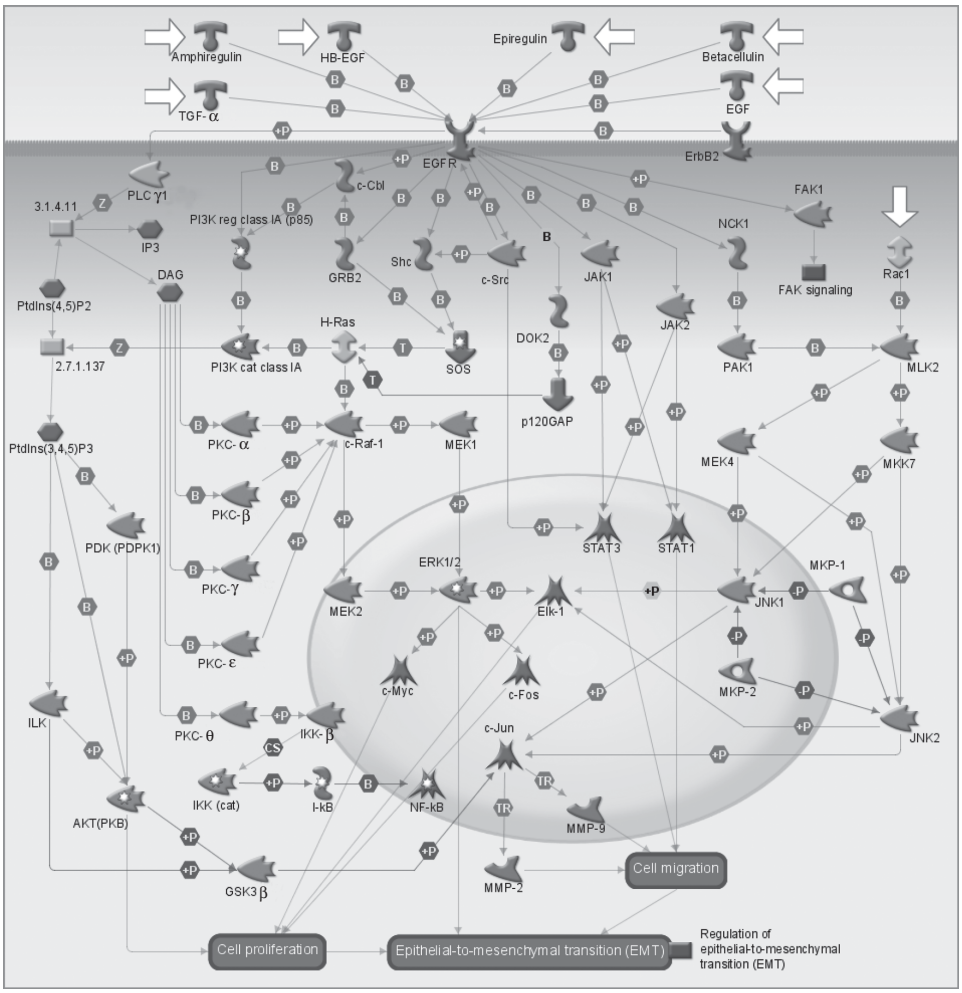

Figure 2.1 EGFR signaling pathway. Binding of ligands such as epiregulin and EGF dimerizes EGFR and activates it. ErbB2 (HER2) does not have to bind to any ligand with high affinity but is a preferred dimerization partner for other ErbB receptors. One component of EGFR signaling acts through the adaptor protein Shc, resulting in activation of RAS-RAF pathway and MAPK cascade. Another component acts through PLCγ and PI3K-Akt axis leading to cell survival. *Source:* Pathway map provided by Genego, Inc. (See color insert.)

cascades, which ultimately affect gene transcription. Gene transcription results in reduced apoptosis, cancer cell proliferation, invasion, and metastasis. These events also stimulate angiogenesis that reacts in relation to stimuli to create new blood supply (neovascularization) to developing or expanding tumors [32]. Major biological pathways mediate downstream effects of EGFR. The first pathway involves the RAS-RAF-MAPK pathway, where phosphorylated EGFR recruits the guanine nucleotide exchange factor via the GRB2 and Shc adaptor proteins, activating RAS and subsequently stimulating RAF and the MAP kinase pathway to affect cell proliferation, tumor invasion, and metastasis. The second pathway involves the PI3K/AKT

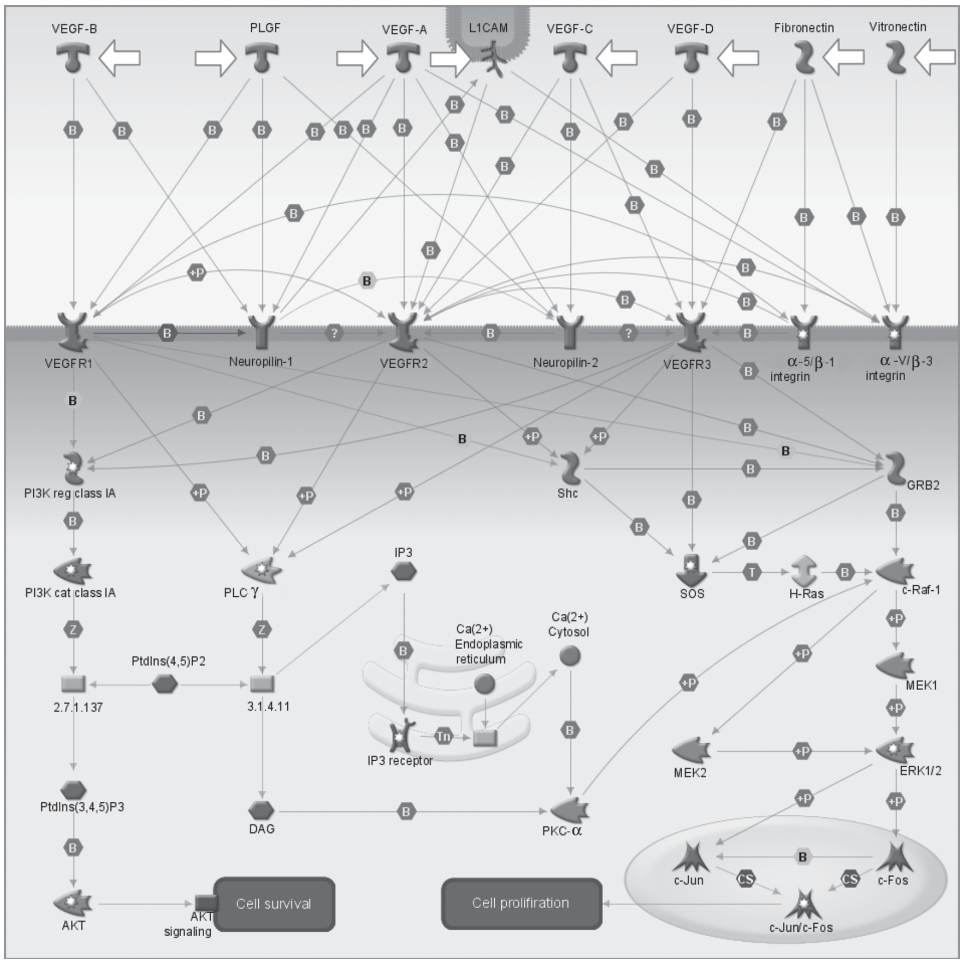

Figure 2.2 VEGF signaling pathway. VEGF-A binding to VEGFR2, a tyrosine kinase, initiates a cascade of signaling including phosphorylation of PLCγ and adaptor protein Shc. PLCγ in turn catalyzes the hydrolysis of phosphatidylinositol ultimately leading to the release of stored Ca. Signaling through Shc activates RAS pathway resulting in MAPK cascade and cell proliferation. Another component of the VEGF signaling pathway acts through the PI3K-Akt axis leading to cell survival. *Source:* Pathway map provided by Genego, Inc. (See color insert.)

pathway, which activates the major cellular survival and antiapoptosis signals by activating various nuclear transcription factors, including NFKB (nuclear factor κB). The third pathway, JAK/STAT, is also implicated in activating gene transcription associated with cell survival [32]. Anti-EGFR antibodies (e.g., cetuximab) bind to the extracellular domain of EGFR monomer and therefore block ligand-induced receptor activation by competing for receptor binding by endogenous ligands. Small-molecule EGFR inhibitors (e.g., gefitinib) compete with ATP to bind the catalytic domain of the kinase, which inhibits EGFR autophosphorylation and subsequent downstream signaling [32].

EGFR inhibitors cause certain types of skin toxicity, typically seen as a papulopustular rash in ~45–100% of patients given these drugs [33,34]. Interestingly, the skin effects have also been used as a biomarker of effect and potential efficacy of these drugs in the clinic. Other toxicities noted with EGFR inhibitors include ingrown nails, paronychia, xerosis [35], and ocular toxicities including changes in eyelids such as squamous blepharitis and in the tear film [36]. Understanding the molecular mechanism of dermal toxicity has led to the design of compounds that potentially rescue the downstream effects of signal inhibition. One such approach is the use of a potent phosphatase inhibitor, menadione, applied topically [33]. Other nonrash toxicities, including skin hyperpigmentation, xerosis, pruritus, hair growth, and color changes have been reported with targeted therapies [37].

2.11.2 Vascular Endothelial Growth Factor

VEGF plays a key role in new blood vessel formation and pathological angiogenesis such as in tumor growth, ischemic diseases, and macular degeneration. The increase in secreted biologically active VEGF protein from cells exposed to hypoxia, a potent inducer of VEGF, is linked to an increased transcription rate, mediated by binding of hypoxia-inducible factor-1 (HIf1) to a hypoxia responsive element in the 5′-flanking region of the VEGF gene. Secreted VEGF protein is a major angiogenic factor that regulates multiple endothelial cell functions, including mitogenesis. Cellular and circulating levels of VEGF are elevated in hematologic malignancies and are adversely associated with prognosis. Angiogenesis is a very complex, tightly regulated, multistep process. Current therapeutic approaches include the inhibition of angiogenesis stimulants (e.g., VEGF) or their receptors, blockade of endothelial cell activation, inhibition of matrix metalloproteinases, and inhibition of tumor vasculature [37].

Compounds that inhibit VEGF affect multiple signaling pathways in cells that regulate and maintain the microvasculature [38]. Side effects from inhibitors of VEGF signaling have ranged from overt cardiac effects to hypertension [15,24,38,39]. Molecules with varying degrees of cardiorelated effects include those with both single and multiple targets. Single-target agents include Avastin and trastuzumab (Herceptin; Genentech/Roche). Multiple-target agents include Nexavar (c/B Raf, VEGFR2/3, PDGFRα/β, KIT, FLT3) and Sutent (VEGFR1/2/3, PDGFRα/β, KIT, FLT3, RET, CSF-1) [22]. Although the mechanism of cardiotoxicity has not been fully elucidated, Fig. 2.3 shows a diagram where three anti-VEGF therapeutics, trastuzumab, imatinib, and sunitinib, share a similar signaling event, translocation of BCL2-associated X protein (BAX) to mitochondria in cardiomyocytes, which can lead to apoptosis [39].

2.12 OTHER TOXICITIES

Targeted therapies can also lead to renal toxicity since most of the biological pathways involved are expressed in the kidney. Related toxicities reported include hypertension and proteinuria [40]. Adverse effects can be manifested through single-target therapies primarily through pathway inhibition such as the p38 mitogen-activated protein kinase (p38MAPK) inhibitors [41] and the type 1 insulinlike growth factor receptor (IGF1R) inhibitors [42]. Multikinase inhibitors induce a number of toxicities

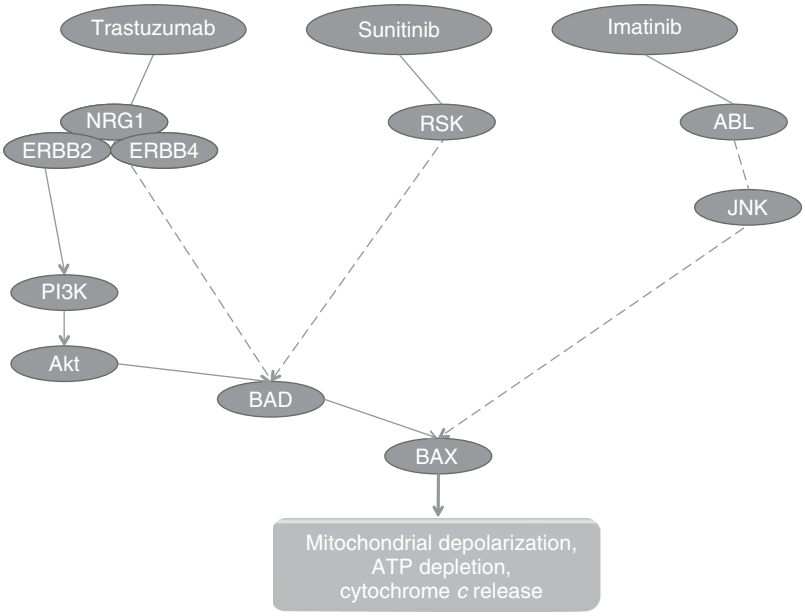

Figure 2.3 Schematic representation of putative molecular mechanisms of cardiotoxicity of three tyrosine kinase inhibitors in cardiomyocytes. A key signaling event common to all three inhibitors is the translocation of BAX to mitochondria, which is known to be involved in mitochondrial depolarization, ATP depletion, and cytochrome *c* release, which is a common mechanism in apoptosis. But in cardiomyocytes, the same mechanism could lead to toxicity. Detailed molecular events of cardiotoxicity due to kinase inhibitors remain to be classified. Trastuzumab is an ERBB2 antibody that reduces oncogenic signaling in breast cancer because of the overexpression of ERBB2(HER2) via PI3K-Akt pathways, ultimately leading to apoptosis. But in cardiomyocytes, pro-apoptotic signals may lead to cardiotoxicity. Imatinib is an inhibitor of ABL and blocks oncogenic signaling in CML through RAF-ERK and PI3K-Akt pathways, leading to apoptosis. But in cardiomyocytes, ABL, Abelson tyrosine kinase may have a survival function, and inhibition of ABL leads to activation of prodeath JNK pathway, ultimately leading to translocation of BAX and apoptosis. Sunitinib is primarily a KDR, kinase insert domain receptor (VEGFR2) inhibitor but also has several off-target effects such as inhibition of ribosomal kinase RSK, perhaps leading to inhibition of BAD, BCL2-associated agonist of cell death phosphorylation and eventual translocation of BAX to mitochondrion. *Source:* Adapted from Force T, Krause DS, Van Etten RA. Molecular mechanisms of the cardiotoxicity of tyrosine kinase inhibitors. Nat Rev Cancer 2007;7:332–344. (See color insert.)

where the commonality of toxicities between certain drugs is associated with common targets within a multikinase profile [43–46]. For instance, sorafenib and sunitinib target PDGFR, FLT3, VEGFR, as well as other kinases (see above), and share certain toxicities including cardiotoxicity. Toxicities of specific drugs are also influenced by the status of the patient and the type of cancer being treated. Several anecdotal reports appear in the literature that involve specific patient characteristics linked to toxicity, such as a case of plantar erythrodysesthesia in a patient being treated for non small-cell lung cancer, who was given low doses of sorafenib [47]. Although toxicities can be broadly based, the multikinase inhibitors are reasonably well tolerated as a class [43] and toxicities are becoming more predictable as use becomes more widespread.

2.13 DO CURRENT PRECLINICAL STUDIES PREDICT HUMAN ADVERSE EVENTS FROM MOLECULAR TARGETED THERAPIES?

Summaries of documents for four drugs submitted to the US FDA for registration purposes were examined to see if cardiovascular toxicity associated with VEGFR inhibitors and/or dermal effects from EGFR inhibitors were identified in *in vitro* systems or animal studies before clinical development [48–51]. Nonclinical studies on sorafenib (Nexavar) indicated a potential for cardiotoxicity primarily detected in safety pharmacology studies. Positive findings were seen in the *in vitro* hERG and action potential assays; however, there were no clear findings in dog telemetry studies or in a chronic one year study in which elevated CK (creatinine kinase) levels were observed. Limited histopathological findings in cardiac tissue were present in some animals in toxicology studies. In clinical trials, there was an increased incidence in hypertension versus control arms and, although rare, valvular disease and heart failure were reported as a cause of death in sorafenib arms. In addition, the drug was found to affect thyroid and parathyroid function and electrolyte imbalance from GI toxicity and diarrhea, both conditions that could increase the risk for cardiovascular toxicity. Force speculates that the overall rate of cardiomyopathy for sorafenib is low. On the other hand, the rate for cardiomyopathy with sunitinib (Sutent) is rated as moderate with hypertension and hypothyroidism seen as contributing factors. In preclinical studies, sunitinib caused both *in vitro* and *in vivo* cardiac effects including increased action potential duration in canine Purkinje fibers, blockage in hERG assays, and QTc prolongation in monkeys. In three- and nine-month studies on monkeys, reductions in heart rate and changes in ECHO parameters were seen, including left ventricular ejection time. There were also histopathological findings in individual animals, including capillary proliferation, myocardium vacuolization, or inflammation of the pericardium. Evidence of hemorrhage in various tissues was seen in both rats and monkeys. In clinical trials, decreases in left ventricular ejection fraction were also seen, which directly correlated with preclinical findings, as did incidences of hemorrhage in several tissues.

Preclinical studies with the EGFR inhibitors erlotinib (Tarceva) and gefitinib (Iressa) showed mechanistic evidence of dermal toxicity. With erlotinib, dermal toxicity was noted in mouse cancer xenograft models and in multiple dose studies in rats and dogs. In addition, ocular toxicity including corneal ulcerations was seen in longer-term dog studies. Dermal toxicity with gefitinib was seen in multiple dose studies in mice and dogs. Other toxicities seen with these compounds were almost universally related to the molecular mechanisms associated with the pharmacology of the drugs. This includes the generalized gastric toxicity leading to diarrhea seen clinically.

A number of other toxicities have been seen in animal studies with both single- and multitargeted compounds, including findings in the hematopoietic system, liver, kidneys, both male and female reproductive systems, bone, teeth, adrenal glands, pancreas, and changes in coagulation parameters. Common findings other than cardiovascular and dermal toxicities include bone marrow hypocellularity, incomplete epiphyseal closing and/or thickening of growth plates particularly in dogs, dentin alteration in juvenile animals, and osteodystrophy of the jaw in rats.

One conclusion that can be drawn from about 10 years of experience with targeted therapies is that rather than front-loading general toxicology studies, it would be more appropriate to design toxicology studies where the molecular mechanisms could be studied and related to potential adverse effects. For the most part, these toxicities

per se can be monitored effectively in early clinical trials, particularly when adequate pharmacokinetic/pharmacodynamic (PK/PD) correlations can be made. If general toxicology studies are not predictive of these molecular mechanisms because of the lack of definitive molecular assays, then additional time should be spent on developing biomarkers to monitor mechanisms in relation to adverse effects. In the ideal situation, assays developed for animal studies can be translated into assays used for clinical trials [5], but as noted earlier, these assays and direct links to toxicity are only starting to emerge.

2.14 EXPLORATORY AND PHASE 0 TRIALS IN CANCER PATIENTS

Several exploratory and phase 0 trial designs have been proposed and issued in a guidance by the US FDA [52]. In addition, a new ICH Guideline S9 is proposing a more abbreviated preclinical resting scheme for FIH oncology trials [10], and several exploratory approaches are currently in use. These include microdosing for preliminary PK information [53], screening studies for more than one compound to judge comparative bioavailability [52], radiolabeled studies at very low doses for distribution and elimination [53], and heavy-water labeling to probe fluxes through various biological pathways [54]. What distinguishes anticancer therapeutics in cancer patients is the ethical consideration that cancer patients should receive doses that have a chance to deliver an efficacious response [55]. Therefore, using low doses that are several multiples below the estimated therapeutic dose is not a preferred approach for FIH dosing in cancer patients but is, in fact, included in the phase 0 oncology designs [55]. In the NCI (National Cancer Institute) proposal [56], the essential rationale is the integration of PD assays into the first trial as a way to explore target modulation as evaluated with PK correlates [56]. These trials have been proposed to be conducted under an Exploratory Investigational New Drug (xIND) application. Since these trials are to be conducted in late-stage cancer patients, standard genotoxicity studies are not required; however, assessment of vital organ function, including cardiovascular, respiratory, and central nervous systems, should be done in animals and would normally be included in the general toxicology studies. Stand-alone safety pharmacology studies are optional under this proposal; however, because of the cardiotoxicity noted with several targeted therapies, cardiovascular safety pharmacology studies would normally be included. In the ICH S9 proposal, the animal toxicology studies essentially mirror the clinical dosing schedule. In the shortest clinical dosing scheme where the drug is given once every three weeks, single-dose toxicology studies will support the FIH trial. When the drug is given once every two weeks, toxicology studies can be designed as two-dose, 14 days apart, studies. The standard determination of an NOAEL is not considered essential in the preclinical toxicology studies, and demonstration of complete reversibility from all adverse effects is not considered an essential part of the supporting data. However, an assessment of reversibility up to a point is always included in supporting toxicology studies. Toxicology studies include rodent and nonrodent species, and a common approach for small-molecule therapeutics, including most targeted therapeutics, is to set a starting dose in humans at one-tenth the severely toxic dose in 10% of the rodents studied. If the nonrodent species (e.g., dog, monkey) is more sensitive to the drug than the rodent model, then the starting clinical dose is one-sixth of the highest nonseverely toxic dose. Since this new guidance is expected to be viewed on a case-by-case basis,

there are potential examples where the toxicology program to support the FIH trial could consist of rodent species only [10,52].

Changes in the requirements needed to initiate clinical trials with oncology therapeutics have occurred for a number of well-publicized reasons. Of major concern is the low success rate in this therapeutic category; only about 5% of INDs for new molecular entities in oncology make it past the investigational phase, which compares to ~10% in other therapeutic areas [56,57]. At issue is certainly the prolonged time line and costs of clinical trials, but at the heart of the problem is the lack of validated preclinical systems that accurately predict the efficacy and toxicity of new therapeutic agents and modalities [6]. These deficiencies include both *in vitro* and *in vivo* assays and models and have led both regulators and investigators to initiate procedures to move new drugs into clinical trials in cancer patients faster and develop both PK and PD endpoints in humans rather than rely on imperfect systems.

2.15 CONCLUSION AND FUTURE PERSPECTIVES

With the changing regulatory view on requirements for FIH dose selection for anticancer drugs, and the increase in discovery and development of targeted therapeutics, the concept of front-loading toxicology studies into the discovery and development programs with the intent on accelerating the development time line can be questioned from a scientific/molecular standpoint. This question is based on the belief that the modulation of specific targets, the goal of targeted therapies, directly relates to both efficacy and toxicity. Therefore, measuring markers of these events as related to exposure via PK/PD relationship modeling may in fact be a more rational way to accelerate a program to phase II, in particular, for phase II dose selection. However, maximizing benefit from this approach will require increased efforts to define and create assays for relevant biomarkers of biological activity and safety as PD endpoints. Currently, this has not been realized to the extent that this model can be applied to a majority of anticancer discovery and development programs. It is possible, however, that current programs could save and preserve (i.e., biobank) samples from nonclinical studies and correlate findings from these samples when molecular markers become established. This approach would create the molecular underpinnings of future anticancer programs related to the same molecular targets. It is tempting to speculate that there will be increased efforts on identifying and qualifying markers seen in preclinical studies with the aim of translating these to humans to provide rational estimates for FIH dose selection. A greater emphasis is expected to be placed on translating effects in animal models into patients in the first trials, and these trials are expected to be initiated with less toxicity testing than seen in the past. The increased use of PK/PD modeling to estimate exposure–response relationship in animal models and clinical trials will take on added importance with targeted anticancer therapeutics. The practice of front-loading toxicity testing to accelerate programs must be tempered with the realization that many toxicities of targeted therapeutics relate to the actual mechanism of action for efficacy. Whether mechanistic assays can be directly linked to toxicity in nonclinical studies has yet to be fully elucidated. Presently, the best approach is to make use of PK/PD relationship modeling across species and biobanking blood and tissue specimens of preclinical as well as clinical studies for future analysis. Early screening for chemical-based toxicity will continue to be performed as part of the

lead optimization process, and cardiotoxicity—other than QTc prolongation—should be incorporated early on with targeted therapeutics because of the complex cross talk apparent in multiple signaling pathways.

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