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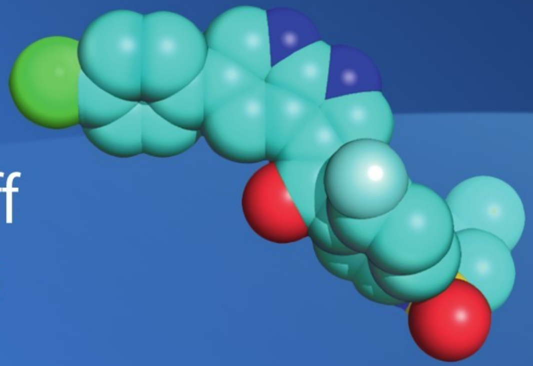

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Editors

Therapeutic Kinase Inhibitors

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Volume 355

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The cover image shows PLX4032 inside the BRAF kinase and separately. Figure provided by G. Bollag.

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Preface

Cancer drug development is currently undergoing a profound shift. Drugs targeting fundamental cellular processes such as DNA–replication and microtubule function, often referred to as "chemotherapy" and still the backbone of most cancer treatment regimens, are increasingly being complemented by or replaced with kinase inhibitors. This new class of drugs targets enzymes which provide growth and survival signals to cancer cells by transferring phosphate groups from Adenosine-5'-triphosphate (ATP) to other proteins, lipids, nucleotides, and carbohydrates.

The earliest roots of kinase inhibitor therapy for cancer can be found in observations several decades ago that mutant kinases are often responsible for tumor inducing properties of certain animal viruses. The idea to develop drugs against these kinases was initially not pursued due to the important physiological functions of many of these enzymes and the concern that kinase inhibitors would be selective enough to provide a sufficiently wide "therapeutic window" between drug activity and drug toxicity. This concern was largely alleviated by the discovery that mutations in kinase encoding genes can render cancer cells uniquely "addicted" to signals provided by the mutant kinase. An early dramatic example for the paradigm of oncogene addiction was the durable remissions of BCR-ABL positive leukemias in response to the ABL kinase inhibitor imatinib (gleevec).

Since the first publication of the gleevec trials in the year 2001, much has happened. Pharmaceutical companies have designed and synthesized inhibitors against virtually all known human kinases and the genomes of many human cancer types have been surveyed for mutations that might result in oncogene addiction. These efforts have been rewarded by the development and regulatory approval of inhibitors against the ABL kinase (chronic myeloid leukemia), EGFR kinase (lung cancer), ALK kinase (lung cancer), PDGFR and KIT kinase (gastrointestinal stromal tumors), VEGFR (kidney cancer) and most recently the BRAF kinase (melanoma). As with any other cancer drug, responses to therapy are often not durable and acquired drug resistance has become a major challenge.

This book summarizes the current state of kinase inhibitor therapy for cancer. Successful drug development relies on the expertise and dedication of many experts. To reflect this team approach to finding new kinase inhibitors and defining

their optimal use for cancer treatment, we invited experts in academia and pharmaceutical industry to share their insights into various aspects of this process, ranging from the first chemical screens, to preclinical testing and disease-focused clinical drug development. We hope these lessons will be instructive for the novice as well as the expert.

Charles L. Sawyers
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Part I
Fundamentals

Setting up a Kinase Discovery and Development Project

Gideon Bollag

Abstract Discovery of novel kinase inhibitors has matured rapidly over the last decade. Paramount to the successful development of kinase inhibitors is appropriate selectivity for validated targets. Many different approaches have been applied over the years, with varied results. There are currently thirteen different small molecule protein kinase inhibitors on the marketplace. Interestingly, a majority of these compounds lack precise selectivity for specific targets. This will change in the coming years, as technology for achieving improved selectivity becomes more widely applied. This chapter will focus on some of the critical considerations in setting up a kinase discovery and development project, citing examples particularly targeting the Raf kinases.

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1 Introduction

It should first be noted that drug discovery and development, like other research, cannot be distilled to a simple recipe. Therefore, the considerations presented in this chapter provide just a few among infinite alternative approaches. Furthermore, the discussion is restricted to small molecule kinase inhibitors dosed orally on a daily schedule. In order to help illustrate the different steps in drug discovery, I will refer to the quite different approaches used to discover two quite different compounds: sorafenib (also known as BAY 43-9006 and marketed as Nexavar) and PLX4720. Both are Raf inhibitors, but sorafenib preferentially targets C-Raf (Wilhelm et al. 2004) while PLX4720 preferentially targets activated Raf enzymes typified by the oncogenic B-Raf^{V600E} kinase (Tsai et al. 2008). PLX4720 is a structurally related analog of vemurafenib (PLX4032), a compound currently undergoing clinical testing in patients with metastatic melanoma. It should be noted that sorafenib was identified and advanced into preclinical studies 3 years before the discovery of the *BRAF* oncogene (Davies et al. 2002; Lyons et al. 2001; Wilhelm et al. 2006). Therefore, the drug discovery effort culminating in sorafenib sought to identify C-Raf inhibitors, while the effort that produced PLX4720 and vemurafenib sought to identify inhibitors of B-Raf^{V600E} kinase activity.

2 Choosing the Drug Target

One of the key considerations to enable a successful drug discovery project involves choosing a good target. There are no standard principles for target selection, but validation in humans is probably the most compelling criterion. In that sense, improving on a currently marketed drug would be a lower risk project, especially if clear limitations exist to the predecessor. One example of this sort of approach involves the development of compounds that target resistant kinase mutations, such as dasatanib (Sprycel) and nilotinib (Tasigna), which show efficacy in cells that are resistant to imatinib (Gleevec) (Quintas-Cardama et al. 2007) (see "JAK-mutant Myeloproliferative Neoplasms"). Targets without pre-existing clinical validation pose a bigger risk, but may provide a more novel therapeutic entry. Sorafenib was discovered as an inhibitor of C-Raf because at the time (in the early 1990s) it was believed that C-Raf may be a critical effector of oncogenic *RAS* (Katz and McCormick 1997). Ideally, genetic data in humans would be available to predict the utility of a target. For example, 'driver' kinase mutations in cancers present intriguing target candidates (Greenman et al. 2007). One target that was identified in this way is the B-Raf kinase (Davies et al. 2002); indeed *BRAF* may be the most common protein kinase oncogene (Greenman et al. 2007). PLX4720 and vemurafenib were identified as oncogenic B-Raf^{V600E} kinase inhibitors because of the genetic validation of the *BRAF* oncogene. This will be discussed in more detail below, and focus on the biology is discussed in "Targeting Oncogenic Braf in Human Cancer" and "Beyond Braf in Melanoma" of this volume.

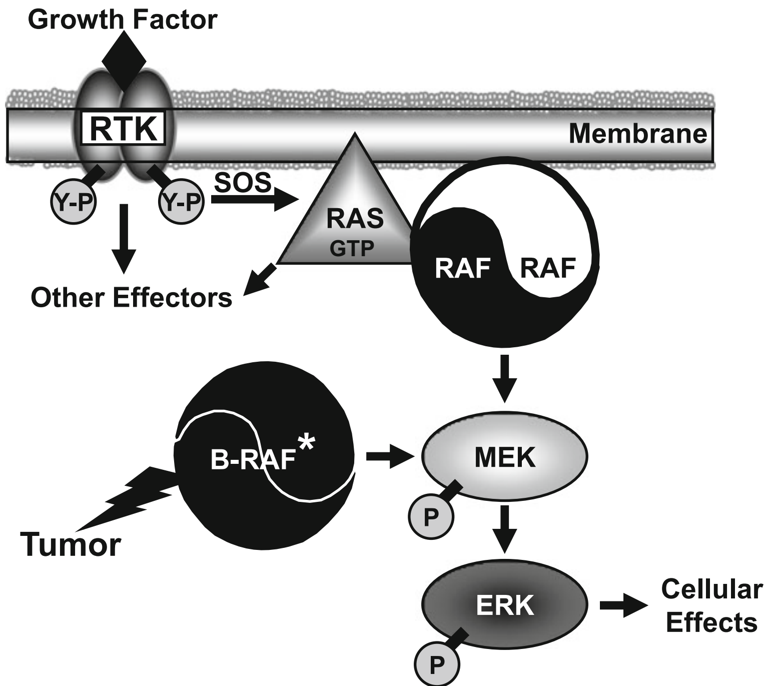

Fig. 1 Diagram of Raf signaling. Normal cellular signals often start with binding of growth factors to their receptors, releasing the receptor tyrosine kinase (RTK) activity to autophosphorylate and phosphorylate downstream substrates (such as SOS) that in turn effect exchange of GDP for GTP on the RAS proteins. Note that alternate effectors are also turned on by the RTK. GTP-bound RAS, which is anchored to the membrane, recruits RAF proteins and activates their ability to phosphorylate MEK on two serine residues. Mounting evidence suggests that growth factor stimulated RAF is dimeric or heterodimeric (comprised of one or more RAF isozymes: A-, B- or C-RAF). Alternatively, tumors bearing the *BRAF* oncogene, predominantly the V600E mutation, unleash constitutive MEK phosphorylation by the B-RAF* (oncogenic) kinase. Phosphorylated MEK in turn phosphorylates ERK, which translocates to the nucleus and phosphorylates a plethora of substrates that include transcription factors. The subsequent reprogramming of transcription results in an assortment of cellular effects that include proliferation, differentiation, survival, motility, or senescence

To illustrate the points made in this chapter, it is helpful to briefly consider some aspects of Raf biology. A diagram depicting the biology and biochemistry of the Ras/Raf pathway is shown in Fig. 1. Normally, the binding of a growth factor to its receptor will activate intrinsic tyrosine kinase activity, resulting in autophosphorylation as well as substrate phosphorylation. Multiple pathways are subsequently activated, including the Ras/Raf pathway. Upon growth factor stimulated exchange of GTP for GDP on the Ras GTPase, Ras-GTP recruits Raf to the membrane. ‘Raf’ could indicate any of the three Raf isoforms, A-Raf, B-Raf, and/or C-Raf, depending on the cell type, and the figure shows a Raf dimer since

recent evidence suggests that dimerization or even hetero-dimerization of Raf is important in its biology (Garnett et al. 2005; Rajakulendran et al. 2009; Rushworth et al. 2006; Wan et al. 2004; Weber et al. 2001). Oncogenically activated B-Raf is independent of upstream signaling, and appears to signal independently of C-Raf. Signaling through the pathway continues as Raf phosphorylates MEK resulting in activation of MEK kinase activity on ERK. Phosphorylated ERK is an active kinase which translocates to the nucleus and phosphorylates nuclear substrates such as transcription factors which in turn effect cellular processes such as proliferation (Schubbert et al. 2007).

While sorafenib was identified with the primary purpose of inhibiting C-Raf kinase activity, it remains unclear—perhaps even doubtful—if C-Raf inhibition bears relevance to the clinical activity demonstrated by sorafenib. To date, sorafenib has been approved by the FDA to treat renal cell carcinoma and hepatocellular carcinoma (Kane et al. 2009; Kane et al. 2006). However, sorafenib is also a potent inhibitor of a selection of tyrosine kinase receptors, including VEGF receptors, PDGF receptors, Kit, Flt3, and Ret; clinical activity may be due to the composite inhibition pattern (Wilhelm et al. 2004). By contrast, PLX4720 and vemurafenib are selective Raf inhibitors, and their preferred affinity for oncogenic B-Raf translates to remarkable selectivity in cellular and in vivo models: anti-tumor activity is evident only if the tumor cell bears the $BRAF^{V600E}$ gene (Tsai et al. 2008). Because of this requirement, clinical development of vemurafenib has utilized the diagnostic selection of patients bearing tumors with the $BRAF^{V600E}$ mutation (Garber 2009).

Note that Raf is a serine-threonine kinase, while most of the targets of marketed kinase inhibitors (including the other targets of sorafenib) are tyrosine kinases. While it was originally believed that distinct chemo types would be required to inhibit these two different types of phosphorylation events, it is now clear that chemotypes can readily crossover to other branches of the kinase family tree.

Once a target is chosen, a series of assays must be developed, and a screening paradigm must be implemented. The assays include biochemical assays for the kinase target and selected counterscreening (non-target) kinases, cell-based assays for showing target inhibition in vitro and appropriate effects on cellular pharmacology, and in vivo models to measure pharmacodynamics and efficacy. Potency and selectivity metrics for each of the assays should be identified that allow filtering through the screening funnel. After proof-of-concept efficacy has been established, the metrics generally become more stringent and additional parameters monitoring pharmacokinetics and Absorption, Distribution, Metabolism, Excretion (ADME) are measured. Often, the bulk of the drug discovery effort focuses on the optimization of pharmacological properties. In other words, potent and selective compounds may be identified quite early in the project, but oral bioavailability is only achieved many months (or even years) later. These steps are described in detail below.

3 Biochemical Assays for Screening and Counter Screening

Determination of kinase inhibitory activity can be achieved in many ways. Thus, inhibition of substrate phosphorylation, inhibition of ATP hydrolysis, or direct binding of compound to kinase can all be employed in setting up a biochemical assay. Furthermore, each of these different biochemical events can be monitored using different technologies (Charter et al. 2006; Eglen and Reisine 2009; Hastie et al. 2006; Li et al. 2008; Riddle et al. 2006; Warner et al. 2004). There are advantages and disadvantages to each of these assays, and—importantly—it is likely that a different compound would emerge depending on the technology used to screen. Fundamentally, the preferred biochemical assay format would most closely mimic the native kinase as it participates in the oncogenic process. In the case of sorafenib, direct enzymatic activity was measured using radioactive ATP as substrate and readout (Wilhelm et al. 2004). For PLX4720, again the primary assay measured direct enzymatic activity, but in this case the readout used an antibody to phospho-MEK and measured the proximity between the labeled antibody and the epitope tag (in this case biotin) on MEK (Tsai et al. 2008; Warner et al. 2004).

Of great importance is the source of the kinase enzyme. Ideally, the enzyme should reflect the native form that exists within the target cell. Often, expression of kinase protein for enzymatic assays is carried out in baculovirus-infected insect cells. Baculoviruses are natural insect pathogens that readily infect cultured insect cells and can be engineered to express very high levels of recombinant protein (Summers 2006). Because insect cells are eukaryotic and possess many of the same post-translational modification systems as human cells, baculovirus-mediated expression is often preferred. Furthermore, co-expression of multiple enzymes is often desirable, and this can be relatively easily executed by co-infecting insect cells with multiple baculoviruses.

The source of Raf enzymes for both the sorafenib and the B-Raf^{V600E}-selective projects derived from baculovirus-based expression, and this was key to the progress. For the sorafenib project, C-Raf protein was purified from insect cells that had been co-infected with three different baculoviruses: epitope-tagged C-Raf, v-Src, and v-Ras (Macdonald et al. 1993). This triple-infection yielded highly active protein, as Ras-aided Src-dependent tyrosine phosphorylation causes significant stimulation of kinase activity. The target protein for guiding discovery of B-Raf^{V600E}-selective compounds was purified from insect cells that had been co-infected with a truncated form of B-Raf^{V600E} (encoding residues D448 through K723) and the co-chaperone CDC37 (Tsai et al. 2008). CDC37 recruits the insect cell-derived HSP90 protein to stabilize a ternary complex with B-Raf^{V600E}, resulting in optimal activity and stability (Wan et al. 2004).

One additional consideration in devising the biochemical assay involves the constitution of the reaction conditions. Typically, buffers, pH, and salts (including magnesium and/or manganese ions) are adjusted for optimal activity. Furthermore, the source and concentration of substrates—both ATP and

phosphate-acceptor—can critically affect the outcome. For example, kinase-inactive MEK protein was used in both screens described above, so that Raf activity would not be complicated by MEK activation. Notably, in the discovery path leading to sorafenib, initial hits from the screens were tested at different ATP concentrations so that compounds with minimal dependence on ATP would be prioritized. This small but significant variation resulted in an initial scaffold that bound outside the adenine binding site of the Raf enzyme, in turn determining that the final lead—sorafenib—itself, would bind to a form of the enzyme that disfavors ATP-binding (Wan et al. 2004).

4 Lead Optimization Using Crystallography and Cellular Assays

The first three-dimensional structure of the kinase domain of B-Raf was published in 2004 (Wan et al. 2004). Interestingly, solution of this X-ray structure was dependent on the presence of sorafenib in the crystallization reaction, and the binding interactions of sorafenib were elegantly revealed. Sorafenib had been discovered without the guidance of co-crystallography, and the solved co-structure nicely rationalized the structure–activity relationships that had been empirically determined (Wilhelm et al. 2006).

By contrast, the discovery effort that led to the identification of PLX4720 and vemurafenib was heavily dependent on the use of X-ray co-crystallography. Indeed, over 100 co-crystal structures of analogs from several different series were solved in the process of optimizing compounds. To aid in this labor-intensive effort, an engineered form of the B-Raf kinase domain was devised by molecular biology and protein informatics techniques. This engineering effort resulted in a B-Raf kinase domain bearing 16 amino acid substitutions, with either valine or glutamate at residue 600 (Tsai et al. 2008).

The solved crystal structure reveals a dimeric architecture. Furthermore, the two protomers generally present in two different conformations, so the structure actually represents a heterodimer. Recent work suggests that this heterodimeric structure may reflect a true physiological entity (Rajakulendran et al. 2009). Indeed, while both subunits in the crystal structure are B-Raf protomers, the relevant form in the tumor may contain Raf homodimers, B-Raf/C-Raf heterodimers, or even other macromolecular complexes of the Rafs with scaffolding proteins or chaperones. Thus, analysis of the binding modes of different compounds in the crystal structure is highly informative. Comparison of the two subunits reveals many key differences, but perhaps most striking is the alternative conformation of a key loop, called the DFG (aspartate-phenylalanine-glycine) loop. When the phenylalanine of this loop points in toward the active site (DFG-in), ATP-binding is favored, and conversely the DFG-out conformation disfavors ATP-binding.

As shown in Fig. 2, sorafenib and PLX4720 each bind to alternate protomers of the heterodimer. In the sorafenib co-structure, electron density for the compound is

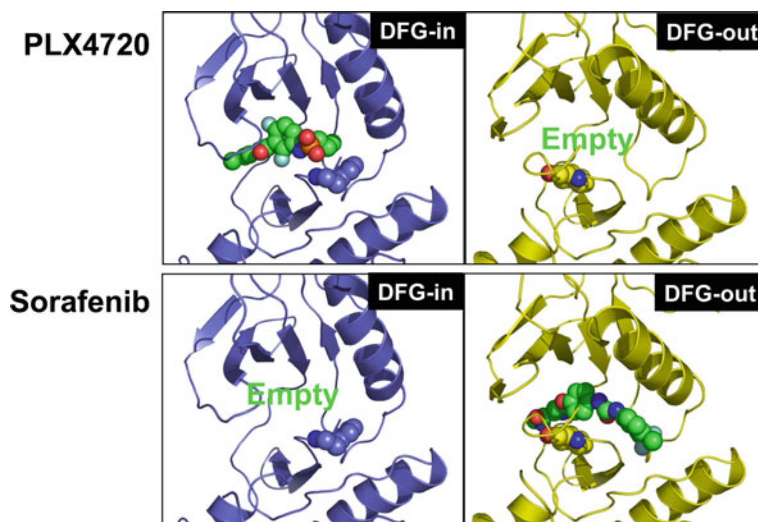

Fig. 2 Three-dimensional co-crystal structures of PLX4720 and sorafenib bound to B-Raf. B-Raf crystallizes to reveal a dimeric X-ray structure. One protomer of the dimer adopts a conformation that could accommodate ATP-binding as reflected by the conformation of the aspartate-phenylalanine-glycine loop (DFG, residues 594-596 of B-Raf). When the phenylalanine of this loop is directed toward the inside of the protein (DFG-in, loop shown using *blue* carbon spheres in the *left* protomers), ATP-binding is feasible. The DFG-out conformation (*right* protomer, loop shown using *yellow* carbon spheres) disallows ATP-binding. Electron density from co-crystals of PLX4720/B-Raf reveals binding exclusively to the DFG-in conformation (*top left*, PLX4720 shown with *green* carbon spheres); by contrast, sorafenib binds solely to the DFG-out conformation (*bottom right*, sorafenib shown with *green* carbon spheres). The DFG-in conformation is stabilized by the V600E mutation, explaining the preferred affinity of PLX4720 for the oncogenically activated form of *BRAF*

found almost exclusively in the DFG-out conformation. By contrast, PLX4720 binds to the DFG-in conformation. Therefore, PLX4720 behaves as a competitive inhibitor of ATP-binding, while sorafenib stabilizes a form of the kinase that disfavors ATP-binding. This property is perhaps fundamental in understanding differences between the two compounds. Since PLX4720 and vemurafenib bind preferably to the more activated form of the kinase—and because the V600E mutation stabilizes this same form—this class of compounds becomes oncogene-selective.

While most of this chapter has focused on sorafenib and PLX4720/vemurafenib to make points about kinase drug development, it should be noted that several additional Raf inhibitors have made it through drug development projects and into the clinic. These include RAF-265, XL281, and GSK2118436. At this time, only the molecular structure of RAF-265 has been disclosed. RAF-265 is derived from a drug discovery effort that used sorafenib as starting point and selected for improved potency on oncogenic B-Raf (Montagut and Settleman 2009; Ramurthy et al. 2008). Like sorafenib, RAF-265 also inhibits growth factor receptors such as VEGFR and PDGFR.

Optimization of RAF-265 was specifically designed to improve the physicochemical properties as well as the potency against B-Raf (Ramurthy et al. 2008). To begin, the urea was tied back into a benzimidazole ring, then substituents were optimized using biochemical assays for B-RafV600E and C-Raf, as well as cellular assays monitoring phosphorylated ERK and proliferation in tumor cells. In addition, improved solubility and overall oral bioavailability were achieved in the final selection of RAF-265. It appears that X-ray crystallography was not part of the optimization plan, but computational modeling based on the published crystal structure (Wan et al. 2004) rationalized the structure–activity relationships.

Two different types of cellular assays are generally essential for guiding lead optimization: pharmacodynamic and phenotypic. A pharmacodynamic assay seeks to determine that the target is inhibited within the cell. A phenotypic assay seeks to demonstrate that target inhibition translates to appropriate cellular efficacy. Typically, a pharmacodynamic assay will determine the phosphorylation state of the target's substrate or alternatively the autophosphorylation of the target directly. Typical phenotypic assays for a kinase inhibitor destined for an oncology indication would be to monitor proliferation, migration, invasion, anchorage-independent growth, or downstream pathway readouts such as transcription or growth factor production. It is important that both sets of assays be configured in a relatively high-throughput format so that many compounds can be analyzed very quickly.

To measure cellular Raf inhibition it is most common to use antibodies to phosphorylated MEK or phosphorylated ERK. This can be done in a variety of compound-treatment formats: unstimulated cells, growth factor stimulated cells, or cells in which Raf proteins are conditionally or constitutively activated. All of these methods were used in the development of both sorafenib and PLX4720/vemurafenib. Although B-Raf mutations had not yet been discovered, a conditionally activated form of B-Raf was used to select compounds that inhibit B-Raf kinase activity using a high-throughput immunoprecipitation assay for MEK activity (Lyons et al. 2001). Note that B-Raf was used, since the activity of B-Raf is basally elevated by two asparates at residues 447 and 448, equivalent to the tyrosines 340 and 341 of C-Raf that require phosphorylation for full activity (Pritchard et al. 1995). The higher-intrinsic activity of B-Raf is likely also the reason that *BRAF* and not *CRAF* is the predominant oncogene. Sorafenib was also shown to inhibit phosphorylation of MEK and ERK in unstimulated and stimulated tumor cell lines (Lyons et al. 2001; Wilhelm et al. 2004).

After the discovery of the BRAF oncogene (Davies et al. 2002) and the determination that *BRAF* oncogene-dependent tumors are highly dependent on the RAF/MEK/ERK pathway (Solit et al. 2006), it became clear that projects focusing on the discovery of oncogenic B-Raf-selective compounds should rely primarily on cell lines that express oncogenic BRAF. Thus, the discovery program that identified PLX4720 and vemurafenib utilized B-Raf^{V600E} cell lines for both pharmacodynamic and proliferation assays. Indeed, PLX4720 displays remarkable selectivity for inhibition of MEK and ERK phosphorylation as well as proliferation in melanoma and colorectal cell lines harboring B-Raf^{V600E}, with essentially no inhibitory activity in cell lines lacking oncogenic *BRAF* (Tsai et al. 2008).

This contrasts sharply with the data for sorafenib; while MEK and ERK phosphorylation are inhibited in many cell lines, there is no selectivity for oncogenic B-Raf, and cellular proliferation is comparably inhibited in most cell lines tested (Wilhelm et al. 2004).

5 Improving Pharmaceutical Properties

An additional set of hurdles encountered during lead optimization address the pharmaceutical properties necessary to safely achieve appropriate systemic compound levels (Wan and Holmen 2009). Typical assays that are used to monitor 'ADME properties' include measures of solubility, ionization potential, lipophilicity, serum protein binding, permeability, and stability to metabolic enzymes from hepatocytes.

Information about absorption can be derived from dissolution, lipophilicity, and permeability. Solubility in the gut is necessary for proper uptake, and balanced lipophilicity aids uptake into cells. For kinase inhibitors generally, improving solubility by adjusting drug product formulation is often critical. For example, ionization potential at different pH values can suggest salt forms that can increase solubility. Cellular permeability, looking at transit in both directions (apical-to-basal and basal-to-apical), can be measured in surrogate gut cells such as Caco-2 cells. High permeability, similar in both directions is desirable. These same parameters also play into determining distribution. The avidity of the compound to serum proteins can markedly affect distribution through circulation and into target tissues. Furthermore, determining whether the compound of interest is a substrate for active transporters can give insight into mechanisms of excretion. Both sorafenib and PLX4720 are poorly soluble and lipophilic, and have very high-serum protein-binding affinity, but they also have good permeability.

In vitro measurements of metabolism deserve special attention, as metabolic stability is a key variable that can distinguish compounds (Bjornsson et al. 2003). Metabolism primarily involves liver enzymes that are grouped into phase I enzymes involved in enzymatic transformations such as oxidation, and phase II enzymes that include transferases that conjugate compounds or their oxidation products to aid their elimination. Obviously, the body has multiple mechanisms to eliminate xenobiotics, and the medicinal chemist attempts to steer around these obstacles. A first measure of metabolism can be determined by exposing compounds to intact hepatocytes or 'S9 fractions' (subcellular fractions enriched for metabolic enzymes). Typically, it is desirable to retain at least half of the parent compound following 30–60 min incubations.

Key phase I enzymes include the family of cytochrome P450 oxidases (CYPs). Determining inhibition of about seven different family members (e.g. isozymes 1A2, 3A4, 2B6, 2C8, 2C9, 2C19, and 2D6) is often monitored during lead optimization. Furthermore, identifying which CYP isozymes can transform the parent compound is generally followed in the clinic. Since many marketed drugs are

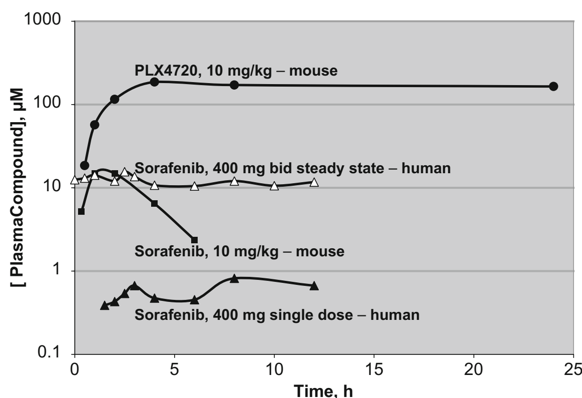

Fig. 3 Pharmacokinetic analysis of PLX4720 and sorafenib. Plasma levels of PLX4720 or sorafenib were measured at the indicated times following compound administration. For PLX4720, compound was administered as a 10 mg/ml suspension in 10% DMSO, and 1% carboxymethylcellulose to mice (Tsai et al. 2008). In mice, sorafenib was administered as a 10 mg/ml suspension in Cremophor EL/95% ethanol/water (12.5/12.5/75, v/v/v) (Sparidans et al. 2009). In humans, sorafenib tosylate was administered as a 200 mg tablet; results are shown for a 400 mg single dose, and for a 400 mg dose taken twice a day (the recommended clinical dose) after reaching steady state (Strumberg et al. 2005)

metabolized by different CYP isozymes, a potent inhibitor has the potential to alter the metabolism of concomitant medications. Sorafenib is a modest inhibitor of CYPs, CYP2B6, and CYP2C8, and is itself transformed by CYP3A4 (Kane et al. 2006, 2009). Nonetheless, clinical studies to evaluate the possibility of drug–drug interactions between sorafenib and concomitant medications have revealed minimal cause for concern (Lathia et al. 2006), perhaps because the high-serum-protein binding serves to shield the compound from the enzymes.

As mentioned above, optimizing for high-oral bioavailability is often the most time-intensive part of a kinase drug development project. Thus, compounds with appropriate potency and selectivity are often poorly active in animal efficacy models. Medicinal and computational chemistry tools help guide this process (Lipinski et al. 2001), but in the end much of the progress relies on empirical iterations.

Examination of the pharmacokinetics of sorafenib and PLX4720 may be instructive to illustrate the challenges of bioavailability. In Fig. 3 compound levels of sorafenib (Sparidans et al. 2009) and PLX4720 (Tsai et al. 2008) in mouse plasma are graphed as a function of time after administration of a 10 mg/kg dose by oral gavage. At this low dose, high-oral bioavailability in a simple formulation is relatively easy to achieve. While bioavailability is high for both compounds, the half-lives are quite different as sorafenib shows a 2–3 h half-life and PLX4720 shows a half-life exceeding 24 h. Note that the relatively modest exposure of sorafenib was nonetheless sufficient for efficacy in multiple models (Wilhelm et al. 2004). While it makes sense that constant high-plasma levels may be required for

optimal efficacy, there is increasing interest in the idea that intermittent high exposures could be sufficient or even preferred (Shah et al. 2008). For such an approach, compounds with shorter half-lives would be preferable.

The exposure of sorafenib in human subjects is markedly higher, as also shown in Fig. 3 in large part due to its substantial half-life of 25–48 h (Kane et al. 2006; Strumberg et al. 2005). Note that a single dose of 400 mg leads to very modest plasma levels, while the current recommended human dose of 400 mg twice daily results in considerable accumulation of the drug in plasma (Strumberg et al. 2005). Since kinase inhibitors like sorafenib and PLX4720 are poorly soluble in water, formulation strategies to improve solubility in the gut are critical. In the case of sorafenib, this was aided by the conversion of the free-base to the crystalline tosylate salt, the current clinical dosage form (Kane et al. 2006).

6 Efficacy Studies in Mice

The standard animal model in oncology drug discovery projects remains the tumor xenograft; this is in spite of the substantial limitations of this model (Sharpless and Depinho 2006). Briefly, human tumor cells are implanted, typically subcutaneously, into immunocompromised mice and these cells grow into a mass that is easily measured. This means that human cells are growing in an environment that is quite different from their native micro-environment. Despite its many imperfections, the tumor xenograft model does help to monitor how effectively compounds can be delivered in vivo. Pharmacodynamic measurements similar to those used to monitor activity in cellular assays can be used to determine the effectiveness of target inhibition, and the rate of tumor growth determines the degree of efficacy.

Both sorafenib and PLX4720 show significant efficacy in xenograft studies. Using careful pharmacodynamic studies, it was shown that in certain models sorafenib-dependent tumor growth inhibition correlates with blockade of the RAF/MEK/ERK pathway, while in other models the inhibition correlates with inhibition of tumor angiogenesis (Wilhelm et al. 2004). By contrast, PLX4720 shows efficacy exclusively in xenografts bearing B-Raf^{V600E} tumors, and the corresponding efficacy correlates with inhibition of the target pathway (Tsai et al. 2008).

During the in vivo efficacy studies, important information about tolerability of the compound can be gleaned from monitoring body weight and mortality. Often, precursor studies determine the maximal tolerated dose (MTD), and efficacy is then determined as a function of dose up to the MTD. A therapeutic index can be estimated by comparing the minimal efficacious dose to the MTD. Indeed, during the drug development process sorafenib was selected from among a series of analogs as having the highest-therapeutic index (Wilhelm et al. 2006).

As described in the next chapter, genetically engineered mouse models are being developed that promise to yield much more predictive efficacy data, but those models are not yet in widespread use during early drug discovery efforts.

7 Toxicology and Safety Studies

Once a kinase inhibitor has cleared all the hurdles discussed above, a series of toxicology and safety pharmacology studies are performed to assess the suitability to advance into clinical studies (Baldrick 2008a, b). Since kinase inhibitors are generally designed to be dosed continuously, multiple dose studies are the norm. Typically, these studies include 28-day general toxicology studies in one rodent species (often rat) and one non-rodent species (often dog), along with studies to determine respiratory safety (often in dogs), cardiovascular safety (often in dogs), and central nervous system safety (often in rats). Since small molecules can affect ion channels as an off-target activity, cardiovascular safety studies are often preceded by in vitro electrophysiology tests of the hERG (human ether-a-go-go) potassium channel. Indeed, this test is sometimes conducted during the lead optimization phase of the project, before selecting compounds for the extensive studies required to submit an IND (Investigational New Drug) application to initiate human clinical studies. Genetic toxicology studies including carcinogenicity testing in vitro (often using the Ames test) and in vivo (often using a micronucleus test in mice) are also performed.

Note that key to conducting these studies is the availability of significant quantities of compound, typically on the kilogram scale. Compounds can be synthesized using carefully controlled standard operating procedures that conform to good manufacturing practices (GMP), and this is required by the FDA for the human studies but not necessarily for IND-enabling studies. At a minimum, the compound purity must be quite high, and the synthetic route must be monitored with detailed analyses using validated protocols at each step. Often, this can take 3–6 months, or more depending on the difficulty of compound synthesis.

Once high-quality compound is available, the toxicology and the safety pharmacology studies must be carried out using good laboratory practices (GLP), again using carefully controlled standard operating procedures. Often, preliminary dose range-finding studies are conducted ahead of the GLP-toxicology studies to help select the most informative doses. While the in-life duration of these studies is carefully defined, much of the time to complete these studies involves preparing tissues and carefully analyzing each critical organ from each animal by histopathology. Often, the GLP-toxicology and safety pharmacology studies can take 6–9 months from first dose to produce the final reports.

8 Future Perspectives

Cancer often causes severe dysregulation of intracellular signaling pathways. Indeed, the more advanced a malignancy, the more mutations and pathways become involved. Thus, it makes good sense to target each of these many signaling pathways with small molecule inhibitors. Since kinases play key roles in